Supersaturation as a driving force for drug absorption from colloidal lipid species in the intestine

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Monash University (Parkville Campus)

by

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Drug Delivery, Disposition and Dynamics
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Saturday, 7 September 2013

To: Prof Shinji Yamashita, Setsunan University

Re: PhD thesis by Yan Yan Yeap

Dear Prof Yamashita,

Please find below the response to questions raised for the thesis entitled:

Supersaturation as a driving force for drug absorption from colloidal lipid species in the intestine

by Yan Yan Yeap

The comments and feedback by Prof Yamashita are highly appreciated. I am honored that the Professor took his time to read the thesis thoroughly, and was interested enough to ask questions. It is my pleasure to provide the answers to the questions here.

1. In Chapter-3, author clearly demonstrated that receptor mediated lipid uptake does not play important roles to promote the absorption of model PWSD, cinnarizine (CIN). However, in Chapter 5, it was concluded that lipid absorption stimulates drug supersaturation and enhance the absorption. It might be possible to consider that the receptor mediated lipid uptake also promotes drug supersaturation by reducing the solibilisation capacity of LBF. Please make some comments on this possibility.

The Professor raises an excellent point where receptor-mediated lipid absorption is potentially an inherent mechanism where drug supersaturation and absorption is promoted. I very much agree with the point raised, and add that the phenomenon will most likely apply for lipids that contribute significantly to colloidal drug solubilization capacity when incorporated into mixed micelles e.g. long-chain fatty acid (LCFA) more so than cholesterol.

The experiments in Chapter 3 were early experiments that were carried out before the possibility of lipid absorption-induced drug supersaturation was hypothesized and tested. Therefore, in those experiments, we only tracked the systemic availability of lipids whose absorption was most likely facilitated by the receptors investigated (i.e. cholesterol for SR-BI and NPC1L1, oleic acid for CD36). For cholesterol, while its presence in vesicles is thought to stabilize the bilayer, it generally is not thought to contribute significantly to the drug solubilization capacity of colloids. Thus, cholesterol absorption is not expected to be a significant trigger for drug supersaturation and absorption. This is reflected in Fig 3.2A & Fig 3.2B of the thesis where cholesterol absorption is almost completely abolished by ezetimibe co-administration, but drug
absorption remains unchanged. On the other hand, we now know that the presence of oleic acid in bile micelles contributes significantly to the colloidal solubilization capacity for a range of lipophilic drugs. Thus, receptor-mediated absorption of oleic acid will likely stimulate drug supersaturation and absorption too. Some evidence that support this notion may be found in Fig 3.2A & Fig 3.2C of the thesis, where the absorption of oleic acid and cinnarizine appeared to be concomitantly reduced (albeit insignificantly) by CD36 inhibitor co-administration. At the time of the experiments, we did not think that the difference was significant enough to warrant further investigation. After the findings in Chapter 5 of the thesis however (i.e. LCFA absorption is a significant trigger for drug supersaturation and absorption), it seems entirely plausible that receptor-mediated LCFA uptake could also enhance drug absorption via the stimulation of drug supersaturation.

At present, the absorption of LCFA is thought to occur mainly via facilitated diffusion at low concentrations, and passive diffusion at high concentrations [1]. Thus, at high LCFA load (as is typically found in the small intestine after lipid ingestion), the physiological significance of active LCFA absorption is questionable. In addition, it is still unclear if the receptors indicated to be involved in LCFA absorption (e.g. CD36 [2], FATP [3], SR-BI [4]) are indeed transporters which facilitate uptake across the apical membrane, or proteins that facilitate intracellular lipid trafficking/modify signalling processes that mediate lipid absorption [5-6]. Given these uncertainties and complexities, we were not able to adequately probe the role of receptor-mediated lipid absorption in drug supersaturation/absorption.

However, in light of the comment made by the professor, it may be interesting to note the data in Fig 5.2A of the thesis, where the attenuation of the UWL acidic microclimate did not completely abolish the absorption of oleic acid, and significant amounts of oleic acid was still absorbed. This could be due to the receptor-mediated absorption component for oleic acid, and it remains highly possible that if this component of lipid absorption was also inhibited, drug absorption may be reduced further.

2. In Fig. 3.7, CIN supersaturation terminated at 1-20min (in the case of Vesicle) or >20 min (in the case of Micelles) after bile addition, however in both cases, decreased concentration of CIN becomes plateau at significantly higher level than equilibrium solubility. Generally, this phenomenon is understood as drug precipitated as amorphous or unstable polymorph. However, using polarized light microscopy, author clearly showed that the CIN precipitated in the crystalline form (Fig. 3.11). Could author explain the reason of the higher plateau concentration of CIN than the equilibrium solubility?

On several occasions, the remaining mixture at the end of the precipitation kinetics experiments (colloids + bile + precipitated drug) was collected, and daily samples (after centrifugation) were taken to track the rate and extent of drug precipitation. We found that while the majority of drug precipitation occurred within the first day of mixing (as shown in Fig 3.7 of thesis), drug concentration in the supernatant took a few days to decrease to equilibrium solubility. At present I do not have an explanation for this phenomenon (where non-instantaneous equilibration to the equilibrium solubility is observed), but speculate that intestinal colloids such as mixed micelles and vesicles may have supersaturation-stabilizing properties. This would be consistent with recent studies from our group that showed that during simulated lipid digestion experiments, stabilization of drug supersaturation was typically possible at and below a supersaturation ratio (SS ratio) of 3, while rapid precipitation was typically observed at SS ratios > 3 [7]. In the experiments depicted in Fig 3.7 of thesis, it is likely that the high degree of drug supersaturation induced immediately after the addition of bile (SS ratio of 6 and 12 for micelles and vesicles,
respectively) was not sustained by the colloidal phases, leading to rapid and significant precipitation. The lower degree of drug supersaturation (SS ratio < 2 for both micelles and vesicles) that resulted after the majority of the precipitation occurred, however, was able to be stabilized for longer periods of time by the colloidal mixture, giving rise to the observation where drug concentration appears to ‘plateau’ at a concentration higher than the equilibrium solubility.

3. Please speculate the possibility of LCFA colloids to interact with the components of mucin layer. I wonder if the LCFA colloids interact with mucin, which might prevent the diffusion of colloids in UWL and affect the absorption of drugs included.

The possibility of LCFA colloids interacting with mucus components is an interesting one and is an area that might be usefully explored in detail. The ability of mucus glycoprotein to bind with a variety of lipids does suggest the possibility that the diffusion of LCFA colloids may be impeded by mucus-LCFA interaction. It follows that the diffusion of colloid-solubilized drugs may be slower in the mucin layer as well, although this will be in contradiction to previous modeling efforts that report enhanced solubilize diffusion across the UWL when solubilized in bile micelles [8-10] (the UWL is often thought to be indistinguishable from the mucus layer).

In the interest of discussion however, if we assume that LCFA colloid-mucin interactions do lead to slower diffusion of solubilized drug, whether or not that is beneficial or detrimental to drug absorption remains unknown. On one hand, if the diffusion of poorly water-soluble drugs through the aqueous mucin layer is indeed the rate-limiting step to drug absorption, mucin-LCFA interaction may then be expected to reduce drug absorption. However mechanistically we understand very little what happens at the mucus/UWL boundary and so it is too early to make predictions. In the presence of mucin-LCFA interaction, it is plausible that enhanced retention time in close proximity to the absorptive membrane could serve to enhance the absorption of somewhat permeability-limited drugs, or, increase the likelihood of acidic microclimate-triggered lipid absorption (which, as shown in Chapter 5 of thesis, is an important driver for drug supersaturation and absorption). In addition, analogous to lipid absorption-induced drug supersaturation, it is also possible that LCFA-mucin interaction serves to deplete LCFA from mixed micelles, and stimulate drug supersaturation as a result. The net effect of LCFA colloid-mucin interaction on drug absorption is likely difficult to predict and may be expected to vary for different drug types.

Nonetheless, available literature and practical experience seem to suggest that co-administration of a lipophilic drug with precursors of LCFA often lead to enhanced drug absorption. I am therefore inclined to think that LCFA colloid-mucin interactions, if present, are in fact beneficial to the absorption of lipophilic drugs, although this judgment is made with reservations due to the lack of available scientific data.

4. Since LBFs performance in the GI tract to produce supersaturation and enhance the absorption of PWSD profoundly depends on the endogenous lipid processing events, conversely, it might become a factor to cause inter- and/or intra individual differences in drug absorption. Please make comments on the possibility of such risks of LBF system.

I agree that it is possible the reliance on endogenous events to produce the necessary enhancement in drug thermodynamic activity may be a contributing factor to intra/inter-individual variability in drug absorption. However, it is well-known that the body is extremely efficient and robust in its ability to process, digest and absorb dietary lipids. Therefore, it is debatable that the
dependence of LBF on endogenous lipid processing events to promote drug absorption is any more variable than other processes that may underpin drug absorption (e.g. dose form disintegration, drug dissolution etc), or any more variable than other formulation approaches.

It is worth remembering that first and foremost, LBF increases drug absorption by circumventing the need for drug dissolution in the GI tract, by increasing the mass of drug that may be present in solution in the small intestine, and by increasing the efficiency of drug presentation to the absorptive membrane (via micellar solubilization). These processes likely vary little between healthy individuals. However, in the interest of discussion, if LBF’s dependence on endogenous lipid processing events to produce drug supersaturation was indeed a significant cause for intra/inter-individual variability, the susceptibility to variability is likely different for different drug types, since different drug types may have different propensity for supersaturation to be induced by endogenous lipid processing events (one of the conclusions in Chapter 4 is that endogenously triggered supersaturation is more apparent for weak bases than neutral compounds). Therefore, for compounds where high variability in absorption may be expected, LBF may be more usefully directed to increase the bioavailability of drugs with broad therapeutic windows.

References


2. Nassir, F.; Wilson, B.; Han, X.; Gross, R. W.; Abumrad, N. A. CD36 is important for fatty acid and cholesterol uptake by the proximal but not distal intestine. Journal of Biological Chemistry 2007, 282, (27), 19493-19501.


I hope my response to the questions have been satisfactory, but please do not hesitate to contact me if I can provide further clarification.

Sincerely,

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Notice 1

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众里寻他千百度，蓦然回首，那人却在灯火阑珊处。

— 王国维《人间词话》“三种境界”
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ABSTRACT

This thesis seeks to elucidate the mechanism of drug absorption from the colloidal micellar and vesicular species that form in the gastrointestinal (GI) tract during lipid digestion. In addition to absorption from the free fraction of drug molecules that exist in equilibrium with drug solubilised in the colloidal reservoir, two alternative models of drug absorption were explored: (i) collisional drug absorption where lipid colloidal phases interact directly with the absorptive membrane, and (ii) supersaturation-enhanced absorption where transient changes to colloid structure and content in situ lead to drug supersaturation, thereby boosting drug thermodynamic activity and rendering solubilised drug more available for absorption via the free fraction. Collisional drug absorption was explored by comparing the intestinal absorptive flux of cinnarizine (CIN) from two distinctly different colloids (micelles vs. vesicles) that were matched for CIN solubilisation capacity and drug loading (and therefore thermodynamic activity). In these systems the number of micellar particles was substantially higher than that of vesicles, and as such collisional absorption of CIN was expected to be higher from micelles. The absorption of CIN from micelles and vesicles, however, was not statistically different, suggesting little collisional involvement in drug absorption. Receptor-mediated collisional absorption was examined by assessing CIN bioavailability from a lipid emulsion in the absence and presence of inhibitors of common lipid uptake transporters (e.g. SR-BI, CD36, NPC1L1). CIN bioavailability was unchanged by inhibitor co-administration. Collectively, the data suggest that collision-mediated uptake is not a significant driver for drug absorption from intestinal lipid colloidal phases, and that drug absorption occurs largely from the free fraction. Subsequently, attention turned to the possibility that drug supersaturation might be stimulated during endogenous processing of intestinal lipid colloidal phases. Two mechanisms were investigated: (i) interaction of lipid colloidal phases with bile secretions, where bile-induced changes to colloid microstructure may lead to reductions in drug solubilisation capacity and (ii) lipid absorption from intestinal colloids, where reductions in colloidal lipid content may reduce drug solubilisation capacity at the intestinal unstirred water layer (UWL). The addition of donor rat bile to CIN-loaded colloids (CIN was loaded at sub-
saturated concentrations) resulted in a decrease in CIN solubilisation and the generation of CIN supersaturation. Bile-induced supersaturation was subsequently shown to increase the intestinal absorptive flux and systemic exposure of CIN from both medium-chain and long-chain lipid containing colloids. To assess the potential for lipid absorption to induce drug supersaturation, the intestinal absorptive flux of CIN from oleic acid-containing colloids was assessed under conditions of normal lipid absorption vs. inhibited lipid absorption (oleic acid absorption was inhibited by co-administration of amiloride, an inhibitor of UWL acidity). When oleic acid absorption was suppressed, the absorption of CIN was dramatically attenuated. Assessment of CIN solubilisation behaviour under conditions that simulate lipid absorption at the UWL subsequently indicated that supersaturation was likely to be responsible for the enhanced CIN absorption observed during normal lipid absorption. In summary, supersaturation appears to be an important driving force for drug absorption from lipid-based intestinal colloids. Two novel mechanisms have been identified by which drug supersaturation may be naturally triggered in the small intestine (bile dilution and lipid absorption). The findings enhance mechanistic understanding of the effects of lipids in food or formulations on drug absorption and are expected to inform the development of more rational design criteria for LBF.
Monash University

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

GENERAL DECLARATION

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master’s regulations the following declarations are made:

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

This thesis includes one original paper published in peer reviewed journals and two submitted publications. The core theme of the thesis is an understanding of the mechanism of drug absorption from intestinal colloidal species following oral administration of lipids. 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 Prof Christopher J. H. Porter and Dr Natalie L. Trevaskis.

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

In the case of Chapter 3 my contribution to the work included the majority of the experimental work, all 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.

In the case of Chapters 4 and 5 my contribution to the work included all 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.

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

Signed: .................................................. Date: .........................................
ACKNOWLEDGEMENTS

I’d first like to thank my supervisors Prof Chris Porter and Dr Natalie Trevaskis for their guidance and support. They are outstanding scientists who still believe in the personal development of their students, for that I could not have asked for two better people to see me through my PhD. Thank you for consistently being there, for the times when you have shown great insight, care, enthusiasm and encouragement. Thank you also for having the foresight and courage to ask an excellent research question that led to the conception of this project – I have had a ball, and these years spent under your supervision will be some of the most profound and memorable experience in my life.

To my parents, I feel truly lucky to be your daughter. You are such resilient individuals, and have strived hard to give us a good life, and importantly, good and free minds. Thank you for always listening and entertaining my thoughts and ideas, even if I was and still am, a little dreamy. I think it has given me the confidence to just be myself. Thank you also for your unconditional love. I didn’t realise the importance of having such unwavering support behind me, but I know now that it’s the reason I do not think twice before undertaking a difficult endeavour. Thank you especially to my mum who, in retrospect, really made the effort to ensure that our upbringing was a well-considered one. To my siblings YXY, YYH (and Alicia and baby Xuan Xuan) and YYY, thank you for always looking out for me and showing an interest in what I do, I hope I have outgrown little sister status over the years.

To Hywel, in the past two years or so I have found it difficult in my mind to disentangle you from the wonderful turn in PhD journey I have had, maybe good things do come in pairs. You have been an absolute inspiration to me, and I am still astounded by the depth of support you continue to provide. Thank you for so many things, from making me dinner to keeping me warm on top of a mountain to showing me how best to import powerpoint figures to always being enthusiastic and insightful when discussing ideas and concepts. I look forward to our future together.

To Shalini and Sifei, how will I complete my PhD a sane person without you two? Shalini you have been a great PhD buddy. Throughout the years you have provided me with lots of laughs, endless conversation, and some serious emotional support through the personally challenging times. I will treasure the friendship and good times we’ve had. Sifei your wise words are worthy of compilation, and your convincing me that breakthrough is near at my lowest point is one of the most important things anyone has said to me. Thank you for always pointing me in the right direction. I am grateful to have known such kind-hearted individuals full of integrity as you two.
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This thesis is a compilation of the following manuscripts:

**Chapter 3:**
Yeap YY, Trevaskis NL, Quach T, Tso P, Charman WN, Porter CJH. Intestinal bile secretion promotes drug absorption from lipid colloidal phases via induction of supersaturation. *Molecular Pharmaceutics, 2013, in press.* (Proofs appended at the end of this thesis)

**Chapter 4:**
Yeap YY, Trevaskis NL, Porter CJH. The potential for drug supersaturation during intestinal processing of lipid-based formulations is enhanced for basic drugs. *Manuscript in submission.*

**Chapter 5:**
Yeap YY, Trevaskis NL, Porter CJH. Lipid absorption triggers drug supersaturation at the intestinal unstirred water layer and promotes drug absorption from mixed micelles. *Manuscript in submission.*
Yeap YY. Rethinking drug absorption from post-digestion lipid colloidal phases: A supersaturation-based model of absorption. Podium presentation, AAPS Annual Meeting and Exposition 2012, Chicago, IL, USA. (Award winning)

Yeap YY, Trevaskis NL and Porter CJH. The acidic microclimate of the intestinal unstirred water layer can promote drug absorption from long-chain mixed micelles via induction of supersaturation at the absorptive site. Poster presentation, AAPS Annual Meeting and Exposition 2012, Chicago, IL, USA.

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Yeap YY, Trevaskis NL and Porter CJH. The role of small intestine lipid uptake transporters in the oral absorption of lipids and poorly water soluble drugs. Poster presentation, APSA Annual Conference 2009, Hobart, Tasmania, Australia.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CV</td>
<td>% coefficient of variation</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BA</td>
<td>bioavailability</td>
</tr>
<tr>
<td>BBMV</td>
<td>brush border membrane vesicles</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistant protein</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceutics Classification System</td>
</tr>
<tr>
<td>BLT-1</td>
<td>Block Lipid Transport-1</td>
</tr>
<tr>
<td>BS</td>
<td>bile salts</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of Differentiation 36</td>
</tr>
<tr>
<td>Ch</td>
<td>cholesterol</td>
</tr>
<tr>
<td>CIN</td>
<td>cinnarizine</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micellar concentration</td>
</tr>
<tr>
<td>DAN</td>
<td>danazol</td>
</tr>
<tr>
<td>DG</td>
<td>diglyceride</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acids</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
<tr>
<td>FABP&lt;sub&gt;pm&lt;/sub&gt;</td>
<td>membrane-associated fatty acid binding protein</td>
</tr>
<tr>
<td>FAT</td>
<td>Fatty Acid Translocase</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport protein</td>
</tr>
<tr>
<td>FF</td>
<td>fenofibrate</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HF</td>
<td>halofantrine</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic-Lipophilic Balance</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LBF</td>
<td>lipid-based formulations</td>
</tr>
<tr>
<td>LC</td>
<td>long-chain</td>
</tr>
<tr>
<td>LCFA</td>
<td>long-chain fatty acids</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LCT</td>
<td>long-chain triglycerides</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LFCS</td>
<td>Lipid Formulation Classification System</td>
</tr>
<tr>
<td>Log D</td>
<td>logarithm of the octanol-water distribution coefficient</td>
</tr>
<tr>
<td>LPC</td>
<td>lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPL</td>
<td>lysophospholipids</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MC</td>
<td>medium-chain</td>
</tr>
<tr>
<td>MCT</td>
<td>medium-chain triglycerides</td>
</tr>
<tr>
<td>MFA</td>
<td>meclofenamic acid</td>
</tr>
<tr>
<td>MG</td>
<td>monoglyceride</td>
</tr>
</tbody>
</table>
µCi microCurie
µg microgram
µL microlitre
µm micrometre
µM micromolar
MRP multidrug resistance-associated protein
nm nanometre
ng nanogram
NMR nuclear magnetic resonance
NPC1L1 Niemann-Pick C1 Like 1
OA oleic acid
o/w oil in water
P_app apparent permeability coefficient
PC phosphatidylcholine
PEG polyethylene glycol
P-gp P-glycoprotein
PL phospholipids
PWSD poorly water-soluble drugs
rpm revolutions per minute
SAXS small-angle X-ray scattering
SDS sodium dodecyl sulphate
sec second
SEDDS self-emulsifying drug delivery systems
SEIF simulated endogenous intestinal fluid
SEM standard error of the mean
SI small intestine
SMEDDS self-microemulsifying drug delivery systems
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-BI</td>
<td>Scavenger Receptor Class B Type 1</td>
</tr>
<tr>
<td>S-SEDDS</td>
<td>supersaturable self-emulsifying drug delivery systems</td>
</tr>
<tr>
<td>SSO</td>
<td>sulfo-N-succinimidyl oleate</td>
</tr>
<tr>
<td>SS ratio</td>
<td>supersaturation ratio</td>
</tr>
<tr>
<td>TBME</td>
<td><em>tert</em>-butyl methyl ether</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TRL</td>
<td>triglyceride-rich lipoproteins</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UWL</td>
<td>unstirred water layer</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>v/v</td>
<td>volume in volume</td>
</tr>
<tr>
<td>w/o</td>
<td>water in oil</td>
</tr>
<tr>
<td>w/v</td>
<td>weight in volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight in weight</td>
</tr>
<tr>
<td>x g</td>
<td>relative centrifugal force</td>
</tr>
</tbody>
</table>
CHAPTER 1 : GENERAL INTRODUCTION
1.1 STATEMENT OF THE PROBLEM
Lipid-based formulations (LBF) are commonly employed to increase the oral bioavailability of poorly water-soluble drugs (PWSD). Confident and widespread application of LBF, however, is limited by the lack of a holistic understanding of the mechanisms by which lipids enhance the absorption of PWSD. Currently, the design and evaluation of LBF is largely predicated on the ability of formulations to maintain drug solubilisation during in vitro simulations of formulation dispersion and digestion in the gastrointestinal (GI) tract. This approach, while generally useful, is largely empirical and does not always adequately predict formulation performance in vivo. In contrast, considerably less attention has been directed towards an understanding of the mechanism(s) of drug absorption from the colloidal phases (e.g. micellar and vesicular species) that form in the small intestine (SI) following lipid ingestion. Improved understanding of the fundamental processes that govern drug absorption from intestinal lipid colloidal phases has the potential to provide enhanced design parameters for LBF, and is the main aim of this PhD thesis.

1.2 LIPIDS FOR THE ENHANCEMENT OF ORAL DRUG ABSORPTION
Growing application of high throughput activity screens and the use of complex chemical scaffolds in drug discovery has led to increasingly frequent identification of poorly water-soluble compounds as prospective drug candidates. Indeed, recent estimates suggest that at least 40% of new chemical entities in development are classified as ‘poorly water-soluble’\(^1\). While these compounds may possess high intrinsic potency at target sites, the development and clinical utility of poorly water-soluble drug candidates are often limited by low systemic exposure after oral administration. This reflects the need for drugs to be molecularly dispersed (i.e. in solution) in order to pass across the absorptive cells that line the GI tract, and for poorly water-soluble
compounds, this prerequisite is limited by slow dissolution rates and low solubility in the aqueous GI milieu.

Several approaches have been employed to improve the dissolution and solubilisation characteristics of poorly-water soluble compounds in the GI tract. These include the isolation of drug candidates in alternate salt forms, polymorphs or co-crystals with improved dissolution and solubility profiles; particle size reduction strategies such as milling or nanomilling; and formulation strategies such as the use of solid dispersions, cyclodextrins, and surfactant or lipid-based drug delivery systems. The common strategies to enhance the dissolution and solubility of poorly water-soluble compounds has recently been reviewed in detail by Williams et al. The focus of the studies in this thesis, however, is the use of LBF to enhance the absorption of PWSD.

1.2.1 Poorly water-soluble drugs and the Biopharmaceutics Classification System

The Biopharmaceutics Classification System (BCS) categorises drugs into four classes according to their water solubility and membrane permeability properties (Table 1.1), and allows for the broad prediction of the rate-limiting steps in drug absorption following oral administration.²

Table 1.1: The biopharmaceutical classification system (BCS) for drugs according to their solubility and permeability properties.

<table>
<thead>
<tr>
<th>Class</th>
<th>Solubility</th>
<th>Permeability</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>High</td>
<td>High</td>
<td>ketoprofen, propranolol, midazolam, paracetamol</td>
</tr>
<tr>
<td>II</td>
<td>Low</td>
<td>High</td>
<td>cinnarizine, danazol, phenytoin, griseofulvin</td>
</tr>
<tr>
<td>III</td>
<td>High</td>
<td>Low</td>
<td>metformin, digoxin, cefotaxamine, cimetidine</td>
</tr>
<tr>
<td>IV</td>
<td>Low</td>
<td>Low</td>
<td>cyclosporin A, lovastatin, amphotericin, paclitaxel</td>
</tr>
</tbody>
</table>
PWSD are contained within Class II and IV of the BCS. In general, the absorption of Class II drugs is limited by solubility alone, whereas the absorption of Class IV drugs is limited by both solubility and permeability. PWSD include examples that have been likened to ‘grease balls’ that are both hydrophobic (i.e. solubility is limited by hydration and interaction with aqueous solvents) and lipophilic (i.e. where lipid solubility is high and melting point is typically low); and compounds that have been likened to ‘brick dust’ that are hydrophobic but not lipophilic (i.e. where solubility is limited by strong intermolecular bonds within the crystal lattice thereby restricting the solubility of compounds in both water and lipids). ‘Grease ball’ like compounds (typically found in Class II) are well-suited to formulation in LBF since the higher lipophilicity of these compounds dictates that they have higher solubility in the formulation when compared to ‘brick dust’ like molecules. As new molecular entities become increasingly lipophilic and poorly water-soluble, LBF are becoming an increasingly effective and popular strategy to improve oral absorption. However, a thorough understanding of the mechanisms by which lipids exert their absorption-enhancing effects must first be obtained before LBF technologies can be confidently applied.

1.2.2 Lipid-based formulations used in oral drug delivery

‘Lipid-based formulation’ is an umbrella term that encompasses a diverse group of formulations including lipid solutions, suspensions, emulsions, microemulsions, nanoemulsions, lipid nanoparticles, self-emulsifying drug delivery systems (SEDDS), liquid crystalline materials such as lamellar, hexagonal, and cubic liquid crystals, and lipid complexes such as cochleates. LBF may be employed for oral or parenteral administration. In oral drug delivery, LBF comprising simple isotropic liquids that may be filled into gelatin capsules are the most widely used. LBF typically comprise a mixture of excipients including triglycerides, mixed mono- and diglycerides, non-ionic surfactants, and cosolvents. The choice of excipients is usually based on the need to
solubilise the drug dose in the formulation, the ability to promote rapid initial emulsification of the formulation in the GI fluids, the capacity to maintain drug solubilisation during dispersion and digestion of the formulation in the GI tract, and the potential to alter metabolic processing pathways such as the stimulation of lymphatic transport or the inhibition of enterocyte-based efflux transporters and metabolism. Some examples of LBF that have been shown to enhance the oral bioavailability of PWSD are listed in Table 1.2.
### Table 1.2: Examples of LBF used to enhance the oral bioavailability (BA) of PWSD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formulation</th>
<th>Study</th>
<th>Observation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnarizine</td>
<td>Oleic acid solution</td>
<td>Oral BA (beagle dogs)</td>
<td>4 fold increase in relative BA c.f. tablets</td>
<td>6</td>
</tr>
<tr>
<td>Penclomedine</td>
<td>Triocanoin, triolein, soybean oil, mineral oil and tributyrin o/w emulsion</td>
<td>Intraduodenal dosing (rats)</td>
<td>3-7 fold increase in absolute BA when dosed in digestible and non-digestible lipids c.f. aqueous suspension</td>
<td>7</td>
</tr>
<tr>
<td>Danazol</td>
<td>Monoolein o/w emulsion</td>
<td>Oral BA (humans)</td>
<td>4 fold increase in relative BA c.f. capsules</td>
<td>8</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Peanut oil, Captex 355 and triacetin oil suspensions</td>
<td>Oral BA (rats)</td>
<td>Griseofulvin: 2-3 fold increase in absolute BA c.f. aqueous suspension</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dexamethasone: 8-9 fold increase in absolute BA c.f. aqueous suspension</td>
<td></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Corn oil o/w emulsion</td>
<td>Oral BA (humans)</td>
<td>2 fold increase in relative BA c.f. aqueous suspension</td>
<td>10</td>
</tr>
<tr>
<td>Ontazolast</td>
<td>Soybean oil o/w emulsion</td>
<td>Oral BA (rats)</td>
<td>15 fold increase in absolute BA c.f. aqueous suspension</td>
<td>11</td>
</tr>
<tr>
<td>Halofantrine</td>
<td>Peanut oil, Captex 355 and tributyrin solutions</td>
<td>Oral BA (rats)</td>
<td>2-3 fold increase in absolute BA c.f. aqueous suspension</td>
<td>12</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>SEDDS (MCT)</td>
<td>Oral BA (beagle dogs)</td>
<td>4 fold increase in relative BA c.f. tablets</td>
<td>13</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>Sandimmune® (SEDDS), Sandimmune Neoral® (SMEDDS)</td>
<td>Oral BA (humans)</td>
<td>Up to 2.4 fold increase in relative BA from SMEDDS c.f. SEDDS</td>
<td>14</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>SMEDDS (PEG)</td>
<td>Oral BA (humans)</td>
<td>1.9 and 1.5 fold increase in BA in fasted and fed states, respectively</td>
<td>15</td>
</tr>
<tr>
<td>Silymarin</td>
<td>SMEDDS (ethyl linoleate)</td>
<td>Oral BA (rabbits)</td>
<td>49 fold increase in relative BA c.f. suspension</td>
<td>16</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>S-SEDDS</td>
<td>Oral BA (rats)</td>
<td>5-fold increase in relative BA c.f. intravenous solution (Taxol®)</td>
<td>17</td>
</tr>
</tbody>
</table>

o/w = oil in water; MCT = medium-chain triglycerides; LCT = long-chain triglycerides; SEDDS = self-emulsifying drug delivery systems; SMEDDS = self-microemulsifying drug delivery systems; S-SEDDS = supersaturable self-emulsifying drug delivery systems.
To assist in the description and comparison of oral LBF, Pouton proposed a classification scheme that originally grouped LBF into three categories\textsuperscript{18} with a fourth recently added\textsuperscript{19}. The Lipid Formulation Classification System (LFCS) allows formulations to be described according to their composition and general behaviour during \textit{in vitro} dispersion and digestion tests (Table 1.3).

\textbf{Table 1.3:} Typical composition of LFCS types I, II, IIIA, IIIB, and IV LBF and their solubilisation capacity for drug before and after dispersion and digestion in the GI tract.

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type II</th>
<th>Type IIIA</th>
<th>Type IIIB</th>
<th>Type IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobic composition</td>
<td>high</td>
<td></td>
<td>low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid: TG, DG, MG (% w/w)</td>
<td>100</td>
<td>40-80</td>
<td>40-80</td>
<td>&lt;20</td>
<td>-</td>
</tr>
<tr>
<td>Water-insoluble surfactants HLB &lt; 12 (% w/w)</td>
<td>-</td>
<td>20-60</td>
<td>-</td>
<td>-</td>
<td>0-20</td>
</tr>
<tr>
<td>Water-soluble surfactants HLB &gt; 12 (% w/w)</td>
<td>-</td>
<td>-</td>
<td>20-40</td>
<td>20-50</td>
<td>30-80</td>
</tr>
<tr>
<td>Hydrophilic cosolvents (% w/w)</td>
<td>-</td>
<td>-</td>
<td>0-40</td>
<td>20-50</td>
<td>0-50</td>
</tr>
<tr>
<td>Solubilisation capacity prior to dispersion</td>
<td>low</td>
<td></td>
<td>high</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle size on dispersion (nm)</td>
<td>coarse</td>
<td>250-2000</td>
<td>100-250</td>
<td>50-100</td>
<td>very fine</td>
</tr>
<tr>
<td>Loss of drug solubilisation capacity upon dispersion</td>
<td>limited</td>
<td></td>
<td>high</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Importance of digestion</td>
<td>crucial</td>
<td></td>
<td>not required</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of drug solubilisation capacity upon digestion</td>
<td>Possible</td>
<td>Possible</td>
<td>Possible</td>
<td>Possible</td>
<td>Possible</td>
</tr>
</tbody>
</table>

TG, DG, MG = triglycerides, diglycerides, monoglycerides; HLB = Hydrophilic-Lipophilic Balance

Type I formulations are comprised of a single lipid vehicle or a blend of lipids typically consisting of common plant oils or fractionated glycerides\textsuperscript{20}. Type I formulations disperse poorly upon contact with aqueous media, but typically maintain drug solubilisation during dilution by GI fluids.
Digestion processes are required to increase the amphiphilicity of formulation lipids, thereby improving dispersion properties and promoting the formation of micellar and vesicular species that are required to maintain PWSD solubilisation and facilitate transfer to the absorptive membrane (see Section 1.3.2 and Section 1.4.2). The requirement for digestion however, may not limit absorption as many studies suggest that formulations containing digestible lipids have superior performance \textit{in vivo} when compared to formulations that contain poorly or non-digestible lipids. For example, Yoshiya \textit{et al.} showed that the systemic exposure of the lipophilic drug SL-512 in rats was significantly higher after oral administration of a 2 mg/kg dose in MCT (readily digested) when compared to administration in N-\textit{a}-methylbenzyllinoleamide (poorly digested)\textsuperscript{21}. Myers \textit{et al.} also noted higher bioavailability of penclomедине when administered intraduodenally in soybean oil, triolein and trioctanoin, when compared to mineral oil\textsuperscript{7}. The major drawback of Type I formulations is their low solvent capacity for many drugs (except for drugs that possess very high lipophilicity).

Type II formulations differ from Type I formulations in that they have improved emulsification properties on dispersion due to the inclusion of lipophilic surfactants (HLB < 12). Type II LBF exhibit typical ‘self-emulsifying’ drug delivery system (SEDDS) behaviour, and self-emulsify on contact with aqueous media to form emulsions with particle sizes of 250-2000 nm. Compared to Type III and IV systems (discussed below), Type II LBF contain limited quantities of hydrophilic excipients and as such drug precipitation out of the formulation is unlikely during formulation dispersion (since most formulation components are water immiscible). The use of Type II LBF, however, has been largely superseded by Type III and IV formulations that have higher drug solubilisation capacities and result in smaller particle sizes on initial dispersion.
Chapter 1: General Introduction

Type III formulations include lipids, water-soluble surfactants (HLB > 12) and cosolvents. They form fine emulsions (particle size of 50-250 nm) on contact with aqueous media, and are often described as self-microemulsifying drug delivery systems (SMEDDS). Type III formulations are arbitrarily divided into IIIA and IIIB to distinguish between formulations that contain a significant proportion of oils (Type IIIA) and those that contain greater quantities of hydrophilic components (Type IIIB). Type III formulations are expected to have widely varying performance characteristics due to the diverse group of formulations that fall under this category. Many marketed LBF are Type III formulations.

Type IV formulations were most recently added to the LFCS\textsuperscript{19}. They contain no classical lipids, and are comprised predominantly of hydrophilic surfactants and cosolvents. The impact of digestion on the performance of Type IV systems is therefore expected to be less pronounced than those containing digestible lipids, although surfactant digestion is still possible in some cases. The advantage of Type IV systems lies in the high solvent capacity of the formulations, which allows for higher drug loading when compared to other types of LBF. However, since the formulations consist of mainly water-miscible excipients, drug precipitation is likely on dilution with GI fluids. Some toxicity concerns have also been raised regarding the chronic use of Type IV formulations containing high quantities of surfactant and cosolvent, however there are many examples of marketed products (e.g. the HIV protease inhibitors) where daily excipient ‘doses’ are high, but seemingly well tolerated.

The selection of excipients for LBF is currently guided primarily by the capacity for the excipients (or combinations of excipients) to solubilise the required drug dose, and to maintain drug solubilisation during \textit{in vitro} dispersion and digestion tests. Other factors that may influence excipient selection include mutual miscibility, toxicity, irritancy, capsule compatibility, purity,
chemical stability and cost. A summary of the common excipients used in LBF is listed in Table 1.4.

**Table 1.4:** Excipients commonly used in oral LBF. The table is adapted with permission from Williams *et al.*

<table>
<thead>
<tr>
<th>Excipient Class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium-chain triglycerides</td>
<td>Coconut or palm seed oil, Miglyol, Captex</td>
</tr>
<tr>
<td>Long-chain triglycerides</td>
<td>Soybean, sesame, safflower, sunflower, corn, cottonseed, olive, palm, peanut (arachis), rapeseed oils; hydrogenated vegetable and soybean oils</td>
</tr>
<tr>
<td>Medium- and/or long-chain monoglycerides, diglycerides, or mixed glycerides</td>
<td>Medium-chain: Capmul, Imwitor, Labrafac, Capmul GMO Long-chain: Maisine 35-1, Peceol, Geleol</td>
</tr>
<tr>
<td>Low HLB surfactants (HLB &lt; 8)</td>
<td>Lipophilic sorbitan fatty acid esters (e.g. Span 85, Span 80); esters of propylene glycol and fatty alcohols (Lauroglycol, Capryol)</td>
</tr>
<tr>
<td>Water-insoluble, lipophilic surfactants (HLB 8-12)</td>
<td>Predominantly oleate esters and include polysorbate 85 (Tween 85) and Tagat TO</td>
</tr>
<tr>
<td>Water-soluble surfactants (HLB &gt; 12)</td>
<td>Include products synthesized by reacting alcohols with ethylene oxide to produce alkyl ether ethoxylates (e.g., Brij, cetomacrogol); sorbitan esters with ethylene oxide to form sorbitan ester ethoxylates (e.g. Tween); ethylene oxide with castor oil to produce castor oil ethoxylates (e.g. Cremophor EL, RH40); vegetable oils with polyethylene glycol (PEG) to produce mixtures of monoglycerides, diglycerides, triglycerides and PEG esters of fatty acid (Labrasol, Labrafil, Gelucire)</td>
</tr>
<tr>
<td>Cosolvents</td>
<td>PEG 400, propylene glycol, propylene carbonate, ethanol</td>
</tr>
</tbody>
</table>

HLB = Hydrophilic-Lipophilic Balance
1.3 OVERVIEW OF LIPID DIGESTION AND ABSORPTION

Ingested dietary or formulation-derived lipids are subjected to a series of digestion and solubilisation processes in the GI tract. These facilitate presentation of poorly water-soluble lipids to the absorptive membrane of the SI at high concentration and in a molecularly dispersed, solubilised form. The main stages of lipid processing from ingestion to uptake into the systemic circulation are described below:

1.3.1 Lipid digestion in the oral cavity and stomach

The digestion of lipids is initiated in the oral cavity by lingual lipase, prior to the passage of food into the stomach via the oesophagus. The presence of lipids in the GI tract leads to the secretion of gastric lipase from gastric mucosa chief cells\textsuperscript{22, 23}. Gastric lipase is acid-resistant, and partially hydrolyses triglycerides (TG) to diglycerides (DG) and fatty acids (FA)\textsuperscript{24, 25} with specific preference for digestion at the sn-3 position on the glycerol backbone\textsuperscript{26, 27}. Gastric lipid digestion is thought to constitute a small portion of total lipid digestion. Indeed, FA concentrations (as an indicator of the extent of lipid digestion) are up to 7-fold higher in the duodenum than in the stomach of humans\textsuperscript{28}, and 600-fold higher in pigs\textsuperscript{29}. Nonetheless, the amphiphilic nature of lipid digestion products (DG, FA), coupled with the shear force exerted on lipid droplets by gastric propulsion, grinding and retropulsion, aid the emulsification of lipids in the stomach and increases the surface area available for digestion. As a result, lipids enter the duodenum via the pyloric sphincter as crude emulsion droplets with diameters less than 0.5 µm\textsuperscript{30}. The rate of gastric emptying is thought to be regulated (slowed) by a feedback mechanism stimulated by the presence of FA in the lower SI\textsuperscript{31, 32}, which further facilitates efficient lipid digestion in the duodenum.
1.3.2 Lipid digestion and solubilisation in the small intestine

The presence of lipids in the duodenum stimulates the secretion of bile from the gall bladder and pancreatic fluid from the pancreas\textsuperscript{33, 34}. Bile contains bile salts (BS), phospholipid (PL), cholesterol (Ch), bile pigments, organic wastes, and bicarbonate ions whereas pancreatic fluid contains enzymes that catalyse the hydrolysis of lipids (lipase and co-lipase), proteins and peptides (trypsin, chymotrypsin and carboxypeptidase) and carbohydrates (amylases). pH change from the stomach environment to the small intestinal environment leads to ionisation of FA, which imparts further amphiphilicity and results in improved emulsification of lipid droplets\textsuperscript{35}.

Pancreatic lipase acts on the oil/water interface of lipid droplets to hydrolyse TG and DG to 2-monoglyceride (2-MG) and FA as the final digestion products while amphiphilic bile constituents such as BS and PL further aid the emulsification process to produce smaller lipid droplets, increasing the effective surface area for lipid digestion. Co-lipase is required to anchor lipase to the oil/water interface of oil droplets in the presence of bile salts\textsuperscript{36}. In addition to the digestion of TG and DG, PL (predominantly phosphatidylcholine (PC)) is hydrolysed to lysophospholipids (LPL) (predominantly lysophosphatidylcholine (LPC)) and FA by pancreatic phospholipase A\textsubscript{2}\textsuperscript{37}; and cholesterol esters (which comprises 10-15% of dietary cholesterol) are hydrolysed to free cholesterol by pancreatic cholesterol esterase\textsuperscript{38}.

Although the aqueous solubility of lipid digestion products is inherently low, the apparent solubility of these molecules in the aqueous environment of the SI may be increased many fold via solubilisation within bile-derived micellar species. In effect, the amphiphilic nature of the biliary lipids (BS, PL, LPL) and the lipid digestion products (MG, FA) enable self-association of endogenous and exogenous sources of lipids to form a series of colloidal structures that maintain lipid digestion products in a solubilised state.
Digesting lipids in the SI display complex phase behaviour depending on the types and quantities of lipid digestion products and biliary components present. Several studies have examined the changes in phase behaviour that occur as lipids are diluted and digested in intestinal fluids and provide insights into the expected phase changes that occur in vivo as lipid digestion products are formed on the surface of a lipid droplet and are progressively diluted (i.e. solubilised) by intestinal fluid. The physical changes to dietary lipids that occur during lipid processing in the SI are summarised in Figure 1.1.
Figure 1.1: Schematic diagram showing the physical changes to dietary lipids during lipid processing in the SI. Crudely emulsified oil droplets enter the duodenum from the stomach and stimulate the secretion of biliary and pancreatic fluids. BS, PL and Ch further emulsify the lipid droplets and increase the surface area available for digestion. Pancreatic lipase and co-lipase catalyse the hydrolysis of one TG molecule to 2 FA molecules and 1 MG molecule. Lipid digestion products accumulate on the surface of digesting oil droplets, where they become increasingly hydrated and eventually slough off as liquid crystalline lamellar, cubic or hexagonal phases. In the presence of raised BS, PL, Ch concentrations in the SI lumen, lipid digestion products are incorporated into bile micelles to form a solubilised system often referred to as the intestinal mixed micellar phase. Continued dispersion of lipid colloidal phases by secreted bile results in the generation of progressively smaller species. Under conditions of low lipid dispersion (i.e. high lipid, low bile), larger multilamellar vesicles are thought to predominate; whereas smaller unilamellar vesicles and mixed micelles co-exist under conditions of high lipid dispersion (i.e. low lipid, high bile). Absorption of MG and FA is thought to occur from mixed micelles and unilamellar vesicles, species with smaller particle sizes (< 500 nm) that are capable of permeating the intestinal unstirred water layer and accessing the absorptive membrane.
During lipid digestion, more polar (but still poorly-water soluble) digestion products accumulate at the surface of digesting oil droplets, where they are thought to ultimately swell (due to hydration) and slough off as liquid crystalline phases ranging from lamellar to cubic and hexagonal phases\textsuperscript{39-41}. The presence of lamellar, inverse hexagonal and micellar/bicontinuous cubic phases during lipid digestion has recently been confirmed by the use of a coupled \textit{in vitro} lipid digestion-synchrotron SAXS (small-angle X-ray scattering) model to study real time evolution of liquid crystalline structures\textsuperscript{40-42}. Currently, there is increasing research effort to understand the impact that these structures have on drug solubilisation and absorption, and the potential for the provision of modified release characteristics\textsuperscript{43, 44}. In the SI lumen, the liquid crystalline phases are diluted by (or solubilised by) intestinal fluids and biliary secretions, leading to the generation of smaller and less lipid-rich colloidal vesicular and micellar phases. Under conditions of low dilution, that is, when the concentration ratio of biliary components:lipid digestion products is low, larger multilamellar vesicles are thought to predominate (an exception to this is seen in the presence of lipids with lower hydrocarbon chain lengths (C\textsubscript{8}) where micelles are the only species observed, presumably due to the ability of C\textsubscript{8} FA to self-aggregate and form micelles at high concentration\textsuperscript{39}). Further dilution of the phases (i.e. increasing the concentration ratio of biliary components:lipid digestion products) leads to colloidal properties that reflect co-existence of smaller mixed micelles and unilamellar vesicles\textsuperscript{39}. Importantly, these colloids (i.e. mixed micelles and unilamellar vesicles) that persist under conditions of high dilution likely represent the phases that are responsible for the presentation of lipid digestion products to the absorptive membrane. Despite the co-existence of vesicular structures with mixed micelles in the aqueous phase of the SI lumen during lipid digestion, the solubilised phase is frequently referred to as the ‘mixed micellar phase’. In reality the intestinal fluids may consist of a range of highly dispersed lipid colloidal species. The properties of different types of intestinal colloidal aggregates are summarised in Table 1.5.
Solubilisation of lipid digestion products in small colloidal species in the mixed micellar phase is also important in the promotion of lipid transport across the intestinal unstirred water layer (UWL). The UWL is an aqueous diffusion barrier for poorly water-soluble compounds that separates bulk intestinal fluid from the absorptive surface of enterocytes. The UWL is estimated to be 500-800 µm wide\cite{45, 46}, has a lower (acidic) pH compared with the bulk intestinal fluid, and is indistinguishable from a viscous mucus layer consisting of water (~ 95%), glycoproteins, lipids, mineral salts and free proteins\cite{45, 47, 48}. Since nano-scale particles are able to permeate intestinal mucus layers\cite{49, 50}, small colloidal phases that persist under conditions of high dilution (such as mixed micelles and unilamellar vesicles) are expected to diffuse more readily across the UWL. Although micellar and vesicular species are larger and slower to diffuse when compared to lipid monomers, colloidal solubilisation enhances the net diffusion rate of lipids across the UWL, due to their ability to shuttle more lipid molecules across the UWL in a given time, when compared to the diffusion of lipid molecules as monomers\cite{33, 51}. 


Table 1.5: Properties of lipid colloidal phases found in the SI during lipid digestion.

<table>
<thead>
<tr>
<th>Colloidal species</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple micelles</td>
<td>Simple micelles are small aggregates of 2-4 bile salt molecules that form spontaneously at concentrations above the critical micellar concentration of bile salts(^{52}). The bile salt molecules orientate in such a way that the aromatic hydrocarbon moieties form the core of the micelle, with the polar head groups facing the aqueous environment. Lipid digestion products are incorporated into the hydrophobic core of micelles, thus are ‘solubilised’ in the aqueous SI environment.</td>
</tr>
<tr>
<td>Mixed micelles</td>
<td>Mixed micelles are micelles that are composed of more than one lipidic species. Simple micelles containing solubilised lipids are mixed micelles by definition. The hydrodynamic radii of mixed micelles are (\sim 3–10 \text{ nm})(^{52}). Some properties of simple micelles may change upon solubilisation of lipids. For example, bile salt-lecithin and bile salt-monoglyceride micelles are swollen (larger) and have greater solubilisation capacity for cholesterol and fatty acids respectively, when compared to simple bile salt micelles(^{52}).</td>
</tr>
<tr>
<td>Vesicles</td>
<td>Vesicles are composed of a single lipid bilayer (unilamellar), or alternating lipid bilayers and layers of water (multilamellar). Simple vesicles are typically formed by phospholipids (phospholipids are capable of forming lamellar liquid crystals in water); while the inclusion of cholesterol in vesicles has been suggested to stabilise the bilayer(^{53}). Phospholipids and cholesterol in bile are thought to be secreted into the duodenum partly as unilamellar vesicles(^{54}). The hydrocarbon chains of the lipid bilayer form the hydrophobic sites in which hydrophobic regions of lipid digestion products can be solubilised in, while the hydrophilic polar groups of the lipid bilayer orientate towards the aqueous layers of water.</td>
</tr>
<tr>
<td>Liquid crystal phases</td>
<td>PL, FA and MG are insoluble swelling amphiphiles that may form liquid crystal phases when dispersed in aqueous media(^{52}). Liquid crystals exhibit both the long range order of crystalline materials and the disorder of liquid systems, and may include lamellar, cubic and hexagonal phases. The relationship between lipid structure and liquid crystal phase formation is defined by the critical packing parameter (p), which is calculated from the effective hydrocarbon volume (v), the fully extended hydrocarbon chain length (l) and the area of the hydrophilic headgroup (a), where (p = v / al)(^\text{55}). Factors that may affect the packing parameter include hydration, pH and temperature. For example, as water is added to anhydrous amphiphilic lipid (thus decreasing the packing parameter), structural changes from inverse cubic and hexagonal phases (w/o systems, (v / al &gt; 1)) to lamellar phase (v / al = 1) to normal cubic and hexagonal phases (o/w systems, (v / al &lt; 1)) may be observed(^\text{55}). During lipid digestion, FA and MG are thought to accumulate at the surface of digesting oil droplets, where they are increasingly hydrated and ultimately sloughed off as liquid crystal phases.</td>
</tr>
</tbody>
</table>

Two types of vesicle may be found in the SI during lipid digestion: larger multilamellar vesicles (hydrodynamic radii \(\sim 50–250 \text{ nm}\)\(^\text{54}\)) under conditions of low bile salt and smaller unilamellar vesicles (hydrodynamic radii \(\sim 40–60 \text{ nm}\)\(^\text{52}\)) at higher bile salt concentrations. Vesicles are typically able to solubilise more lipid molecules per particle when compared to micelles due to their larger size.
1.3.3 Lipid absorption across the enterocyte apical membrane

Realising that micellar structures act as carriers of lipids across the UWL, Westergaard and Dietschy suggested a model of absorption where micelle-solubilised lipids served as a reservoir to replenish the passively absorbed free fraction via an equilibrium relationship. Later, Shiau and colleagues postulated that absorption of FA (specifically LCFA) was further facilitated by the acidic microclimate of the intestinal UWL. They suggested that exposure of LCFA to pH values below their pKₐ promoted LCFA protonation and led to an attenuation in LCFA amphiphilicity. This in turn was suggested to enhance LCFA absorption via (i) enhanced LCFA partitioning across the lipophilic absorptive membrane, and (ii) enhanced LCFA thermodynamic activity (due to reduced LCFA solubility in bile micelles). In both cases, however, uptake of FA across enterocyte brush border membrane was thought to occur via passive diffusion.

In contrast, in a seminal paper by Chow and Hollander, linoleate (a LCFA) uptake across the absorptive membrane was shown to be concentration dependent, and facilitated diffusion was suggested to be the main mechanism of absorption at low lipid concentrations, whereas simple passive diffusion was thought to dominate at high concentrations. In subsequent years, findings from several authors have supported the concept of dual, concentration-dependent mechanism of absorption for LCFA, and efforts have been made to identify the plasma membrane lipid transporters responsible for facilitated LCFA absorption. Transporters that have been identified include CD36, FATP4, SR-BI, and FABPₚₘ, although conclusive evidence to support the role of these receptors in the intestinal absorption of LCFA remains elusive. A number of transporters including CD36, SR-BI, and NPC1L1 have also been implicated in cholesterol uptake across intestinal brush border membrane. The mechanisms of lipid absorption into enterocytes are summarised in Figure 1.2.
1.3.4 Lipid transport into the systemic circulation

The GI tract is supplied by an extensive network of blood and lymphatic vessels. Absorbed FA and MG may be transported to the systemic circulation via either the portal blood, or the intestinal lymph (Figure 1.2 (iv)). Lipids that are destined for transport into the portal vein diffuse directly across enterocytes and gain access to the blood capillaries via the lamina propria. Lipids that are destined for transport into the lymphatic vessels are trafficked to the endoplasmic reticulum where they are re-synthesised to TG via either the 2-monoglyceride pathway (which predominates in the fed state)\textsuperscript{69, 70} or glycerol-3-phosphate pathway (which predominates in the fasted state)\textsuperscript{71, 72}. Re-synthesised TG, together with cholesterol esters, constitutes the primary core lipids of intestinal lipoproteins. Intestinal lipoproteins are large colloidal particles with a hydrophobic core and a hydrophilic surface (primarily consisting phospholipid, free cholesterol and apolipoproteins). In the fasted state, very-low-density lipoproteins (VLDL) are preferentially produced by the enterocytes whereas after the ingestion of lipids, both VLDL and chylomicrons (larger but less dense lipoproteins) are produced. Intestinal lipoproteins that are assembled in the endoplasmic reticulum of enterocytes are subsequently exocytosed into the lamina propria where they preferentially access the lymphatic vessels rather than the blood capillaries. Preferential lymphatic access occurs because the diffusion barrier of the vascular endothelium to large lipoprotein is significant (resulting from the presence of tight junctions between adjacent endothelial cells, and an underlying basement membrane), whereas the lymphatic endothelia barrier is considerably more permeable (due to the presence of wider intercellular spaces between lymphatic endothelial cells (Figure 1.2 (iv))). In general, FA and MG with hydrocarbon chain lengths of 14 and above are primarily transported into the intestinal lymph whereas the more water-soluble short-chain and medium-chain FA and MG are primarily transported by the portal blood\textsuperscript{73-76}. Absorbed LPC may be re-synthesised to PC\textsuperscript{77-79} (and incorporated into the hydrophilic surface of lipoproteins) or hydrolysed to glycerol-3-phosphorylcholine that is transported via the portal blood\textsuperscript{80, 81}. In the fed
state, absorbed cholesterol is esterified to cholesterol esters\(^{82-84}\), prior to incorporation into the hydrophobic core of lipoproteins.

**Figure 1.2:** Schematic diagram outlining the mechanisms of lipid absorption into enterocytes (i, ii, iii) and lipid transport into the systemic circulation (iv). (i) Solubilisation of lipid digestion products within mixed micelles or unilamellar vesicles enhances the net diffusion rate of lipids across the UWL and maximises the concentration of lipids adjacent to the absorptive membrane. Lipid absorption occurs via the free fraction, which is in equilibrium with the solubilised fraction. (ii) Diffusion of colloids across the acidic UWL leads to protonation of LCFA (depicted as a loss of head groups on lipid molecules), which reduces LCFA solubility in bile micelles (thus swelling micelles), and enhances LCFA absorption via enhanced partitioning across the absorptive membrane and enhanced thermodynamic activity. (iii) LCFA absorption at low concentration is thought to be mediated by a saturable, facilitated process. (iv) Absorbed lipids are transported to the systemic circulation either via the portal blood, or the intestinal lymph. The intestinal lymph drains via the thoracic lymph directly into the systemic circulation at the junction of the left subclavian vein and left jugular vein, therefore avoiding first-pass through the liver.
1.4 MECHANISMS BY WHICH LIPIDS ENHANCE THE ORAL BIOAVAILABILITY OF DRUGS

In the absence of carrier-mediated uptake, flux per unit area (F) across the intestinal absorptive membrane can be represented by the following equation:

\[
F = P \times C
\]  

Equation 1.1

where P is the permeability coefficient of drug across the apical membrane; and C is the concentration of drug on the abluminal side of the apical membrane.

For solubilised drug, and assuming that drug absorption is driven only by the free concentration in the GI lumen, the maximum flux (per unit area) that can be obtained across the apical membrane is a function of the permeability coefficient and the maximum free concentration. In the absence of supersaturation, the maximum free concentration that can be obtained in equilibrium with a solubilised reservoir is the equilibrium solubility (C_s) of a drug in the inter-micellar phase of the SI content (a value that approximates the solubility of drug in water buffered to intestinal pH). The rate of drug absorption is therefore limited by permeability across the absorptive membrane and/or its solubility in the SI lumen. Furthermore, although drug solubilisation increases the total mass of drug in solution, the free drug concentration is not expected to be raised above that of drug in simple solution. Based on Equation 1.1, therefore, drug solubilisation would not be expected to increase drug absorption. In contrast, a large number of studies detail the utility of solubilising formulations in enhancing the oral bioavailability of PWSD. This apparent anomaly may be explained by the impact of drug solubilisation on dissolution (as described in Section 1.4.1 below) and the potential for formulations to promote supersaturation, where the free concentration can be
raised above the equilibrium aqueous solubility (see Section 1.5.3). The latter is the major topic of this thesis.

After oral administration, the dissolution rate of a drug in the GI fluids (i.e. the rate at which drug passes into solution in the GI tract) may also be an important determinant of drug absorption. Since transit time along the GI tract is finite, the rate of drug dissolution must be sufficiently rapid to allow drug to pass into solution, and be available for absorption prior to transit past the absorptive site. The dissolution rate of a drug is related to its solubility according to the Noyes-Whitney equation:

$$\frac{dc}{dt} = \frac{D A}{h} (C_s - C)$$

where $\frac{dc}{dt}$ is the dissolution rate of the drug; $D$ is the diffusion rate of the drug in water; $A$ is the surface area of contact of the solid with the dissolution fluid; $h$ is the width of the diffusion layer; $C_s$ is maximum drug concentration at the surface of the dissolving fluid i.e. drug solubility; and $C$ is the concentration of drug in the well-stirred bulk.

The low aqueous solubility of PWSD (i.e. low $C_s$) dictates that the dissolution rate is also low and that the absorption of many PWSD is limited by low solubility and/or slow dissolution in the GI fluids. In general, co-administration with lipids provides PWSD with the opportunity to intercalate into endogenous lipid digestion and absorption pathways (stimulated by the ingestion of lipids), ultimately leading to enhancements in drug dissolution, solubilisation, absorption and systemic exposure as described below.
1.4.1 Enhancement of drug dissolution in gastrointestinal fluids

Dosing PWSD in LBF offers significant dissolution advantages when compared to a conventional solid dose form. LBF typically comprise lipid solutions or emulsion-preconcentrates that are filled into soft or hard gelatin capsules prior to administration. In this cases, drug is pre-solubilised within the formulation, thus obviating the need for traditional solid-liquid drug dissolution. Ideally, as these lipidic formulations are dispersed, digested and solubilised in the intestinal content, drug remains solubilised in a monomolecular form and in equilibrium with drug free in solution. In effect therefore, dissolution is replaced with a (rapid) partitioning process between solubilised drug and drug in free solution. Bearing in mind the intrinsically slow rate of dissolution of PWSD from solid dose forms, drug solubilisation in LBF therefore provides significant dissolution rate advantages.

However, drug precipitation frequently occurs during formulation dispersion and digestion. Slow drug re-dissolution from the precipitated solids into GI fluids may therefore limit drug absorption even though the dose is initially solubilised in the formulation. Nonetheless, the co-administration of lipids may also enhance the dissolution rate of solid drug in the SI via improvements in wetting, and enhancements in drug solubilisation capacity. Specifically, raised concentrations of amphiphilic biliary lipids (BS, PL) in the SI after lipid ingestion reduces the contact angle between solid drug and intestinal fluid, thereby increasing the surface area available for dissolution. This mechanism has been documented to increase the dissolution rate of phenylbutazone and hydrocortisone. Solubilisation of drugs within fed state intestinal mixed micelles (which may consist BS, PL, FA, MG) also significantly increases the apparent solubility of many PWSD in intestinal fluids when compared to the fasted state, thereby increasing dissolution via increases in solubility according to the Noyes-Whitney equation (Equation 1.2). Increases in dissolution afforded by enhanced solubilisation however, may be off-set by the larger sizes of
micellar species which have slower diffusion rates (hence lower D value in Equation 1.2), which explains the less-than-proportional increase in dissolution rate (in relation to solubility) observed in many studies\textsuperscript{93, 94}.

**1.4.2 Enhancement of apparent drug solubility in intestinal fluids**

After the ingestion of lipids, biliary secretions combine with lipid digestion products to generate a series of intestinal lipid colloidal phases including emulsion droplets, vesicles and mixed micelles that have markedly increased solubilisation capacities for PWSD. Although increases in the concentrations of BS, PL, Ch in the fed state GI lumen alone results in significantly increased solubilisation capacity of a wide range of PWSD\textsuperscript{95-98}, incorporation of digested lipids into bile salt micelles has been shown to afford further enhancements in drug solubilisation capacity\textsuperscript{39, 91}, presumably due to the ability of lipid digestion products to swell endogenous bile micelles and to generate a range of lipid colloidal phases with increased solubilisation capacity (discussed in Section 1.3.2). The solubilisation enhancement afforded by the incorporation of exogenous lipids is dependent on the nature of the digestion products and the structures of the colloidal species formed. For example, medium-chain FA and MG are more amphiphilic than long-chain FA and MG and more readily combine with endogenous BS, PL and Ch. Thus, at the same lipid load, medium-chain lipids typically form smaller, more optically clear micellar dispersions while long-chain lipids form slightly turbid systems containing both vesicular and micellar species. Drug solubilisation enhancement afforded by the two systems at similar lipid loads are therefore significantly different, with a less than 3-fold enhancement (relative to drug solubility in endogenous BS, PL, Ch species) observed in the case of medium-chain lipids and up to 20-fold enhancement observed in the case of long-chain lipids\textsuperscript{39, 99}. Thus, the mass of drug that may be solubilised in fed state GI fluids is markedly higher than that in the absence of lipid co-administration.
Analogous to the mechanism by which micellar solubilisation increases FA transport across the intestinal UWL (outlined in Section 1.3.2), micellar solubilisation may also increase drug transport across the UWL, thereby increasing the mass of solubilised drug that is presented to the absorptive membrane. This concept has been applied to good effect in mathematical models simulating the absorption of progesterone\textsuperscript{100} and danazol\textsuperscript{101} from bile micelles. Thus, current understanding suggests that drug absorption across the apical membrane of enterocytes occurs via the free drug fraction that is in equilibrium with the solubilised fraction; and that upon drug absorption from the free fraction, drug rapidly partitions out of the solubilised reservoir to replenish the free concentration and maintain solubilisation equilibrium\textsuperscript{2}. In turn, the presence of drug-rich micelles at the absorptive membrane is likely maintained by the continuous diffusion of micelles from the lumen where lipid digestion, dispersion and drug solubilisation is on-going. Therefore, although the concentration of drug in the free fraction (the fraction available for absorption) is not raised by micellar solubilisation (the maximum attainable inter-micellar free concentration remains at the solubility limit of drug in water), micellar solubilisation overcomes the solubility limitations of PWSD on the abluminal side of the apical membrane via the provision of a solubilised reservoir which replenishes free drug concentration during on-going absorption.

### 1.4.3 Enhancement of intestinal permeability and inhibition of enterocyte-based efflux transporters and metabolism

Many LBF components (pre- or post-digestion) have been suggested to enhance passive paracellular (i.e. between cells) and transcellular (i.e. across cells) membrane diffusion\textsuperscript{102, 103}. Excipients that enhance transcellular permeability are of relevance for PWSD, as PWSD are likely to be absorbed via the transcellular route rather than the paracellular route due to their hydrophobicity. Medium-chain FA and glycerides\textsuperscript{104, 105}, long-chain FA and MG\textsuperscript{103}, and a variety of surfactants\textsuperscript{106, 107} have been shown to increase the transcellular permeability of the intestinal
epithelial membrane in vitro as well as in Caco-2 cells. The ability of excipients to increase membrane permeability has been attributed to their amphiphilic/surfactant properties, which disrupt the organisation of the lipid bilayer. Generally, surfactants that have large, charged or hydrophilic head groups and a relatively small hydrophobic tail are most likely to increase transcellular permeability as they are taken up rapidly by the outer membrane leaflet but are slow to translocate to the inner membrane leaflet\(^2\). The increased residence time of surfactant molecules between the bilayers, coupled with the inability of the hydrophobic tail to fill the void between adjacent membrane lipids, leads to monolayer/bilayer curvature strain, and the generation of a more permeable membrane. In extreme cases, membrane solubilisation is possible where the curvature strain is sufficiently high and membrane lipids are expelled. Bile salts are strong membrane solubilisers\(^{103}\), however, the presence of phospholipid in endogenous bile is expected to reduce the permeability enhancement effects of bile salts by decreasing bile salt thermodynamic activity in mixed bile salt-phospholipid micelles\(^{108}\). Although many drugs that are formulated in LBF have high membrane permeability (e.g. typical BCS Class II compounds), the permeability-enhancing effects of lipid excipients may be of relevance to BCS Class IV drugs where absorption is likely limited by both poor solubility and membrane permeability.

An increasing number of LBF excipients have recently been described to enhance drug permeability via inhibition of intestinal efflux transporters such as P-glycoprotein (P-gp), breast cancer resistant protein (BCRP) and multidrug resistance-associated protein (MRP)\(^2, 109-112\). Intestinal efflux transporters are present on the apical membrane of enterocytes, and actively pump absorbed drugs back to the intestinal lumen, thus limiting the absorption of their substrates. Well-known substrates of efflux transporters include digoxin\(^{113}\), doxorubicin\(^{114}\), vincristine\(^{114}\), cyclosporine A\(^{115}\), verapamil\(^{116}\) and the HIV protease inhibitors\(^{117}\). Examples of common LBF excipients that inhibit P-gp, BCRP and MRP have been compiled by Williams et al.\(^2\). The
mechanisms of efflux inhibition are thought to include competitive or allosteric inhibition of drug binding sites, changes to membrane fluidity leading to protein destabilisation, and reductions in transporter expression.

The CYP3A family of metabolic enzymes (also present in enterocytes) share similar substrate specificity to P-gp, and have been suggested to be functionally linked with P-gp to limit the systemic exposure of their substrates. Although the mechanism of interaction between P-gp and CYP3A is not well-understood, it is postulated to be due to the function of the efflux transporter in increasing substrate exposure to the metabolising enzyme in a given time (via repeated cycles of drug efflux and reabsorption which may also modulate the degree of enzyme saturation), or increased affinity of CYP3A-generated metabolites for P-gp when compared to the parent compound. Thus, inhibition of intestinal efflux might also be expected to reduce enterocyte-based first-pass metabolism, and increase the oral bioavailability of drugs. Dietary lipids and LBF components may also affect pre-systemic metabolism via direct effects on enzyme activity and indirect effects on enzyme expression levels. Examples of lipids or solubilising excipients that increase drug exposure by altering pre-systemic metabolism may be found in reviews by Patel et al. and Buggins et al., respectively. Systemic metabolism may be altered by lipid co-administration via the alteration of systemic lipoprotein levels (therefore changing the extent of drug binding in the blood which may decrease or increase uptake into metabolising compartments), although this mechanism is less likely for formulation lipids due to the small lipid doses contained.

Although the use of LBF excipients as permeability enhancers and metabolic and efflux inhibitors is receiving increasing interest, relatively few studies, to this point, have shown significant in vivo
effects for transcellularly transported drugs. As such the major effects on bioavailability enhancement appear to be driven by differences in solubility and dissolution.

### 1.4.4 Promotion of lymphatic drug transport

As described in Section 1.3.4, ingestion of lipids promotes the assembly of triglyceride-rich lipoproteins (TRL) in the enterocyte and these are transported from the enterocyte to the systemic circulation via the intestinal lymph. Drug transport into the systemic circulation may occur via either the portal blood, or the intestinal lymph. Since the blood flow of the portal vein is approximately 500-fold higher than the lymph flow in the mesenteric lymph duct\(^{137}\), the majority of absorbed drugs are transported to the systemic circulation via the portal blood\(^{76}\). However, for highly lipophilic (log P > 5) and highly lipid soluble (LCT solubility > 50 mg/g) drugs, the potential for lymphatic transport is increased due to the increased likelihood of drug association with developing TRL in the enterocyte\(^{138}\). Since intestinal lymph drains directly into the systemic circulation (by-passing the liver), co-administration with lipids may increase the bioavailability of lymphatically transported drugs via the avoidance of first-pass metabolism in the liver.

Enhancements in lymphatic drug transport have been observed for many drugs after co-administration with dietary or formulation lipids\(^{137}\). Significantly, studies with halofantrine have shown that even small amounts of long-chain lipid (e.g. quantities available within a single capsule) are capable of supporting substantial lymphatic drug transport in the fasted state. In this way, LBF have the potential to increase oral drug bioavailability for compounds that are highly first-pass metabolised and that are lymphatically transported\(^{139}\). In general, lymphatic drug transport increases with administered lipid load\(^{138-140}\), and is more effectively promoted by long-chain and monounsaturated glycerides than their medium-chain/short-chain and saturated counterparts\(^{12, 138, 139}\).
1.5 PROPOSED MECHANISMS OF DRUG ABSORPTION FROM INTESTINAL MIXED MICELLES AND VESICLES

1.5.1 Current model of drug absorption and its limitations

In contrast to the wealth of data describing lipid absorption from intestinal mixed micelles (discussed in Section 1.3.3), the mechanism of absorption of PWSD from mixed micelles remains poorly understood. Currently, the absorption of PWSD is believed to occur via passive diffusion of drug monomers across the enterocyte apical membrane (see Figure 1.3(A), solid black arrow), and drug in the solubilised fraction serves as a reservoir to replenish absorbed drug via re-establishment of the solubilisation equilibrium:

\[
C_{\text{colloid}} \leftrightarrow C_{\text{free}}
\]

where \(C_{\text{free}}\) and \(C_{\text{colloid}}\) represent the free concentration and the concentration solubilised in micelles, respectively. The total solubilised drug concentration \(C_{\text{total}}\) is the sum of \(C_{\text{free}}\) and \(C_{\text{colloid}}\):

\[
C_{\text{total}} = C_{\text{free}} + C_{\text{colloid}}
\]  
\[\text{Equation 1.3}\]

In addition to passive diffusion, some drugs may be actively absorbed by specific transporters present on the apical membrane (see Figure 1.3 (A), dotted black arrow), usually as a result of structural similarities of the drug to endogenous substrates of the transporter\(^{141, 142}\). Some examples of active transporters in the intestine and their drug substrates are listed in Table 1.6.
Table 1.6: Examples of active transporters in the intestine with specific reference to drug substrates\textsuperscript{141}.

<table>
<thead>
<tr>
<th>Transporter Family and Example</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>amino acid – large amino acid transporter</td>
<td>L-dopa, baclofen, gabapentin</td>
</tr>
<tr>
<td>oligopeptide – PepT1</td>
<td>lisinopril, cefadroxil, bestatin, cephradine</td>
</tr>
<tr>
<td>Na\textsuperscript{+} dependent phosphate transporter</td>
<td>foscarnet, fosfomycin</td>
</tr>
<tr>
<td>monocarboxylic acid – MCT1</td>
<td>pravastatin, salicylic acid, carindacillin</td>
</tr>
<tr>
<td>reduced folate transporter (RFT)</td>
<td>methotrexate</td>
</tr>
<tr>
<td>monosaccharide transporters – SGLT1</td>
<td>glucose, p-nitrophenyl-\textbeta-D-glucopyranoside</td>
</tr>
<tr>
<td>apical Na\textsuperscript{+} dependent bile acid transporter (ASBT)</td>
<td>S3744 (investigational compound)</td>
</tr>
<tr>
<td>nicotinic acid transporter</td>
<td>valproic acid, penicillins</td>
</tr>
</tbody>
</table>

Importantly, regardless of active or passive transport, the maximum driving concentration for drug absorption (i.e. maximum $C_{\text{free}}$) is not typically increased by micellar solubilisation and is defined by drug solubility in the inter-micellar fluid (essentially the aqueous solubility). From the equilibrium shown above, the relationship between the free fraction ($f$) and the solubilised fraction ($s$) may be described by the following equations\textsuperscript{143}:

\[
\begin{align*}
s &= \frac{C_m - C_s}{C_m} & \text{Equation 1.4} \\
f &= 1 - s & \text{Equation 1.5}
\end{align*}
\]

where $C_m$ is the solubility of drug in the solution with micelles and $C_s$ is the solubility of drug in the same medium without micelles (essentially the aqueous solubility). It is assumed that $s$ is
constant and independent of the drug concentration for a particular concentration of micelles, and that only one type of micelles exist. Thus,

\[ C_{\text{total}} = fC_{\text{total}} + sC_{\text{total}} \]  

Equation 1.6

Since the solubilised and free drug concentration are in equilibrium, the degree of drug saturation in the free fraction and solubilised fraction are expected to be equal. For example, when a micellar system is loaded with drug at its full solubilisation capacity, \( C_{\text{free}} \) will be the saturated solubility of the drug in the colloid-free medium (i.e. \( C_s \)), while \( C_{\text{micelle}} \) will be the saturated solubility of the drug in the colloids (i.e. \( C_m - C_s \)).

Therefore, even if micelles are efficient in promoting drug transport across the intestinal UWL and replenishing the absorbed free fraction, the inter-micellar drug concentration at the absorptive membrane is at best maintained at the solubility limit of free drug in water, and is very low. Since solubilisation does not increase (and may reduce) \( C_{\text{free}} \), and drug flux across an absorptive membrane is the product of the free drug concentration and the drug permeability across the membrane, lipid co-administration may not be expected to enhance the absorption of drugs that are solubility- (but not dissolution rate) limited. Indeed, many authors have shown that increasing the total concentration of solubilised drug within bile salt or synthetic micelles does not necessarily lead to proportional enhancements in intestinal absorptive flux\(^{100, 144, 145} \). These observations recently led Miller et al. and Dahan et al. to suggest that formulations that enhance apparent drug solubility via solubilisation may do so at the expense of overall drug absorption due to decreases in the free fraction\(^{100, 146, 147} \).
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Co-administration with lipids, however, remains an effective means to enhance the oral absorption of PWSD, as exemplified by the myriad drugs that demonstrate positive food effect and bioavailability enhancement when administered in a LBF. In addition, mathematical models of intestinal drug absorption that utilise $C_{\text{free}}$ as the driving concentration frequently underestimate the absorption of BCS Class II compounds\textsuperscript{101, 148, 149}. Indeed, the use of total solubilised concentrations (i.e. $C_{\text{free}}$ plus $C_{\text{micelle}}$) often led to better predictions\textsuperscript{101, 148}. These observations suggest that the absorption of lipophilic, poorly water-soluble compounds from lipid-based colloids may not be solely dictated by membrane permeability and free solubility, and that instead additional mechanisms may contribute to overall flux.

Therefore, the principle hypothesis in this thesis is that after lipid co-administration, the solubilised fraction (i.e. the fraction where the majority of drug in solution resides) is not simply an inert reservoir that replenishes $C_{\text{free}}$, but may contribute to drug flux across absorptive membrane via enhancements in apparent drug permeability or apparent drug solubility. The proposed mechanisms where the solubilised fraction may contribute to drug absorption are discussed below:
Figure 1.3: Schematic diagram outlining the mechanisms of drug absorption from intestinal mixed micelles or vesicles. (A) Current understanding of drug absorption where uptake across the enterocyte apical membrane is driven by the free fraction (black solid arrow) while the solubilised reservoir replenishes absorbed free drug via an equilibrium relationship. Additionally, the absorption of drugs that possess structural similarities to endogenous substrates may be facilitated by specific transporters present on the apical membrane of enterocytes (black dotted arrow). (B) Proposed mechanism of drug absorption where in addition to passive diffusion of free drug, absorption may also occur via collisional uptake (red dotted arrows), where micelles or vesicles interact directly with the apical membrane to mediate selective transfer of solubilised drug or endocytosis of colloid-receptor complex. Collisional uptake may or may not be receptor-mediated. (C) Proposed mechanism of drug absorption where the stimulation of drug supersaturation in a previously stable drug-containing colloid leads to transient increases in the thermodynamic activity (free concentration) of drug, thereby increasing the number of free drug molecules that are available for passive diffusion across the apical membrane (red dotted arrows). These alternative mechanisms of drug absorption from colloidal species are examined in detail in this thesis.
1.5.2 Collisional uptake of solubilised drug

The transfer of FA between model cell membranes, proteins (receptors) and intestinal lipid colloidal phases via collisional transfer has been extensively investigated by Storch and colleagues\textsuperscript{150-153}. Collisional transfer refers to the transfer of solubilised content from donor to acceptor compartments via direct interactions (collisions). For example, Narayanan et al. showed that FA transfer between mixed micelles occurred as a consequence of micelle collisions rather than a diffusion process where FA first dissociate from donor micelles into the aqueous phase prior to association with acceptor micelles\textsuperscript{152}. Similar findings have also been demonstrated for the transfer of LCFA from phospholipid vesicles to proteins such as brain-FABP\textsuperscript{151}.

In the SI, since lipid digestion products and PWSD are also compounds with limited free solubility in water, it is possible that the absorption of these compounds could occur via direct collisional transfer from mixed micelles or vesicles to the absorptive membrane (see Figure 1.3(B), red dotted arrows). Collisional transfer may or may not be receptor-mediated, however recent interest in the role of intestinal lipid uptake transporters such as CD36\textsuperscript{61, 154, 155}, SR-BI\textsuperscript{63, 67, 155, 156}, NPC1L1\textsuperscript{157}, caveolin-1\textsuperscript{158-160}, FATP4\textsuperscript{62} and FABP\textsubscript{pm}\textsuperscript{64, 65} in lipid absorption, and reports of direct interactions of colloidal or macromolecular species in the blood (e.g. albumin, HDL) with one or more of these receptors have led to the hypothesis that absorption of solubilised lipids and drugs may also occur via a similar mechanism. Thus colloid-receptor interactions between mixed micelles or vesicles and lipid uptake transporters in the brush border membrane of the SI may facilitate lipid and/or drug absorption (Figure 1.3(B), red dotted arrows). In this model, after initial collision, monomeric lipids and PWSD may be selectively transferred into cells, or the entire colloid-receptor complex may be endocytosed. A brief description of the relevant receptors and their role in lipid uptake is given below:
1.5.2.1 SR-BI

SR-BI (Scavenger Receptor Class B Type 1) is an 83 kDa glycoprotein that can bind to a variety of ligands such as anionic phospholipids, apoptotic cells and modified/native lipoproteins in vitro\textsuperscript{161}. In vivo, SR-BI is a physiologically relevant receptor for HDL and plays a key role in cholesterol transport into and out of cells. SR-BI acts as a docking receptor for plasma HDL (high density lipoprotein), and mediates the selective uptake of cholesterol esters into hepatocytes and steroidogenic tissues\textsuperscript{161}. In the GI tract, SR-BI is expressed on the apical surface of intestinal villi in the proximal small intestine\textsuperscript{67}. However, the physiological ligand(s) and mechanisms of action of SR-BI at this site remain unclear. Whilst a study by Hauser et al. reported inhibition of sterol collisional transfer into brush border membrane vesicles (BBMV) and Caco-2 cells by anti-human SR-BI IgG\textsuperscript{67}, and there has been evidence of SR-BI receptor endocytosis in pig enterocytes\textsuperscript{156} during fat absorption, the role of SR-BI in lipid absorption is yet to be fully elucidated.

1.5.2.2 CD36

CD36 (Cluster of Differentiation 36), also known as Fatty Acid Translocase (FAT), is also a class B scavenger receptor (88 kDa) that shares many ligands (such as native and modified lipoproteins, anionic phospholipids and serum HDL) with SR-BI\textsuperscript{154, 155, 161}. The distribution of CD36, however, favours tissues with high metabolic needs for FA e.g. adipose tissue, heart muscle, skeletal muscle and the intestine\textsuperscript{162}, consistent with its established role as a FA transporter. The role of CD36 in native LCFA uptake into cells has been well-studied especially in muscle\textsuperscript{162} and adipose tissue\textsuperscript{163}. For example, CD36 expression and FA transport are increased during chronic muscle stimulation\textsuperscript{162}, and CD36 knockout mice show deficits in cellular FA uptake i.e. elevated plasma FA levels and reduced FA accumulation in muscle and adipose tissue\textsuperscript{163}. Even though the role of CD36 as a FA transporter is well-known, the exact mechanism of transport under normal physiological conditions i.e. where FA are mostly bound to albumin, is less clear. Indeed, whether
CD36 interacts with free FA or the entire FA-albumin complex in the plasma, and whether it acts as a docking receptor or an endocytotic receptor to facilitate cellular uptake is not yet known. In the SI, CD36 is most highly expressed on the apical side of intestinal villi of the duodenum and jejunum, and is believed to facilitate FA and cholesterol absorption. For example, FA and/or cholesterol absorption is lower in vitro and in vivo in CD36 knockout mice when compared to wild type mice, and one study which investigated the inhibitory effect of CD36 antibodies on radiolabelled sterol uptake into human BBMV concluded that CD36 is important in free cholesterol uptake in the small intestine. In contrast, in an investigation of FA uptake into the SI of CD36 knockout mice, CD36 was found to be unimportant in FA uptake but instead played a role in chylomicron formation and secretion in the enterocytes. A potential role for CD36 in intestinal lipid absorption is therefore well established. However, the mechanism whereby CD36 facilitates lipid uptake into enterocytes is not yet known.

1.5.2.3 NPC1L1

Ezetimibe, a cholesterol absorption inhibitor, is widely used in practice and has been shown to have modest efficacy in lowering plasma cholesterol. Although the exact mechanism of action of ezetimibe remains unknown, pursuit of potential sites of ezetimibe activity helped to reveal the role of Niemann-Pick C1 Like 1 (NPC1L1) protein in cholesterol absorption. NPC1L1 is a 145 kDa membrane protein that is highly expressed in the SI, with detectable levels in tissues such as the liver, gallbladder, testis, and stomach. In the SI, NPC1L1 is predominantly expressed on the apical side of the proximal small intestine, with the highest levels found in the jejunum. Unlike other lipid transporters, little is known about NPC1L1, especially its role outside the intestine, in large part reflecting the relatively recent (2000) identification of NPC1L1 as a potential lipid transporter. What is known however, is the seemingly important role of NPC1L1 in cholesterol absorption and involvement in the ezetimibe-sensitive cholesterol absorption
Chapter 1: General Introduction

pathway. Thus, cholesterol absorption is reduced by more than 70% in NPC1L1 knockout mice when compared to wild type mice\textsuperscript{167}, and ezetimibe treatment does not reduce cholesterol absorption further in the same knockout animals\textsuperscript{167}. The available data therefore lend strong support to a potential role of NPC1L1 in cholesterol absorption, however, the mechanism by which NPC1L1 facilitates cholesterol absorption is still not established\textsuperscript{165}. Data suggesting that NPC1L1 aids cholesterol absorption by facilitating intracellular cholesterol transport\textsuperscript{167} (rather than absorption directly) further complicate the issue of whether NPC1L1 is an authentic cholesterol transporter, or a protein that facilitates intracellular trafficking, or both.

1.5.2.3 Endocytosis

Endocytosis can be mediated by several pathways including clathrin coated pits, caveolae-mediated pathways, and non-clathrin, non-caveolae mediated endocytosis\textsuperscript{168}. Briefly, clathrin-mediated endocytosis occurs ubiquitously and involves the deformation of the plasma membrane into a coated pit, a process mediated by surface proteins such as clathrin and adaptor protein-2. Lipid rafts are domains of the plasma membrane that have a higher degree of order due to enrichment by cholesterol, sphingolipids and phospholipids. Due to their less fluid (more rigid) structure, lipid rafts are able to spatially segregate membrane lipids and proteins such that membrane processes are also functionally separated\textsuperscript{169}. Lipid rafts have received increasing recent attention as plasma membrane platforms with involvement in the binding and uptake of LCFA\textsuperscript{160}. Caveolae are subsets of lipid rafts and are characterised by flask-like invaginations in the plasma membrane that resemble vesicles that are poised to bud\textsuperscript{170}. The expression of the scaffolding protein, caveolin, is essential to the formation of caveolae\textsuperscript{170}. Evidence also exists to suggest a role for endocytotic pathways mediated by clathrin coated pits and caveolae in lipid uptake. For example, the uptake of LCFA by human microvascular endothelial cells has been shown to involve endocytosis mediated by clathrin-coated vesicles and caveolae\textsuperscript{171}, and the lipid uptake
transporters CD36 and SR-BI are found preferentially located in caveolae on the apical membrane of enterocytes\textsuperscript{161, 172}. Caveolin-1, the scaffolding protein of caveolae, binds FA with strong affinity\textsuperscript{169}, and may be a potential lipid uptake transporter itself. These observations suggest that an endocytotic component to lipid uptake in the SI is possible.

### 1.5.3 Supersaturation-enhanced drug absorption

Supersaturation is a transient solution state that precedes precipitation, where the concentration of solute in a system is higher than the equilibrium solubility of the solute in the same system. Supersaturation results in an increase in thermodynamic activity, and therefore a potential means by which drug absorption can be enhanced.

The potential for supersaturation to enhance the oral bioavailability of PWSD has received increasing recent interest\textsuperscript{173, 174}, and delivery systems that aim to generate supersaturated drug concentrations in the GI lumen, and to stabilise the metastable supersaturated state (i.e. delaying drug precipitation) are increasingly common. The attainment of intraluminal drug concentrations in excess of its equilibrium solubility may be achieved by delivering drugs in a solubilised form that lose solubilisation capacity \textit{in situ} (e.g. cosolvent systems, LBF), and the delivery of high-energy crystal forms or amorphous materials that provide accelerated dissolution and/or higher solubility than their most stable form\textsuperscript{173}, and where precipitation does not occur immediately.

In the context of LBF, Gao and Morozowich first reported on “supersaturable SEDDS” (S-SEDDS), where dispersion of the formulation in the GI fluids led to drug precipitation, but where addition of a precipitation inhibitor resulted in transient stabilisation of a supersaturated state\textsuperscript{17}.
They subsequently showed that the oral bioavailability of a number of drugs was significantly improved after dosing of S-SEDDS when compared to a conventional suspension or SEDDS\textsuperscript{17, 175}.

Recent data, however, suggests that many LBF, whether they are labelled as ‘supersaturable’ or not, frequently lose drug solubilisation capacity during GI processing and have the potential to trigger drug supersaturation. Solubilisation capacity can be lost via initiation of the digestion of glyceride lipids\textsuperscript{91, 176} and/or surfactants\textsuperscript{177}, and the dilution of co-solvents and surfactants\textsuperscript{5, 178}. LBF that lose solubilisation capacity \textit{in vivo} may therefore benefit from supersaturation-enhanced drug absorption, provided that precipitation is sufficiently delayed. In this regard, a range of polymers have been explored for their utility in supersaturation stabilisation during GI processing of LBF, with cellulose-based polymers appearing to be the most useful to date\textsuperscript{179}.

In contrast, the possibility that supersaturation-enhanced absorption may play a role in the absorption of solubilised drug from intestinal micellar and vesicular species has not been explored in detail. Importantly, for a solubilised system, supersaturation is expected to increase $C_{\text{free}}$ above drug aqueous solubility. This may provide a means to reverse the reduction in drug thermodynamic activity resulting from solubilisation, and explain the paradox that is the ability of solubilising LBF to increase the absorption of many PWSD despite apparent reductions in drug thermodynamic activity (see Figure 1.3(C), red dotted arrows). In essence, the stimulation of supersaturation in stable colloidal systems may provide a means to mobilise the solubilised drug reservoir such that solubilised drug is more readily available for absorption. Since lipid-based micelles and vesicles are dynamic structures that are in constant interaction with lipid dispersion, digestion and absorption processes in the GI environment, changes to solubilisation capacity (and therefore supersaturation opportunities) are likely to exist within endogenous lipid processing pathways. This thesis has examined these possibilities in detail.
1.6 STRUCTURE OF THIS THESIS

Chapters 3, 4 and 5 of this thesis have been directly reproduced from published or submitted manuscripts. The constraints of journal publication dictate that these chapters focus on the results, discussions, and conclusions of the experiments, but include relatively limited detail of the methods employed and validation studies. A general methods chapter (Chapter 2) has therefore been added to further describe the method development and validation studies conducted. The thesis concludes with a final summary and perspectives chapter (Chapter 6).
CHAPTER 2 : GENERAL METHODS
2.1 INTRODUCTION

This general methods chapter contains further information on the *in situ* autoperfused rat jejunum preparation, brief outline of the surgical procedures required for the conduct of *in vivo* bioavailability studies, and a detailed description and validation of the analytical methods used throughout this thesis. Other experimental methods have been included in the experimental chapters (3 through 5) which are reproduced from published or submitted manuscripts.

2.2 AUTOPERFUSED RAT JEJUNUM

2.2.1 Materials

Sodium chloride (NaCl), D-mannitol, antipyrine, D-mannitol, [1-\(^{14}\)C] (46.6 mCi/mmol), antipyrine, [3-\(^{14}\)C] (5.4 mCi/mmol) (Sigma-Aldrich, Australia), sodium hydroxide pellets (NaOH) and ethanol 96% v/v (Merck, Australia), disodium hydrogen orthophosphate (Na\(_2\)HPO\(_4\)) and sodium dihydrogen orthophosphate (NaH\(_2\)PO\(_4\).2H\(_2\)O) (Ajax Finechem, Australia), 0.9% Sodium Chloride Intravenous Infusion BP (Baxter, Australia), polyethylene glycol, [1,2-\(^{3}\)H] (1.50 mCi/g), Irga-Safe Plus\textsuperscript{TM}, SOLVABLE\textsuperscript{TM} (Perkin Elmer Life Sciences, MA, USA), oleic acid, [9,10-\(^{3}\)H(N)] (60 Ci/mmol) (American Radiolabeled Chemicals, MO, USA), heparin sodium injection BP (1000 I.U./mL, Hospira, Australia), xylazine (100 mg/mL, Troy Laboratories, Australia), acepromazine (10 mg/mL, Ceva Delvet, Australia), ketamine (100 mg/mL, Provet, Australia) and pentobarbitone sodium (325 mg/mL, Virbac, Australia) were obtained from listed suppliers. Water was obtained from a Millipore milliQ Gradient A10 water purification system (Millipore, MA, USA).
2.2.2 Methods

2.2.2.1 Surgical equipment and accessories

The surgical instruments required for the setup of the autoperfused rat jejunum include a scalpel, a pair of sharp straight scissors, a pair of iridectomy scissors, two curved anatomical forceps and one fine tip curved forceps. A portable, multi-head, flexible cold fibre-optic illuminating system (Microlight 150®, Fibre optic light guides, Australia) was used to obtain adequate lighting for surgery. A heated surgical board (Ratek Instruments, Australia) was used to maintain body temperature of the animals throughout surgery and experiments.

2.2.2.2 Animal care and anaesthesia

All rat studies were approved by the institutional animal ethics committee, and were conducted in accordance with the guidelines of the Australian and New Zealand Council for the Care of Animals in Research and Teaching. Male Sprague-Dawley rats (280–330 g) were used in all experiments, and were allowed to acclimatize in the institutional animal housing facility for at least 7 days with free access to standard chow and water. All animals were fasted overnight (12–18 h) prior to surgery. Anaesthesia was induced in rats by subcutaneous injection of 1.0 mL/kg of ‘Cocktail I’ (37.3 mg/mL ketamine, 9.8 mg/mL xylazine, 0.4 mg/mL acepromazine in saline), and maintained throughout the study with subcutaneous doses of 0.44 mL/kg of ‘Cocktail II’ (90.9 mg/mL ketamine, 0.9 mg/mL acepromazine) when required. To prepare for surgery, rats were shaved and cleaned aseptically with 70% v/v ethanol on both the abdomen and the skin overlaying the trachea, and placed in dorsal recumbency. Throughout surgery and experiment, the body temperature of rats was maintained at 37 °C.
2.2.2.3 Surgical technique

The autoperfused rat jejunum model involves the perfusion of an isolated jejunal segment and simultaneous blood collection from the corresponding mesenteric vein branch (see Figure 2.1). The surgical procedures for the perfusion studies are similar to those described by Singhal et al. with slight modifications\textsuperscript{180}. Firstly, the right jugular vein was cannulated to enable infusion of donor rat blood (the surgical procedures for jugular vein cannulation is detailed in Section 2.3.2.5). The small intestine was then exposed by a longitudinal midline incision to the abdomen, and a piece of jejunal segment (~ 10 cm) carefully isolated and externalised. Access to the lumen was made at the proximal and distal ends by electrocautery, and jejunal contents were flushed out with warm perfusion buffer (150 mM Na\textsuperscript{+}, 18 mM H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−}, 12 mM HPO\textsubscript{4}\textsuperscript{2−}, 108 mM Cl\textsuperscript{−}, adjusted to pH 6.30 ± 0.01 with NaOH solution). The segment was then cannulated at the proximal and distal ends with sections of Teflon tubing (0.03-inch i.d. proximal/inlet, Upchurch Scientific, Oak Harbor, WA, Australia; 0.0625-inch i.d. distal/outlet, Shimadzu, Kyoto, Japan) which were secured with surgical sutures. The mesenteric vein that drains the cannulated jejunal segment was then identified, and prepared for catheterisation by careful blunt dissection of the surrounding connective tissue under a dissecting microscope. The rat was then heparinised (90 I.U./kg) via the jugular vein using an appropriate volume of a 100 I.U./mL heparinised saline solution, and the mesenteric vein immediately catheterised under the dissecting microscope with a 24G intravenous catheter (Angiocath\textsuperscript{TM}, BD, Australia), which had the top 1.5 cm tip cut so that the tip remained in the mesenteric vein after the guide needle was withdrawn. A drop of instant cyanoacrylate glue was placed over the site of catheterisation, the guide needle removed, and a 30 cm piece of silicone tubing (0.025-inch i.d., Helix Medical, CA, USA) attached to the catheter tip for the collection of venous blood. Immediately following catheterisation of the mesenteric vein and for the remainder of the experiment, the jugular vein cannula was connected to a peristaltic pump (Adelab Scientific, SA, Australia), and rats were infused with heparinised donor rat blood.
I.U./mL) at a rate of 0.3 mL/min (this rate was selected to match the outflow of the mesenteric blood). Donor rat blood was collected from donor rats via cardiac puncture under isoflurane anaesthesia (IsoFlo®, Abbott Laboratories, IL, USA) immediately prior to surgery. The temperature of the animal and preparation were maintained by a heated surgical board and lamp, and the exposed jejunal segment was covered by saline-soaked gauze.

**Figure 2.1:** Autoperfused rat jejunum preparation depicting mesenteric venous blood collection from the perfused jejunal segment (~10 cm). The model allows for simultaneous perfusion of the jejunal segment and collection of mesenteric venous blood, which enables analysis of compound absorption in a closed system.

### 2.2.2.4 Variations in the setup of the autoperfused rat jejunum

Various perfusion techniques may be used to conduct the autoperfused rat jejunum experiments, including single-pass, recirculating, oscillating and closed loop perfusion\textsuperscript{181}. Among the techniques mentioned, single-pass and recirculating perfusion are most commonly employed\textsuperscript{182, 183}, and were established to determine their suitability as model systems to collect the data required for this thesis. During single-pass perfusion, perfusate from a reservoir is continuously flowed
through the jejunal segment and outflowing perfusate continuously collected (Figure 2.2A). Thus, the jejunal segment is exposed to fresh perfusate containing a constant concentration of compound at all times (although the concentration of compound decreases during intestinal transit due to absorption). On the other hand, during recirculating perfusion, outflowing perfusate is recirculated back to the perfusate reservoir, and re-perfused into the jejunal segment (Figure 2.2B). Thus, assuming that the compound of interest is absorbed, the intestine is exposed to a gradually declining concentration of compound. As a result, the mathematical modelling for single-pass and recirculating techniques are slightly different. Both techniques, however, have been satisfactorily employed to study drug absorption kinetics in the small intestine\textsuperscript{182}.

2.2.2.5 Experimental protocol for single-pass jejunal perfusion

The animals were equilibrated for 30 min after surgery. During this time, blood that was collected from the cannulated mesenteric vein (~ 0.3 mL/min) was mixed with donor rat blood and re-infused via the jugular vein. The inlet jejunal cannula was connected to a second peristaltic pump and perfusion buffer was pumped through the jejunal segment at a rate of 0.1 mL/min. Outflowing buffer during the equilibration period was discarded to waste. After the equilibration period and immediately before the experiment was commenced (i.e. immediately before the solution of interest was perfused), air was pumped through the jejunal segment for 10 sec at 0.1 mL/min, to create an air pocket in the outflowing perfusate which differentiated outflowing perfusion buffer and outflowing solution of interest during the first perfusate sampling interval. Outflowing perfusate was continuously collected into 1.5 mL polypropylene tubes at 10-min intervals whilst venous blood draining the perfused jejunal segment was collected into pre-weighed 1.5 mL polypropylene tubes at 5-min intervals and weighed. The setup of the single-pass autoperfused rat jejunum preparation is shown in Figure 2.3.
At the end of the experiment, the animal was euthanised via an intravenous injection of 100 mg sodium pentobarbitone into the jugular vein. The jejunal segment was flushed with 10 mL warm perfusion buffer followed by 10 mL air, excised, and dissected longitudinally for accurate determination of surface area (calculated by multiplying the diameter by the length of the perfused intestinal segment). The jejunal segment was then weighed and stored at -20 °C for future analysis of compound content.

### 2.2.2.6 Experimental protocol for recirculating jejunal perfusion

The experimental protocol for recirculating jejunal perfusion was similar to single-pass perfusion (described in Section 2.2.2.5), except that perfusate was passed through the jejunal segment at a rate of 0.6 mL/min, and outflowing perfusate was recirculated back to a reservoir (typically 10 mL) and re-perfused into the jejunal segment (see Figure 2.2B). During the 30-min equilibration period, perfusion buffer was recirculated through the jejunal segment at a rate of 0.6 mL/min. Immediately before the experiment was commenced (i.e. immediately before the solution of interest was perfused), air was pumped through the jejunal segment for 5 sec at 0.6 mL/min. The perfusate reservoir was sampled at 10-min intervals (typically 100 µL aliquots) whilst venous blood draining the perfused jejunal segment was collected into pre-weighed 1.5 mL polypropylene tubes at 5-min intervals and weighed.
Figure 2.2: Schematic of autoperfused rat jejunum with (A) single-pass perfusion, and (B) recirculating perfusion. In single-pass perfusion, perfusate is flowed through the isolated jejunal segment once and collected. In recirculating perfusion, outflowing perfusate is redirected back to the perfusate reservoir and re-perfused into the isolated jejunal segment. Diagrams are adapted from Cummins et al.\textsuperscript{184}. 
Figure 2.3: Setup of the single-pass autoperfused rat jejunum preparation.

2.2.3 Data analysis

2.2.3.1 Permeability calculations for single-pass perfusion

In the single-pass perfusion model, permeability coefficients were calculated after attainment of steady state drug absorption, and using steady state compound concentrations in perfusate and plasma. Two permeability coefficients were calculated to describe compound absorption from the jejunal lumen into the mesenteric venous blood as described previously\textsuperscript{184} and as follows:

\[
\text{‘Disappearance’} \quad P_{\text{app}} = -\frac{Q}{A} \ln \frac{C_1}{C_0} \quad \text{Equation 2.1}
\]

\[
\text{‘Appearance’} \quad P_{\text{app}} = \frac{\Delta M_B/\Delta t}{A <C>} \quad \text{Equation 2.2}
\]
where ‘Disappearance’ $P_{app}$ is the apparent permeability coefficient calculated from compound loss from the perfusate (cm/sec); ‘Appearance’ $P_{app}$ is the apparent permeability coefficient calculated from compound appearance in the mesenteric blood (cm/sec); $Q$ is the perfusate flow rate (mL/sec); $A$ is the surface area of the perfused jejunal segment (cm$^2$), which is calculated by multiplying the diameter and the length of the perfused intestinal segment; $C_1$ is the average steady state compound concentration exiting the perfused jejunal segment (ng/mL); $C_0$ is the compound concentration entering the jejunal segment (ng/mL); $\Delta M_b/\Delta t$ is the average rate of compound mass appearance in mesenteric venous blood at steady state (ng/sec); and $<C>$ is the logarithmic mean compound concentration in the lumen (ng/mL), where $<C> = (C_1 - C_0) / (\ln C_1 - \ln C_0)$.

### 2.2.3.2 Permeability calculations for recirculating perfusion

Similar to single-pass perfusion, two permeability coefficients were calculated in the recirculating perfusion model to describe compound absorption from the jejunal lumen into the mesenteric venous blood. The disappearance permeability coefficient was assessed from the rate of compound loss from the perfusate reservoir$^{185}$, while the appearance permeability coefficient was calculated from cumulative compound appearance data in the mesenteric venous blood:

\[
'\text{Disappearance}' P_{app} = \frac{V}{A} \cdot k_u \quad \text{Equation 2.3}
\]

\[
'\text{Appearance}' P_{app} = \frac{1}{AC_o} \cdot \frac{dM}{dt} \quad \text{Equation 2.4}
\]

where ‘Disappearance’ $P_{app}$ is the apparent permeability coefficient calculated from compound loss from the perfusate (cm/sec); ‘Appearance’ $P_{app}$ is the apparent permeability coefficient calculated from compound appearance in the mesenteric venous blood (cm/sec); $V$ is the effective
volume of recirculating perfusate (mL); $A$ is the surface area of the perfused jejunal segment (cm$^2$); $k_u$ is the first order disappearance rate constant (sec$^{-1}$), calculated from the slope of a log$_e$ perfusate concentration vs. time plot; $C_o$ is the initial perfusate concentration (ng/mL); and $dM/dt$ (ng/sec) is the appearance flux of compound in the mesenteric venous blood, calculated from the slope of a cumulative mass transported vs. time plot.

### 2.2.4 Validation of the autoperfused rat jejunum

#### 2.2.4.1 Passive permeability markers

The autoperfused rat jejunum was validated via comparison of calculated disappearance and appearance permeability coefficients for the model compounds antipyrine and mannitol to published data using the recirculating perfusion technique. Antipyrine is passively absorbed via the transcellular route (i.e. diffusion across the apical and basolateral membranes of enterocytes) whereas mannitol is passively absorbed via the paracellular route (i.e. diffusion through the tight and gap junctions between enterocytes). Antipyrine and mannitol thus act as markers for passive permeability via the transcellular and paracellular route. Since paracellular transport comprises $\sim 0.01\%$ of the total surface area available for absorption in the small intestine$^{186, 187}$, the intestinal permeability of mannitol is expected to be significantly lower than antipyrine. Comparison of the disappearance and appearance permeability coefficients of antipyrine and mannitol to literature values enables assessment of the reproducibility of the autoperfused rat jejunum model and the integrity of the intestinal segments during perfusion experiments$^{180, 182, 188}$.

In the validation experiments, the perfusate reservoir contained 10 mL of 1 mM mannitol in perfusion buffer spiked with 1 µCi $^{14}$C-mannitol or 10 mL of 1 mM antipyrine in perfusion buffer spiked with 1 µCi $^{14}$C-antipyrine. The experiments were conducted over 60 min, during which
time 100 µL aliquots of perfusate were sampled from the reservoir at t = 0 and at 10 min intervals whilst blood draining the jejunal segment was collected every 5 min and plasma separated by centrifugation (10,000 xg, 5 min). At the end of the experiment, the perfused jejunal segment was removed and longitudinally dissected for accurate determination of surface area and quantification of radiolabel. For quantification of antipyrine or mannitol in perfusate and plasma, 100 µL of perfusate samples and 500 µL of plasma samples were placed in liquid scintillation (LSC) vials containing 2 mL Irga-safe Plus scintillation fluid, and vortexed for 10 sec. For quantification of antipyrine or mannitol in the perfused jejunal segment, the segment was placed in a 20 mL glass vial, 2 mL of SOLVABLE™ added, and the vial was incubated at 40 ºC for 48 h. The dissolved tissue solution was divided into two equal portions where 12 mL of Irga-safe Plus was added to each vial followed by a 1-min vortex. Radioactivity of the perfusate, plasma, and intestine samples were quantified via scintillation counting on a Packard Tri-Carb 2000CA liquid scintillation analyser (Packard, Meriden, CT, USA). Blood concentrations were calculated by multiplying the plasma concentrations of antipyrine and mannitol and their respective blood:plasma concentration ratios, which are determined in Section 2.2.4.2.

The perfusate disappearance and blood appearance profiles for antipyrine and mannitol are shown in Figure 2.4, and calculated permeability coefficients are in Table 2.1. The apparent permeability coefficient of antipyrine based on disappearance from the perfusate (~ 45 x 10^{-6} cm/sec) and the apparent permeability coefficient of mannitol based on appearance in the mesenteric venous blood (~ 1.9 x 10^{-6} cm/sec) were comparable to reported values in the literature (47-73 x 10^{-6} cm/sec\textsuperscript{182, 183} and 1 x 10^{-6} cm/sec\textsuperscript{180}, respectively). The permeability coefficient of antipyrine based on appearance in the mesenteric blood is not available in the literature to date, and the permeability coefficient of mannitol based on disappearance from the perfusate was not calculated due to its low disappearance from the perfusate (see Figure 2.4A) which precluded accurate assessment of
the permeability coefficient. The intestinal permeability of antipyrine was therefore significantly higher than mannitol, consistent with their respective mode of absorption as discussed earlier. The total recoveries (i.e. the sum of recovery percentages in the perfusate, blood, and the perfused jejunal segment) of antipyrine and mannitol in the autoperfused rat jejunum preparation were 93.37% ± 8.25% and 97.02% ± 0.20% (mean ± SEM, n = 3), respectively. A summary of the mass balance for mannitol and antipyrine is shown in Table 2.2.

Thus, the permeability data obtained using mannitol and antipyrine validate the reproducibility of the autoperfused rat jejunum and the integrity of the intestinal segments during perfusion experiments. Although the validation experiments were conducted using the recirculating perfusion model, the data also apply for the single-pass perfusion model, since previous studies that compared the permeability of antipyrine using both techniques reported statistically equal apparent permeability coefficients\(^3\), and the apparent permeability coefficient of mannitol obtained in this thesis (using the recirculating model) was comparable to literature value that employed the single-pass model\(^{180}\).
Figure 2.4: (A) Disappearance profiles of antipyrine (filled circles) and mannitol (open circles) from the perfusate and (B) Appearance profiles of antipyrine (filled circles) and mannitol (open circles) in mesenteric venous blood during recirculating perfusion of an isolated ~10 cm segment of rat jejunum. Data represent mean ± SEM of n = 3 experiments, linear regression lines utilised in calculation of permeability coefficients are shown as solid (antipyrine) or dotted (mannitol) lines.
Table 2.1: Permeability coefficients of antipyrine and mannitol based on disappearance from the perfusate and appearance in mesenteric venous blood during recirculating perfusion of an isolated rat jejunal segment (~ 10 cm). Data represent mean ± SEM of n = 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>Disappearance $P_{\text{app}}$ (x 10$^6$ cm/sec)</th>
<th>Appearance $P_{\text{app}}$ (x 10$^6$ cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antipyrine</td>
<td>45.49 ± 7.86</td>
<td>38.27 ± 5.56</td>
</tr>
<tr>
<td>Mannitol</td>
<td>n/a $^a$</td>
<td>1.94 ± 0.42</td>
</tr>
</tbody>
</table>

$^a$ The disappearance $P_{\text{app}}$ for mannitol could not be determined accurately due to low disappearance from the perfusate

Table 2.2: Mass balance for mannitol and antipyrine in the autoperfused rat jejunum preparation. Total mass balance (as a %) is the sum of percentages recovered in the perfusate, mesenteric venous blood and perfused jejunal segment at the end of the perfusion experiments. Data represent mean ± SEM values for n = 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>Antipyrine</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery in perfusate</td>
<td>75.24 ± 6.89</td>
<td>94.38 ± 0.43</td>
</tr>
<tr>
<td>% Recovery in blood</td>
<td>16.78 ± 2.32</td>
<td>1.13 ± 0.20</td>
</tr>
<tr>
<td>% Recovery in jejunal segment</td>
<td>1.35 ± 0.14</td>
<td>1.50 ± 0.16</td>
</tr>
<tr>
<td>Total Mass Balance</td>
<td>93.37 ± 8.25</td>
<td>97.02 ± 0.20</td>
</tr>
</tbody>
</table>
2.2.4.2 Blood:plasma ratio determination

Calculation of appearance permeability coefficients requires accurate quantitation of total compound transport into mesenteric venous blood. Since compound concentrations were assayed in plasma (and not whole blood), the partitioning behaviour of compounds between whole blood and plasma was determined such that concentrations of compound in whole blood could be back calculated. Blood:plasma concentration ratios were therefore determined for mannitol and antipyrine (for validation experiments), and for cinnarizine, danazol and oleic acid (for experiments described in Chapters 3, 4 and 5), to enable conversion of plasma concentrations into blood concentrations.

Briefly, in triplicate, 0.5 mL of blank blood was spiked with known amounts of compound to achieve low, medium, and high concentrations (the concentrations were chosen based on the likely amount of compound transported into mesenteric venous blood during permeability experiments) and gently mixed. The final blood concentrations were 1, 10, 20 µM for mannitol and antipyrine; 20, 100, 500 ng/mL for cinnarizine and danazol; and 1, 5, 10 µg/mL for oleic acid. Traces of radiolabelled mannitol, antipyrine and oleic acid were also spiked into blank blood to facilitate quantitation. Blood samples were centrifuged at 10,000 xg for 5 min, and plasma concentrations of compound were assayed by scintillation counting (mannitol, antipyrine and oleic acid), HPLC (cinnarizine), or LC-MS (danazol). The blood:plasma ratio was calculated from the ratio of known concentration in spiked blood to the concentration measured in plasma separated from spiked blood. The blood:plasma ratios for mannitol, antipyrine, cinnarizine, danazol and oleic acid were 0.48 ± 0.02, 1.23 ± 0.03, 0.68 ± 0.03, 0.65 ± 0.01, and 1.24 ± 0.00 (mean ± SEM, n = 3), respectively, and were not concentration-dependent within the tested range.
2.2.4.3 Assessment of net water flux

Changes in perfusate volume (due to water absorption from or water secretion into the lumen of the perfused jejunal segment) affect the concentration of compound in the perfusate, and therefore impact accurate calculation of disappearance permeability coefficients. For example, water secretion into the lumen increases perfusate volume and lowers the concentration of compound in the perfusate. If correction factors were not applied to the measured concentrations, compound loss from the perfusate (i.e. mass of compound assumed to be absorbed) would be overestimated, leading to overestimation of disappearance permeability. Therefore, net water flux across the perfused jejunal segment is often determined during individual experiments via the inclusion of a non-absorbable marker, such as PEG 4000\(^{189}\), in the perfusate. The concentration of the non-absorbable marker is only expected to change when there is net water flux into or out of the perfused jejunal segment. Thus, changes in the concentration of a non-absorbable marker directly reflect volume changes in the perfused jejunal segment, and correction factors may be subsequently calculated to adjust the concentrations of compound of interest in the perfusate at each sampling point.

In the experiments conducted in this thesis however, the routine inclusion of a radiolabelled marker of water flux (e.g. \(^{14}\)C- or \(^{3}\)H-PEG 4000) in each experiment was impractical since other radiolabelled compounds (e.g. \(^{14}\)C-cholesterol and \(^{3}\)H-oleic acid) were also often included in the perfusate. Net water flux was therefore determined in separate validation experiments, where \(^{14}\)C-PEG 4000 was perfused through isolated jejunal segments using the recirculating perfusion model (a total of five experiments were conducted). The recirculating perfusate reservoir consisted of 10 mL perfusion buffer spiked with 0.25 µCi \(^{14}\)C-PEG 4000. The experiments were conducted over 60 min, and 100 µL aliquots of perfusate were sampled from the reservoir at \(t = 0\) and at 10 min
intervals. The perfusate samples were analysed for radiolabelled PEG 4000 content as described in Section 2.2.4.1.

A plot of mean calculated perfusate volume vs. time is shown in Figure 2.5. The plot shows that significant volume gain mainly occurred in the first 10 min of the experiment, and the perfusate volume was relatively constant for the remainder of the experiment (relative to the calculated volume at 10 min, percent volume changes from 10-60 min were < 2%, a value below experimental error limits). The volume gain in the first 10 min likely reflects the incorporation of small amounts of perfusion buffer that remained in the jejunal segment after the equilibration period. The PEG 4000 experiments therefore suggested that no significant water flux occurred between 10 and 60 min. As such, rather than applying correction factors at each sampling point, the analysis of perfusate compound disappearance was modified slightly such that calculations of disappearance permeability coefficients were performed using compound concentrations obtained between 10 and 60 min (instead of between 0 and 60 min) for recirculating perfusion experiments. For single-pass perfusion, since the analysis of perfusate compound disappearance was performed after drug loss from perfusate reached steady state (a process that typically takes > 30 min as exemplified by perfusate disappearance profiles in the experimental chapters), no modifications to the method of analysis was required.
Figure 2.5: Mean plot of calculated perfusate volume vs. sampling time points for five perfusion experiments where 0.25 µCi of $^{14}$C-PEG 4000, a non-absorbable marker, was included in the perfusate and recirculated through ~ 10 cm segments of rat jejunum. Broken line represents theoretical volume of perfusate in the absence of net water flux. A trend of water gain was evident at all sampling time points. Data represent mean ± SEM values.

2.2.5 Adsorption of cinnarizine onto the recirculating perfusion apparatus

Validation of the autoperfused rat jejunum preparation was performed using the recirculating perfusion model (see Section 2.2.4) since the model provides better simulations of the absorption process in vivo, where compound absorption is continuous along the length of the small intestine. However, in subsequent experiments where drug-containing lipid colloidal phases were perfused, the model poorly water-soluble drug (PWSD), cinnarizine, was found to adsorb extensively to the recirculating perfusion apparatus. Specifically, in sham experiments where cinnarizine was solubilised in intestinal lipid colloidal phases (composition shown in Figure 2.6) and pumped through the recirculating perfusion apparatus only (i.e. through the perfusion tubing but not through the intestinal segment of an animal) at a rate of 0.6 mL/min, cinnarizine concentration in
the perfusate declined appreciably, with > 60% loss over 2 h. Since the stability of cinnarizine in the phases was validated for a period of 24 h (data not shown), the decrease in perfusate concentration of cinnarizine was attributed to adsorption onto the recirculating apparatus, presumably to the relatively lengthy silicone tubing that was required for the recirculating model setup.

To overcome the problem of cinnarizine adsorption onto the recirculating apparatus, the duration of the sham experiment was increased to 6 h in an attempt to saturate the tubing with cinnarizine, so that a constant cinnarizine perfusate concentration could be achieved before perfusate was passed through the rat jejunal segment. This approach, however, did not prove viable as the concentration of cinnarizine decreased to ~ 10% of initial after 5 h (Figure 2.6A), and was too low to be used in the experiments. Thus, the recirculating model was deemed unsuitable for the jejunal perfusion of cinnarizine in intestinal lipid colloidal phases, and the single-pass model was trialled as an alternate method instead. The single-pass perfusion setup required shorter lengths of polyethylene tubing, and perfusate is only passed through the tubing once. Therefore, cinnarizine adsorption was expected to occur to a lesser extent than with the recirculating perfusion setup. Indeed, a repeat sham experiment using the single-pass perfusion setup where cinnarizine was solubilised in the same lipid colloidal phase and pumped through the perfusion apparatus at a rate of 0.1 mL/min revealed negligible cinnarizine adsorption, and the concentration of cinnarizine in the perfusate was within ± 10% of initial throughout the experiment (Figure 2.6B). Similar outcomes were later obtained for another model PWSD, danazol, where no adsorption was observed during the sham perfusion of danazol containing-intestinal lipid colloidal phase (composition shown in Figure 2.7) through the perfusion apparatus at a rate of 0.1 mL/min (Figure 2.7).
Therefore, while validation of the animal model was carried out using the recirculating perfusion setup, the single-pass perfusion setup was the method employed to investigate the absorption kinetics of cinnarizine and danazol from intestinal lipid colloidal phases.

Figure 2.6: Plot of percent initial cinnarizine concentration in perfusate vs. time during an adsorption test experiment where an intestinal lipid colloidal phase* containing 10 µg/mL cinnarizine was flowed through (A) the recirculating perfusion apparatus for 6 h, and (B) the single-pass perfusion apparatus for 2 h. In the recirculating perfusion setup, samples were taken at t = 0, at hourly intervals for the first 5 h, and at 10 min intervals thereafter to reflect the approach of attempting to saturate the adsorption of cinnarizine onto the perfusion apparatus for 5 h prior to commencement of a 1 h jejunal perfusion experiment. Even though steady state (i.e. constant) concentrations appeared to have been reached after 5 h, the concentration (~ 10% of initial) was deemed too low for the perfusion experiments. In the single-pass perfusion setup, samples were taken at t = 0 and at 10 min intervals for 2 h, and negligible cinnarizine adsorption was observed during the sham experiment.

* Intestinal lipid colloidal phase consisted of 0.025 %w/v oleic acid and 0.016 %w/v monoolein solubilised in 4 mM sodium taurocholate, 1 mM lysophosphatidylcholine and 0.25 mM cholesterol at pH 6.50.
Figure 2.7: Plot of percent initial danazol concentration in perfusate vs. time during an adsorption test experiment where an intestinal lipid colloidal phase** containing 14.4 µg/mL danazol was flowed through the single-pass perfusion apparatus for 70 min. Samples were taken at t = 0 and at 10 min intervals, and negligible danazol adsorption was observed during the sham experiment.

** Intestinal lipid colloidal phase consisted of 0.10 %w/v oleic acid and 0.064 %w/v monoolein solubilised in 4 mM sodium taurocholate, 1 mM lysophosphatidylcholine and 0.25 mM cholesterol at pH 6.30.

2.3 IN VIVO BIOAVAILABILITY STUDIES

To determine whether absorption trends observed using the in situ autoperfused rat jejunum preparation could be extended to the in vivo setting, the bioavailability of cinnarizine and danazol was assessed in rats following intraduodenal administration in intestinal lipid colloidal phases. In the case of danazol, intraduodenal administration did not lead to detectable systemic plasma concentrations (limit of quantification for danazol plasma assay was 5 ng/mL) despite significant absorption into the mesenteric venous blood during perfusion experiments (suggesting significant hepatic first-pass metabolism). Thus, bioavailability data for danazol is not included in this thesis. The experimental protocol for the bioavailability studies is described in detail in Chapters 3 and 4. This section contains details of the surgical procedures required for the setup of the animal model.
2.3.1 Materials

Heparin, saline, ketamine xylazine, acepromazine were sourced from suppliers listed in Section 2.2.1.

2.3.2 Methods

The right carotid artery, right jugular vein, common bile duct and duodenum were cannulated for the purpose of: blood sample withdrawal, intravenous administration of formulation or saline, pancreatobiliary fluid diversion and intraduodenal administration of formulation, respectively.

2.3.2.1 Conscious state

Studies that assess the bioavailability of drugs in oral formulations are most often conducted using conscious animals since the rate of gastric emptying, intestinal mixing and digestion are likely to be reduced in anaesthetised animals. The formulations used in the experiments throughout this thesis, however, were highly dispersed intestinal lipid colloidal phases assembled from fully digested lipids, and as such, drug absorption from the formulations is less likely to be affected by the conscious state of the animals. In addition, the protocol for the bioavailability studies necessitated abdominal incisions to be made to the animals (for the diversion of pancreatobiliary fluids as well as the intraduodenal administration of formulations - to avoid the effects of gastric mixing since the formulations were designed to simulate colloidal phases found in the small intestine). These surgical interventions were expected to significantly enhance the degree of post-operative pain and stress in the animals. Therefore, bioavailability studies were conducted using anaesthetised rats to reduce the logistical and ethical burden of the experiments, and to match the conditions used in the in situ jejunal perfusion experiments.
2.3.2.2 Surgical equipment and accessories

The surgical instruments required for the cannulation of the carotid artery, jugular vein, common bile duct and duodenum include a scalpel, a pair of sharp straight scissors, two curved anatomical forceps, one fine tip curved forceps, one fine tip straight forceps, a pair of iridectomy scissors, one fine tip haemostat and one small ‘alligator’ clamp. A portable, multi-head, flexible cold fibre-optic illuminating system (Microlight 150®, Fibre optic light guides, Australia) was used to obtain adequate lighting for surgery. A heated surgical board (Ratek Instruments, Australia) was used to maintain body temperature of the animals during surgery.

2.3.2.3 Animal care and anaesthesia

The care and anesthesia of animals have been described in Section 2.2.2.2.

2.3.2.4 Cannulation of the right carotid artery

A longitudinal incision (1.5 cm) was made to the right of the central neck muscle, starting from above the right breast bone towards the head. The carotid artery (visualised as a red, pulsating vessel running parallel to the white vagus nerve ~ 1 cm below the skin surface) was isolated by blunt dissection of surrounding connective tissue and muscle layers. Particular care was taken in separating the carotid artery from the vagus nerve. Blood flow to the artery from the heart was temporarily halted by the placement of a pair of fine tip straight forceps (bridged over a 10 mL syringe to provide leverage) underneath the vessel. A small incision was made on the top surface of the vessel using iridectomy scissors. A bevelled 30 cm piece of polyethylene tubing (0.50 mm i.d., 0.80 mm o.d., Microtube Extrusions, Australia; attached to a 25G syringe containing 2 I.U/mL heparinised saline) was then inserted into the incision, advanced 2.5 cm into the vessel, and secured in place with surgical sutures (with the aid of a haemostat if needed). To ensure
cannula patency, the cannula was flushed with 0.2 mL heparinised saline and sealed using a naked flame. The neck incision was closed with surgical suture.

2.3.2.5 Cannulation of the right jugular vein

A longitudinal incision (1.5 cm) was made to the right of the central neck muscle, starting from above the right breast bone towards the head. The jugular vein (visualised as a bluish-grey vessel close to the skin surface) was isolated by blunt dissection of surrounding connective tissue and muscle layers. A ligature was placed around the vessel (using surgical suture), anterior to the site of cannulation, to halt blood return from the brain via the right jugular vein. A small incision was made on the top surface of the vessel using iridectomy scissors. A bevelled 30 cm piece of polyethylene tubing (0.50 mm i.d., 0.80 mm o.d., Microtube Extrusions, Australia; attached to a 25G syringe containing 2 I.U/mL heparinised saline) was then inserted into the incision, advanced 2 cm into the vessel, and secured in place with surgical suture. To ensure cannula patency, the cannula was flushed with 0.2 mL heparinised saline and sealed using a naked flame. The neck incision was closed with surgical suture.

2.3.2.6 Cannulation of the common bile duct

The abdominal muscle wall was opened by a latitudinal incision (~ 3 cm) 1-2 cm below the ribcage, approximately 0.5 cm to the left side of the animal from the midline. The duodenal loop was isolated and exteriorised to expose the common bile duct, which may be visualised as a small, yellow vessel originating from the liver and draining into the duodenum. The duct was cleared of surrounding connective tissue using forceps, and a small puncture hole made on the top surface, close to the duodenum, with a 25G needle. A bevelled 15 cm piece of polyethylene tubing (0.28 mm i.d., 0.68 mm o.d., Microtube Extrusions, Australia) was then inserted into the duct and secured in place with surgical sutures. The position of the cannula was selected to be below the
entry points of all exocrine branches from the liver and pancreas. The cannula was externalised through the abdominal incision which was subsequently closed with surgical suture.

2.3.2.7 Cannulation of the duodenum

The abdomen muscle wall was opened by a latitudinal incision (~2 cm) 1-2 cm below the ribcage, approximately 0.5 cm to the left side of the animal from the midline. The duodenum (identified as the brighter pink section of the small intestine, which upon gentle downward pulling reveals the stomach) was isolated and a small puncture hole made in the duodenum, 1 cm below the pylorus, with a 23G needle. A bevelled, J-shaped (previously heat-moulded with a cigarette lighter) piece of polyethylene tubing (0.58 mm i.d., 0.96 mm o.d., Microtube Extrusions, Australia) was then inserted into the hole and glued in place using instant cyanoacrylate adhesive. The cannula was externalised through the abdominal incision which was subsequently closed with surgical sutures.

2.4 VALIDATION OF ANALYTICAL METHODS

2.4.1 HPLC assays for quantification of cinnarizine, halofantrine, fenofibrate, danazol and meclofenamic acid in intestinal lipid colloidal phases

The concentration of cinnarizine, halofantrine, fenofibrate, danazol and meclofenamic acid in intestinal lipid colloidal phases was determined by High Performance Liquid Chromatography (HPLC). The HPLC system used for the quantification of cinnarizine consisted of a Waters 610 fluid unit, Waters 717 autosampler, Model 600 fluid controller (Waters, Milford, MA, USA) and a RF-10A XL fluorescence detector supplied by Shimadzu (Shimadzu Corp., Kyoto, Japan). Data was recorded and integrated using Empower 2 personal chromatography data software (Waters,
Milford, MA, USA). The HPLC system (Shimadzu Corp., Kyoto, Japan) used for the quantification of halofantrine, fenofibrate, danazol and meclofenamic acid consisted of a LC-20AD binary pump, a SIL-20A HT autosampler, and a temperature-controlled column compartment (CTO-20A) coupled with a RF-10A XL fluorescence detector and a SPD-20A UV/Vis detector. The column compartment was maintained at 40 °C. The chromatographic data were recorded and integrated using LabSolutions software package (Shimadzu). The injection volume for all samples was 50 µL. The HPLC assay conditions for the five compounds are described in Table 2.3.

The assays were validated on three separate days by analysing drug standards (prepared at low, medium and high concentrations) in quadruplicate and comparing against a linear standard curve. The assays for all five compounds were found to be accurate (to within ± 10% of target concentration except for the lower limit of quantitation which was ± 15%) and precise (% CV < 10 for all concentrations except the lower limit of quantification which was < 15) for the concentration ranges reported in Table 2.3. The validation results are summarised in Table 2.4.
Table 2.3: HPLC assay conditions for cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) in intestinal lipid colloidal phases.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Flow rate (mL/min)</th>
<th>Detection</th>
<th>Retention time (min)</th>
<th>Validated concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN</td>
<td>Waters Symmetry® C18, 5 µm, 3.9 x 150 mm</td>
<td>50% v/v Acetonitrile : 50% v/v 20 mM NH₄H₂PO₄</td>
<td>1.0</td>
<td>Fluorescence λ = 249/311 nm</td>
<td>5.7</td>
<td>20–1000 ng/mL</td>
</tr>
<tr>
<td>HF</td>
<td>Phenomenex Luna® C8(2), 5 µm, 4.6 x 250 mm</td>
<td>75% v/v Acetonitrile : 25% v/v H₂O (with 0.2% w/v SDSa &amp; 0.2% v/v acetic acid)</td>
<td>1.5</td>
<td>UV λ = 254 nm</td>
<td>4.2</td>
<td>0.5–25 µg/mL</td>
</tr>
<tr>
<td>FF</td>
<td>Waters Xbridge™ C18, 5 µm, 4.6 x 150 mm</td>
<td>80% v/v Acetonitrile : 20% v/v H₂O (total 0.01% v/v formic acid)</td>
<td>1.0</td>
<td>UV λ = 286 nm</td>
<td>3.9</td>
<td>0.5–25 µg/mL</td>
</tr>
<tr>
<td>DAN</td>
<td>Waters Xbridge™ C18, 5 µm, 4.6 x 150 mm</td>
<td>75% v/v Methanol : 25% v/v H₂O</td>
<td>1.0</td>
<td>UV λ = 286 nm</td>
<td>4.4</td>
<td>0.25–50 µg/mL</td>
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<tr>
<td>MFA</td>
<td>Phenomenex Luna® C8(2), 5 µm, 4.6 x 250 mm</td>
<td>80% v/v Acetonitrile : 20% v/v 10 mM H₃PO₄</td>
<td>1.0</td>
<td>UV λ = 285 nm</td>
<td>4.8</td>
<td>0.5–25 µg/mL</td>
</tr>
</tbody>
</table>

a SDS is sodium dodecyl sulphate
Table 2.4: Precision (% coefficient of variation) and accuracy (% of the target value) of the HPLC assays for cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) in intestinal lipid colloidal phases.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target concentration</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Precision</td>
<td>Accuracy</td>
<td>Precision</td>
</tr>
<tr>
<td>CIN</td>
<td>50 ng/mL</td>
<td>1.9</td>
<td>107.7</td>
<td>1.5</td>
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<td></td>
<td>200 ng/mL</td>
<td>1.0</td>
<td>95.1</td>
<td>0.5</td>
</tr>
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<td></td>
<td>1000 ng/mL</td>
<td>0.2</td>
<td>101.8</td>
<td>0.2</td>
</tr>
<tr>
<td>HF</td>
<td>0.5 µg/mL</td>
<td>0.5</td>
<td>102.4</td>
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<td></td>
<td>5 µg/mL</td>
<td>0.1</td>
<td>100.4</td>
<td>0.2</td>
</tr>
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<td></td>
<td>25 µg/mL</td>
<td>0.2</td>
<td>100.1</td>
<td>0.2</td>
</tr>
<tr>
<td>FF</td>
<td>0.5 µg/mL</td>
<td>0.2</td>
<td>94.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5 µg/mL</td>
<td>0.2</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>25 µg/mL</td>
<td>0.2</td>
<td>100.4</td>
<td>0.0</td>
</tr>
<tr>
<td>DAN</td>
<td>0.25 µg/mL</td>
<td>0.3</td>
<td>110.0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>5 µg/mL</td>
<td>0.2</td>
<td>99.4</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>50 µg/mL</td>
<td>0.1</td>
<td>100.0</td>
<td>0.1</td>
</tr>
<tr>
<td>MFA</td>
<td>0.5 µg/mL</td>
<td>0.5</td>
<td>99.7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>5 µg/mL</td>
<td>0.6</td>
<td>98.1</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>25 µg/mL</td>
<td>0.6</td>
<td>101.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>
2.4.2 HPLC assay for quantification of cinnarizine in rat plasma

Cinnarizine plasma samples were prepared for HPLC analysis using a validated extraction procedure. Standards for the assay were prepared by spiking 100 μL aliquots of blank plasma (in 4 mL polypropylene tubes) with 20 μL of 50, 100, 200, 400, 800, 1600 ng/mL cinnarizine in mobile phase solution, which provided spiked plasma concentrations in the range of 10-320 ng/mL cinnarizine. 20 μL of an internal standard solution (500 ng/mL flunarizine in mobile phase) was also added, and tubes briefly vortexed. 20 μL of trichloroacetic acid was added to precipitate plasma protein, and the tubes vortexed for 1 min. 2 mL of tert-butyl methyl ether (TBME) was added to extract cinnarizine and flunarizine into the organic phase. The tubes were vortexed for 1 min, allowed to equilibrate for 10 min, and vortexed for a further minute before being centrifuged at 10,000 xg for 15 min. 1.6 mL aliquots of the supernatant were then transferred into new polypropylene tubes, and TBME evaporated using a nitrogen evaporator at 40 °C. Cinnarizine and flunarizine in the tubes were reconstituted with 150 μL mobile phase and assayed via HPLC.

The HPLC assay conditions for the quantification of cinnarizine in rat plasma were similar to that for the quantification of cinnarizine in intestinal lipid colloidal phases described in Section 2.4.1, with slight modifications. The mobile phase used in the plasma assay consisted 45% v/v acetonitrile : 55% v/v 20 mM NH₄H₂PO₄, i.e. the organic solvent content was lowered to enable better separation of cinnarizine and flunarizine peaks. The injection volume was 100 μL, and the retention time for cinnarizine and flunarizine were 8.9 min and 11.3 min, respectively. Unknown concentrations were determined by comparing the unknown cinnarizine:flunarizine area under the curve (AUC) ratio against a standard curve of cinnarizine:flunarizine AUC ratio vs. cinnarizine concentration. The assay was validated on three separate days by analysing drug standards (prepared at low, medium and high concentrations) in quadruplicates and comparing against a linear standard curve. The assay was found to be accurate (to within ± 10 % of target
concentration except for the lower limit of quantitation which was ± 15%) and precise (% CV < 10 for all concentrations except the lower limit of quantification which was < 15) for cinnarizine plasma concentrations between 10-320 ng/mL. The validation results are shown in Table 2.5.

**Table 2.5:** Precision (% coefficient of variation) and accuracy (% of the target value) of the plasma cinnarizine HPLC assay.

<table>
<thead>
<tr>
<th>[Cinnarizine] (ng/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precision</td>
<td>Accuracy</td>
<td>Precision</td>
</tr>
<tr>
<td>10</td>
<td>11.5</td>
<td>91.0</td>
<td>6.6</td>
</tr>
<tr>
<td>80</td>
<td>2.2</td>
<td>91.4</td>
<td>6.3</td>
</tr>
<tr>
<td>320</td>
<td>9.0</td>
<td>100.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>

### 2.4.3 LC-MS assay for quantification of danazol in rat plasma

Danazol plasma samples were prepared for LC-MS analysis using a validated precipitation procedure. Standards for the assay were prepared by spiking 125 µL aliquots of blank plasma with 5 µL of 125, 250, 1250, 2500 and 6250 ng/mL danazol in acetonitrile, which provided spiked plasma concentrations in the range of 5-250 ng/mL danazol. 5 µL of an internal standard solution (2000 ng/mL progesterone in acetonitrile) was also added to each sample, and tubes vortexed for 30 sec. Following that, 62.5 µL of saturated ammonium sulphate solution was added, tubes vortexed for 30 sec; 125 µL ACN was added, and the tubes vortexed again for 30 sec. The tubes were left to stand for 20 min at room temperature prior to centrifugation at 35,000 xg for 5 min. 100 µL aliquots of the organic phase (top layer of supernatant) were then transferred into autosampler vials and 10 µL injected onto the LC-MS. Unknown concentrations were determined
by comparing the unknown danazol:progesterone peak height ratio against a standard curve of danazol:progesterone peak height ratio vs. danazol concentration.

The LC-MS system (LCMS 2020, Shimadzu, Japan) consisted a LC-20AD binary pump, a SiL-20AC Ht refrigerated autosampler, a mobile phase vacuum degassing unit (DGU-20A3), and a temperature-controlled column compartment (CTO-20A) coupled with a single-quadrupole mass spectrometric (MS) detector (Shimadzu LCMS 2020) equipped with an electrospray ionisation source. The autosampler was maintained at 4 °C and the column at 40 °C. The stationary phase was a Phenomenex Gemini® C6 phenyl column (50 × 2.0 mm, 3 µm). Samples were eluted using a gradient at a total flow rate of 0.3 mL/min. The mobile phases consisted a mixture of solvent A (95% v/v water : 5% v/v methanol ) and solvent B (5% v/v water : 95% v/v methanol) both containing 1 mM ammonium formate and 0.1% v/v formic acid. The initial percentage of solvent B was 60% and was linearly increased to 100% over 6.5 min and was held at 100% for 7 min. After 14.25 min, the gradient was returned to 60% solvent B which was held until the end of the run (17 min). The MS conditions were as follows: drying gas flow 20 L/min; nebulising gas flow 1.5 L/min; drying gas temperature 200 °C; interface voltage 3.5 kV; detector voltage 1.0 kV. Selected-ion monitoring was accomplished at m/z +338.2 for danazol [M+H]⁺ and m/z +314.9 for progesterone [M+H]⁺. The chromatographic data were acquired and analysed using LabSolutions software package (Shimadzu).

The assay was validated on three separate days by analysing drug standards (prepared at low, medium and high concentrations) in quadruplicates and comparing against a linear standard curve. The assay was found to be accurate (to within ± 10 % of target concentration except for the lower limit of quantitation which was ± 15%) and precise (% CV < 10 for all concentrations except the
lower limit of quantification which was < 15) for cinnarizine plasma concentrations between 5-250 ng/mL. The validation results are shown in Table 2.6.

**Table 2.6:** Precision (% coefficient of variation) and accuracy (% of the target value) of the plasma danazol LC-MS assay.

<table>
<thead>
<tr>
<th>[Danazol] (ng/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precision</td>
<td>Accuracy</td>
<td>Precision</td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>96.0</td>
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</tr>
<tr>
<td>250</td>
<td>6.4</td>
<td>97.6</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**2.4.4 Enzymatic colorimetric assay for quantification of total bile salt in whole rat bile**

Total bile salt concentration in whole rat bile was analysed using an enzymatic colorimetric assay (Total Bile Acids kit # 431-15001; Wako Pure Chemical Industries, Osaka, Japan). Standards for the assay were freshly prepared 50, 100, 200, 400, 500 µM sodium glycocholate in water. Rat bile samples were diluted 1:100 with water. Two 20 µL aliquots of sample were added to two wells of a 96-well plate. 50 µL of ‘enzyme reagent’ and 50 µL of ‘enzyme reagent for blank test’ were added to the first and second sample, respectively. The plate was then incubated at 37 °C for 10 min. At the end of the incubation period, 50 µL of ‘stopper solution’ was added to both samples. Sample absorbance was then measured at a wavelength of 540 nm on plate reader (Fluostar Optima plate reader, BMG Labtechnologies, Germany). The difference between the specific absorption of the sample treated with ‘enzyme reagent’ and the sample treated with ‘enzyme reagent for blank test’ was used in the determination of total bile salt concentration. The assay was
validated on three separate days by analysing three standards (prepared at low, medium and high concentrations) in quadruplicates and comparing against the linear standard curve. The assay was found to be accurate (to within ± 10 % of target concentration except for the lower limit of quantitation which was ± 15%) and precise (% CV < 10 for all concentrations except the lower limit of quantification which was < 15) for total bile salt concentrations between 50-500 µM. The validation results are shown in Table 2.7.

**Table 2.7:** Precision (% coefficient of variation) and accuracy (% of the target value) of the total bile salt enzymatic colorimetric assay.

<table>
<thead>
<tr>
<th>[Glycocholate] (µM)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precision</td>
<td>Accuracy</td>
<td>Precision</td>
</tr>
<tr>
<td>50</td>
<td>13.2</td>
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<td>200</td>
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</tr>
<tr>
<td>500</td>
<td>4.0</td>
<td>101.9</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

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

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concept and design of studies, planning and execution of experimental work, data analysis and interpretation, formulation of conclusions and hypotheses resulting from the relevant studies, drafting and revision of manuscript</td>
<td>60%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.J.H. Porter</td>
<td>Project supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
<tr>
<td>N.L. Trevaskis</td>
<td>Project co-supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
<tr>
<td>T. Quach</td>
<td>Synthesis of sulfo-N-succinimidyl oleate</td>
<td>NA</td>
</tr>
<tr>
<td>W.N. Charman</td>
<td>Manuscript review</td>
<td>NA</td>
</tr>
<tr>
<td>P. Tso</td>
<td>Manuscript review</td>
<td>NA</td>
</tr>
</tbody>
</table>

Candidate’s Signature

Declaration by co-authors

The undersigned hereby certify that:

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

Location(s) Monash Institute of Pharmaceutical Sciences
Department of Pathology and Laboratory Medicine, University of Cincinnati
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<td>Signature 4</td>
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<tr>
<td>Signature 5</td>
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</table>
CHAPTER 3 : INTESTINAL BILE SECRETION PROMOTES DRUG ABSORPTION FROM LIPID COLLOIDAL PHASES VIA INDUCTION OF SUPERSATURATION

Yan Yan Yeap\textsuperscript{1}, Natalie L. Trevaskis\textsuperscript{1}, Tim Quach\textsuperscript{2}, Patrick Tso\textsuperscript{3}, William N. Charman\textsuperscript{1}, Christopher J. H. Porter\textsuperscript{1}

\textsuperscript{1} Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria, 3052, Australia
\textsuperscript{2} Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria, 3052, Australia
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\textit{Molecular Pharmaceutics (2013); In press.}
3.1 ABSTRACT

The oral bioavailability of poorly water-soluble drugs (PWSD) is often significantly enhanced by co-administration with lipids in food or lipid-based oral formulations. Co-administration with lipids promotes drug solubilisation in intestinal mixed micelles and vesicles, however, the mechanism(s) by which PWSD are absorbed from these dispersed phases remain poorly understood. Classically, drug absorption is believed to be a product of the drug concentration in free solution and the apparent permeability across the absorptive membrane. Solubilisation in colloidal phases such as mixed micelles increases dissolution rate and total solubilised drug concentrations, but does not directly enhance (and may reduce) the free drug concentration. In the absence of changes to cellular permeability (which is often high for lipophilic, PWSD), significant changes to membrane flux are therefore unexpected. Realising that increases in effective dissolution rate may be a significant driver of increases in drug absorption for PWSD, we explore here two alternate mechanisms by which membrane flux might also be enhanced: (1) collisional drug absorption where drug is directly transferred from lipid colloidal phases to the absorptive membrane, and (2) supersaturation-enhanced drug absorption where bile mediated dilution of lipid colloidal phases leads to a transient increase in supersaturation, thermodynamic activity and absorption. In the current study, collisional uptake mechanisms did not play a significant role in the absorption of a model PWSD, cinnarizine, from lipid colloidal phases. In contrast, bile-mediated dilution of model intestinal mixed micelles and vesicles led to drug supersaturation. For colloids that were principally micellar, supersaturation was maintained for a period sufficient to promote absorption. In contrast, for primarily vesicular systems, supersaturation resulted in rapid drug precipitation and no increase in drug absorption. This work suggests that on-going dilution by bile in the gastrointestinal tract may invoke supersaturation in intestinal colloids and promote absorption, and thus presents a new mechanism by which lipids may enhance the oral absorption of PWSD.
3.2 INTRODUCTION

The potential for lipid-based formulations (LBF) to enhance the oral bioavailability of poorly water-soluble drugs (PWSD) has been recognised for over 40 years\(^2\). Lipid co-administration is thought to enhance the oral absorption of PWSD by providing mechanisms to overcome both slow dissolution and low solubility of PWSD in the aqueous gastrointestinal (GI) milieu. Firstly, LBF present drug to the GI tract in a molecularly dispersed form (i.e. in solution in the formulation), thereby circumventing the need for dissolution from the solid to the liquid state. Subsequently, the intercalation of formulation lipids into endogenous lipid digestion pathways results in the generation of intestinal lipid colloidal phases (such as vesicular and micellar species) that enhance the solubilisation capacity of the small intestine, promote drug solubilisation and reduce the risk of drug precipitation.

Solubilisation within lipid colloidal phases therefore increases the apparent solubility of PWSD in the intestinal fluids and promotes dissolution. However, in the absence of solid drug, solubilisation also results in a reduction in thermodynamic activity\(^{143}\). In simple micellar systems this reduction in thermodynamic activity is manifest in a decrease in the free concentration of drug. Where solubilised drug exists in equilibrium between the free concentration ($C_{\text{free}}$) and the concentration solubilised in intestinal colloids ($C_{\text{colloid}}$), the total solubilised drug concentration ($C_{\text{total}}$) is the sum of $C_{\text{free}}$ and $C_{\text{colloid}}$,

$$C_{\text{total}} = C_{\text{free}} + C_{\text{colloid}}$$  \hspace{1cm} \text{Equation 3.1}

Under these circumstances, the solubility of drug in the inter-micellar phase (effectively the aqueous solubility of the drug) provides the upper limit for $C_{\text{free}}$ and the presence of solubilising colloidal species typically increase the total drug concentration but do not increase, and often
reduce, $C_{\text{free}}$. Classical models of passive drug absorption suggest that drug flux across an absorptive membrane is the product of the free drug concentration and the drug permeability across the membrane. Therefore, where solubilisation reduces $C_{\text{free}}$ (but does not alter permeability), absorption is expected to be reduced. Indeed, in solubilised systems, even at saturation, $C_{\text{free}}$ does not exceed the equilibrium solubility of drug in (non-micellar) aqueous solution. Solubilisation in intestinal colloidal phases therefore provides no practical advantage in free drug concentration, and in the absence of changes to permeability, is unlikely to lead to appreciable increases in membrane flux when compared to an aqueous solution containing drug at close to saturated solubility. In support of this suggestion, many authors have shown that increasing the total concentration of a PWSD by solubilisation does not necessarily result in proportional increases in absorptive flux\textsuperscript{100,143-146}. Dahan \textit{et al.} and Miller \textit{et al.} recently proposed a model to quantify this phenomena and referred to the existence of a ‘solubility-permeability interplay’ where potential increases in membrane flux due to increases in solubilised drug concentration were off-set by a reduction in the apparent permeability, the latter being, in large part, a function of decreases in free fraction\textsuperscript{100,146}.

The dispersion and digestion of LBF therefore creates a solubilised reservoir that is in rapid equilibrium with drug in free solution and provides significant advantage in the effective rate of dissolution of a PWSD when compared to traditional dissolution from a solid dose form. In contrast, the inherent solubility limitations to flux (rather than dissolution-rate limitations) are seemingly unchanged when viewed from the perspective of the free concentration or may be made worse. This appears at odds with the wealth of experimental and practical observations that suggest the ability of lipids (either formulation- or dietary-derived) to enhance the oral absorption of a range of PWSD\textsuperscript{2}. A possible explanation for this anomaly is that the traditional view of drug absorption from colloidal dispersions may not adequately describe the dynamic manner in which
LBF exert absorption-enhancing effects in the GI tract. Two alternative potential mechanisms of drug absorption are therefore examined here (Figure 3.1).

**Figure 3.1:** Alternative mechanisms of drug absorption from intestinal lipid colloidal phases. In collisional drug absorption (left panel), in addition to the diffusion of free drug molecules (a), lipid colloidal phases collide with the absorptive membrane and facilitate direct transfer of solubilised drug into absorptive cells (b). Collisional transfer may or may not be receptor-mediated. In supersaturation-enhanced drug absorption (right panel), the interaction between secreted bile and lipid colloidal phases leads to drug supersaturation (possibly via stimulation of phase changes to less lipid-rich colloids with lowered drug solubilisation capacity). The increase in thermodynamic activity manifests in increases in free drug concentration, and enhanced diffusional flux (a) across the absorptive membrane.

The first mechanism evaluated was the potential for drug absorption to occur via direct collisional transfer from lipid colloidal phases to the absorptive membrane, and thus to be mediated not only
by $C_{\text{free}}$ but also by the solubilised fraction, $C_{\text{colloid}}$. Previously, studies by Storch and colleagues have shown that the transfer of poorly water-soluble fatty acids between model cell membranes and proteins may occur via collisional transfer\textsuperscript{150, 151}. More recently, the possibility of drug absorption via collisional uptake has been suggested by Yano et al. and Gao et al.\textsuperscript{190, 191}. Collisional uptake may or may not be receptor-mediated\textsuperscript{192}, however, lipid uptake receptors such as CD36\textsuperscript{61}, SR-BI\textsuperscript{67} and NPC1L1\textsuperscript{68} have been suggested to facilitate the absorption of cholesterol and fatty acids; poorly water-soluble molecules that are also solubilised in intestinal colloidal phases. In the case of SR-BI and CD36, direct interaction of the receptor with colloidal structures such as HDL (high-density lipoprotein), bile salt micelles and phospholipid vesicles\textsuperscript{66, 67}, has also been suggested, raising the possibility that lipid uptake receptors may interact directly with intestinal lipid colloidal phases to facilitate collisional absorption of solubilised contents, including PWSD.

The second mechanism evaluated was the potential for endogenous lipid processing pathways to lead to drug supersaturation in lipid colloidal phases. Supersaturation increases the thermodynamic activity of solubilised drug, and in the solubilisation model described by Equation 3.1, will increase $C_{\text{free}}$ above the equilibrium aqueous drug solubility. This in turn is expected to enhance drug flux. The potential for supersaturation to enhance the oral bioavailability of PWSD has received increasing recent interest\textsuperscript{173, 191}. For LBF, supersaturation may be generated by the loss of drug solubilisation capacity resulting from the digestion of triglycerides\textsuperscript{91, 176, 193} and/or surfactants\textsuperscript{177}, and the dilution of co-solvents\textsuperscript{5} during GI processing\textsuperscript{176}. In contrast, the possibility that supersaturation may result directly from interactions between lipid colloidal phases and biliary fluids has been almost entirely ignored. Although traditional micellar solubilisation models suggest that increases in bile salt concentrations increase drug solubilisation, previous studies have also shown that dilution of lipid colloidal phases with model intestinal fluids (containing bile salts,
phospholipid and cholesterol) may lead to the generation of less lipid-rich colloidal phases with lowered solubilisation capacities\textsuperscript{194}. This provides a plausible mechanism for supersaturation generation in the small intestine and has been examined in detail here.

The data suggest that under the conditions explored, collisional drug absorption has little impact on drug absorption from intestinal colloidal species. In contrast, supersaturation-enhanced drug absorption, mediated by the interaction between secreted bile and lipid colloidal phases, may provide an endogenous mechanism to promote supersaturation and to facilitate drug absorption from lipid colloidal phases.

### 3.3 METHODS

#### 3.3.1 Materials

Cinnarizine, flunarizine dihydrochloride, monensin sodium, sodium taurocholate, sodium taurodeoxycholate, sodium glycocholate, sodium glychenodeoxycholate, cholesterol, \(L-\alpha\)-lysophosphatidylcholine (LPC, from egg yolk), \(L-\alpha\)-phosphatidylcholine (PC, from dried egg yolk), oleic acid, caprylic acid, monocaprylin, \(N\)-hydroxysulfosuccinimide sodium, dicyclohexylcarbodiimide solution (60%w/v in xylenes), \(N,N\)-dimethylformamide, TWEEN\textsuperscript{®} 80, potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}) and sodium chloride (NaCl) were obtained from Sigma-Aldrich, Australia. Sodium taurochenodeoxycholate, sodium glycodeoxycholate, orthophosphoric acid 85% (H\textsubscript{3}PO\textsubscript{4}), sodium hydroxide pellets (NaOH), \textit{tert}-butyl methyl ether (TBME), dimethyl sulfoxide (DMSO), glacial acetic acid and absolute ethanol were from Merck, Australia. Disodium hydrogen orthophosphate (Na\textsubscript{2}HPO\textsubscript{4}), sodium dihydrogen orthophosphate (NaH\textsubscript{2}PO\textsubscript{4}.2H\textsubscript{2}O) and ammonium dihydrogen orthophosphate (NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}) (Ajax Finechem,
Australia), cholesterol, \([4^{14}C]\)- (49.8 mCi/mmol) and Irga-Safe Plus™ (Perkin Elmer Life Sciences, MA, USA), oleic acid, \([9,10-^3H(N)]\) (60 Ci/mmol) (American Radiolabelled Chemicals, MO, USA), Block Lipid Transport-1 (BLT-1, \textit{i.e.} 2-hexyl-1-cyclopentanone thiosemicarbazone) (Chembridge, CA, USA), ezetimibe (Jai Radhe Sales, AMD, India), heparin sodium injection BP (1000 I.U./mL, Hospira, Australia), xylazine (100 mg/mL, Troy Laboratories, Australia), acepromazine (10 mg/mL, Ceva Delvet, Australia), ketamine (100 mg/mL, Provet, Australia) and pentobarbitone sodium (325 mg/mL, Virbac, Australia) were obtained from listed suppliers. Acetonitrile, methanol, and chloroform used were analytical reagent grade. Water was obtained from a Millipore milliQ Gradient A10 water purification system (Millipore, MA, USA).

3.3.1.1 Sulfo-N-succinimidyl oleate (SSO) synthesis

SSO synthesis was adapted from the procedure of Harmon \textit{et al.} Briefly, dicyclohexylcarbodiimide (1.26 mmol) and \(N\)-hydroxysulfosuccinimide (sodium salt, 1.20 mmol) were added to a solution of oleic acid (1.20 mmol) dissolved in \(N,N\)-dimethylformamide (2 mL) and the reaction stirred at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and ethyl acetate (2 mL) added to the filtrate, which was left to stand at 4° C overnight. SSO (precipitate) was then collected by filtration and dried under vacuum (1 mmHg). SSO identity was confirmed by NMR and mass spectrometry.

3.3.2 Experimental outline

To assess the role of \textit{receptor-mediated collisional drug absorption}, cinnarizine bioavailability was assessed after administration of a lipid emulsion formulation in the absence and presence of BLT-1, SSO, and ezetimibe. BLT-1, SSO and ezetimibe are chemical inhibitors of SR-BI, CD36, and NPC1L1, respectively. The possibility of endocytosis-mediated uptake was also investigated by the use of a general endocytosis inhibitor, monensin.
The role of collisional drug absorption was assessed more generically using an in situ rat jejunum perfusion model to compare the absorptive flux of cinnarizine from two distinctly different lipid colloidal phases (micelles vs. vesicles) with matched drug solubilisation capacities. Colloidal systems with the same total solubilisation capacity, loaded with drug at the same concentration have the same thermodynamic activity and therefore C_{free} is the same in both cases. Under these circumstances, comparison of the flux profiles obtained from two structurally different colloids, but with identical C_{free}, provides a means of determining whether the nature of the colloid, or C_{free}, is the principal determinant of absorption. Generation of identical flux profiles from both systems would therefore confirm the dependence of flux on thermodynamic activity and free concentration, whereas a significant difference in flux would indicate a role for factors beyond C_{free} in determining flux. These include the potential for collisional drug absorption mechanisms since collision rates are a statistical function of particle number and are expected to be markedly higher for micelles (where the smaller particle size results in higher particle numbers) when compared to vesicles.

To assess the potential for intestinal fluids to enhance drug absorption from lipid colloidal phases via the induction of drug supersaturation, whole bile was collected from fasted rats, and mixed with model micelles and vesicles to simulate the process of interaction with bile in vivo. The potential for bile to generate drug supersaturation was evaluated in vitro by assessing changes in cinnarizine solubility, and by monitoring the kinetics of cinnarizine solubilisation and precipitation, following bile addition to cinnarizine-loaded micelles and vesicles. Subsequently, the impact of drug supersaturation on the intestinal absorptive flux of cinnarizine from micelles and vesicles was assessed in an in situ rat jejunum perfusion model, with and without co-perfusion of donor bile. Finally, the relevance of bile-induced drug supersaturation in vivo was assessed via
examination of changes to cinnarizine bioavailability after administration of drug-loaded micelles and vesicles (with matched thermodynamic activity) in bile-intact vs. bile-diverted rats.

### 3.3.3 Formulation preparation

#### 3.3.3.1 Lipid emulsion

The lipid emulsion (3 mL per dose) consisted of 1 mg cinnarizine and 49 mg oleic acid solubilised in 8 mM sodium taurocholate, 2 mM phosphatidylcholine, 2 mM cholesterol and trace amounts of $^{14}$C-cholesterol (1 µCi/3 mL) and/or $^3$H-oleic acid (3 µCi/3 mL). The emulsion was prepared in 7.5 mL batches by weighing appropriate masses of cinnarizine in oleic acid stock solution (20 mg/g), phosphatidylcholine and cholesterol into a glass vial, and the mixture made up to volume with a buffered sodium taurocholate solution (buffer consisted 18 mM Na$_2$H$_2$PO$_4$.2H$_2$O and 12 mM Na$_2$HPO$_4$). Appropriate volumes of $^{14}$C-cholesterol, $^3$H-oleic, 5 mg/mL BLT-1 in ethanol (for SR-BI inhibition experiments only), 25 mg/mL SSO in DMSO (for CD36 inhibition experiments only), and 10 mg/mL monensin in ethanol (for endocytosis inhibition experiments only) were spiked into the vial, and vortexed for 1 min. The formulation was emulsified by ultrasonification with a Misonix XL 2020 ultrasonic processor (Misonix, Farmingdale, NY, USA) equipped with a 3.2-mm microprobe tip running at an amplitude of 240 µm and a frequency of 20 kHz for 1.5 min. The total solvent concentration in the emulsion was ≤ 2.5% v/v. The emulsion was used within 4 h of preparation, and the concentration of drug and labelled cholesterol and/or oleic acid assayed before dosing (in duplicate) to confirm compound content in the emulsion and to allow for dose normalisation between rats.
3.3.3.2 Intravenous formulation

The formulation (1 mL per dose) used for intravenous administration of cinnarizine and $^{14}$C-cholesterol comprised 0.5% w/v Tween® 80 in buffer (36 mM Na$_2$HPO$_4$ and 22 mM KH$_2$PO$_4$, adjusted to pH 4 with acetic acid). Cinnarizine and cholesterol were added to the formulation by spiking 5% v/v DMSO (containing 10 mg/mL cinnarizine) and 5% v/v ethanol (containing 5 mg/mL cholesterol and 40 µCi/mL $^{14}$C-cholesterol) into the micellar solution. The formulation was mixed by vortexing, and the concentration of drug and labelled cholesterol assayed before dosing to confirm compound content in the formulation and to allow for dose normalisation between rats. The formulation was used within 1 h of preparation.

3.3.3.3 Model micelles and vesicles

The preparation of model micelles and vesicles was guided by the methods and phase diagram published by Kossena et al.$^{39}$ Medium-chain lipid containing colloids were chosen over long-chain systems since the former have previously been shown to generate monophasic micellar and vesicular systems$^{39}$. Relatively high lipid concentrations were chosen to reflect the species that are expected to initially form during the digestion of medium-chain triglycerides$^{91}$. The model colloids consisted of tricaprylin digestion products (caprylic acid and monocaprylin) solubilised in simulated endogenous intestinal fluid (SEIF). SEIF comprised the six most prevalent bile salts in human bile$^{199}$, lysophosphatidylecholine (LPC), and cholesterol. The total bile salt:LPC:cholesterol molar ratio was maintained at 16:4:1, reflecting known ratios within fasted human bile$^{200,201}$. The combination of bile salts used here comprised 25 mol% sodium glycocholate, 17.5 mol% sodium glycodeoxycholate, 25 mol% sodium glycochenodeoxycholate, 12.5 mol% sodium taurocholate, 7.5 mol% sodium taurodeoxycholate and 12.5 mol% sodium taurochenodeoxycholate. The concentration ratios of the bile salts were chosen based on average concentrations of the six most prevalent bile salts found in human bile$^{199}$. The caprylic acid:monocaprylin molar ratio was kept at
Chapter 3: Bile-induced supersaturation

2:1, reflecting the ratio of digestion products expected on digestion of 1 mole of triglyceride. The concentration of micellar and vesicular components was varied by trial and error (but maintaining the ratios described above) to identify systems with similar drug solubilisation capacities. It has previously been shown that the thermodynamic activity (i.e. free concentration) of drug in a solubilised system may be estimated via assessment of solubility behaviour, such that different colloidal solutions containing drug at a fixed proportion of the saturated solubility, results in matched free concentrations\(^{202}\). Thus, drug was loaded into either micellar or vesicular systems at the same concentration (and the same proportion of saturated solubility) and was therefore present at the same thermodynamic activity (i.e. \(C_{\text{total}}, C_{\text{colloid}}\) and \(C_{\text{free}}\) were the same in both micellar and vesicular systems). The compositions of the identified micellar and vesicular systems are shown in Table 3.1.

### Table 3.1: Composition of model micelles and vesicles.\(^\wedge\)

<table>
<thead>
<tr>
<th></th>
<th>Total bile salt(^#) (mM)</th>
<th>LPC(^$) (mM)</th>
<th>Cholesterol (mM)</th>
<th>Caprylic acid (mM)</th>
<th>Monocaprylin (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelles</td>
<td>8</td>
<td>2</td>
<td>0.5</td>
<td>69.3</td>
<td>34.7</td>
</tr>
<tr>
<td>Vesicles</td>
<td>2</td>
<td>0.5</td>
<td>0.125</td>
<td>52.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

\(^\wedge\) Micelles and vesicles also consist of 18 mM NaH\(_2\)PO\(_4\).2H\(_2\)O and 12 mM Na\(_2\)HPO\(_4\). Sodium strength was adjusted to 150 mM with NaCl. Final pH of phases was adjusted to 6.30 ± 0.01

\(^\#\) Total bile salt consist of 25 mol% sodium glycocholate, 17.5 mol% sodium glycdeoxycholate, 25 mol% sodium glycodeoxycholate, 12.5 mol% sodium taurocholate, 7.5 mol% sodium taurodeoxycholate, 12.5 mol% sodium taurochenodeoxycholate

\(^$\) LPC is lysophosphatidylcholine

SEIF (8 mM total bile salt:2 mM LPC:0.5 mM cholesterol) was prepared in 50 mL batches. Briefly, LPC and cholesterol were dissolved in 1 mL chloroform in a round bottom flask, followed
by solvent evaporation under vacuum. The thin film formed by solvent evaporation was reconstituted with buffered bile salts solution (8 mM total bile salt, 18 mM NaH₂PO₄.2H₂O and 12 mM Na₂HPO₄, 100 mM NaCl), vortexed for 1 min, and allowed to equilibrate at room temperature overnight. When vesicles were prepared, a similar procedure was adopted, but in this case SEIF was diluted 4-fold with buffer (18 mM NaH₂PO₄.2H₂O and 12 mM Na₂HPO₄, 108 mM NaCl) to reduce the bile salt:lipid concentration ratio. Micelles and vesicles were prepared in 10 mL batches by adding caprylic acid and monocaprylin (quantities in Table 3.1) to SEIF, followed by pH adjustment to 6.30 with solid NaOH and vortexing for 1 min. The phases were then ultrasonicated as described earlier (30 sec continuous ultrasonication followed by pulsatile, 1 sec-on/1 sec-off ultrasonication for 5 min). When included in the colloids, cinnarizine was pre-dissolved in caprylic acid, and the drug/fatty acid solution allowed to equilibrate overnight prior to micelle/vesicle preparation.

### 3.3.4 Particle sizing

The particle size of the model micelles and vesicles was determined by photon correlation spectroscopy (Malvern Instruments Nano-ZS Zetasizer, Malvern, UK) at 37 °C. Light scattering cells were cleaned by rinsing with ultrapure laboratory grade water (Milli-Q, Millipore, MA, USA), and allowed to dry while inverted on lint-free wipes (Kimwipes®, Kimberly-Clark, Australia). Samples were transferred into the cells using sterile 1 mL syringes, and analysed without dilution to prevent structural changes to the phases. CONTIN analysis method was used to analyse the autocorrelation functions and to provide a calculated polydispersity index. Micelles had a mean particle size of 9 ± 1 nm [polydispersity index 0.128 ± 0.040], and vesicles had a mean particle size of 443 ± 32 nm [polydispersity index 0.456 ± 0.038]. Data reported are mean ± SEM of n = 3 determinations.
3.3.5 Equilibrium solubility of cinnarizine in the model micelles and vesicles

Excess solid cinnarizine was added to 2 mL micelles or vesicles in glass vials. Vials were briefly vortexed, incubated at 37 °C, and samples taken every 24 h over a period of 120 h. During sampling, vials were centrifuged (2,200 x g, 10 min, 37 °C), 50 µL of supernatant sampled, and vials re-vortexed. Equilibrium solubility was defined when drug concentrations in consecutive samples varied by ≤ 5%, and was determined on three separate occasions. Equilibrium solubility of cinnarizine was also determined after 1:1 addition of bile/bile pH 6.30/buffer pH 6.30 to the different colloidal phases. The pH of fresh bile was adjusted to 6.30 with H₃PO₄. Buffer pH 6.30 consisted of 18 mM NaH₂PO₄.2H₂O, 12 mM Na₂HPO₄, and 108 mM NaCl.

3.3.6 Kinetics of cinnarizine precipitation

The kinetics of cinnarizine precipitation was monitored after addition of bile to the micellar and vesicular phases, to determine whether a period of drug supersaturation existed prior to drug precipitation. In a temperature and stirring rate-controlled vessel, 2.5 mL of bile was added to 2.5 mL micelles or vesicles containing 0.2 mg/mL cinnarizine (~ 80% saturated solubility). Samples (100 µL) were taken before the addition of bile, and at 1, 10, 20, 30, 40, 50, 60, 80, 100, 120 min after bile addition. Samples were immediately centrifuged (2,200 x g, 5 min, 37 °C) to separate precipitated drug, and 50 µL of supernatant assayed for drug content. The proportion of the initial solubilised cinnarizine concentration, that remained solubilised after bile addition, was assessed as the percent of the drug mass remaining in solution (i.e. concentration in the supernatant multiplied by the volume remaining in vessel) relative to the total drug mass in the vessel at each time point.

3.3.7 Solid-state analysis of the cinnarizine precipitate

Selected cinnarizine pellets from the precipitation kinetics experiments were analysed using a Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarising
filters. At the end of the precipitation kinetics experiments, 1.5 mL of remaining bile + colloid mixture were centrifuged (2,200 xg, 10 min, 37 °C), the supernatant was discarded, and a small amount of pellet was carefully placed on a microscope slide. Samples were analysed under cross-polarised light, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan).

3.3.8 Animals

Animal care and anaesthesia have been described in Section 2.2.2.2. All animals were fasted overnight (12–18 h) prior to surgery. At the end of all experiments, rats were euthanized via an intravenous or intracardiac injection of 100 mg sodium pentobarbitone.

3.3.9 Surgical procedures

3.3.9.1 Cinnarizine bioavailability studies following intraduodenal administration

The surgical procedures for the conduct of bioavailability studies included cannulations of the right carotid artery, right jugular vein, duodenum (1 cm below pylorus), and common bile duct (only for bile-diverted rats). The surgical procedures for the cannulations have been described in Section 2.3.2.

3.3.9.2 Fasted rat bile collection

The bile duct was cannulated near the hilum of the liver (where the duct is free of pancreatic tissue) in order to facilitate the collection of bile fluid without contamination by exocrine pancreatic secretions. Rats were rehydrated via saline infusion (1.5 mL/h) into a cannula inserted into the right jugular vein, and bile continuously collected for 5 h. The concentration of
total bile salt in collected bile was assayed using a validated enzymatic colorimetric assay (Total Bile Acids kit #431-15001; Wako Pure Chemical Industries, Osaka, Japan) on a plate reader (Fluostar Optima plate reader, BMG Labtechnologies, Germany) measuring absorbance at a wavelength of 540 nm. In all subsequent experiments, bile was used within 24 h of collection.

3.3.9.3 Single-pass rat jejunum perfusion

The model employed to assess flux across rat jejunum involved *in situ* perfusion (single-pass) of an isolated jejunal segment and simultaneous blood collection from the corresponding mesenteric vein branch. The surgical procedures for the setup of the single-pass rat jejunum perfusion model have been described in Section 2.2.2.3.

3.3.10 Cinnarizine bioavailability studies

A 30-min equilibration period was allowed between the end of surgery and drug dosing. To examine the impact of lipid uptake receptors on drug absorption, studies were conducted in the presence or absence of lipid uptake inhibitors. To examine the impact of bile-induced drug supersaturation on drug absorption, studies were conducted in bile-intact or bile-diverted rats. Colloidal systems (lipid emulsion, micelles, vesicles) containing cinnarizine were infused into the duodenum of rats at a rate of 1.5 mL/h for 2 h. When the dose infusion was complete, saline was infused at a rate of 1.5 mL/h for 10 min to flush any remaining formulation in the tubing into the duodenum. Blood samples (0.3 mL) were collected via the carotid artery cannula up to 8 h after infusion initiation into tubes containing 3 I.U. heparin. The sampling intervals were: t = 0, 1, 2, 2.5, 3, 4, 6, 8 h for the receptor inhibition studies; and t = 0, 1, 1.5, 2, 3, 4, 6, 8 h for the bile-induced drug supersaturation studies. After each blood sample was taken, the cannula was flushed with 0.3 mL of 2 I.U./mL heparinised saline to ensure cannula patency, and to replace the volume of blood
removed. Plasma was separated by centrifugation (10,000 g, 5 min) to enable analysis of drug and labelled lipid content.

3.3.10.1 Administration of lipid uptake inhibitors (BLT-1, SSO, ezetimibe) and endocytosis inhibitor (monensin)

Ezetimibe has previously been dosed at 0.3 mg/kg intravenously into rats, and has been shown to inhibit cholesterol absorption (from an intraduodenally dosed lipid emulsion) without reports of toxicity\(^{204}\). Therefore, in our study, intravenous administration of ezetimibe (0.3 mg/kg via the jugular vein) was selected as the route to administer the inhibitor at the beginning of the 30-min equilibration period. An appropriate volume of 5 mg/mL ezetimibe in ethanol was spiked into blank rat plasma, and 0.8 mL of resultant plasma dosed into rats as an intravenous bolus. The total ethanol concentration was less than 2.5% v/v. In the case of BLT-1 and SSO, the inhibitors had not been previously administered intravenously, therefore local (i.e. intestinal) administration was selected to limit the systemic effects of the inhibitors. Monensin was also administered directly into the intestine to minimise systemic endocytosis inhibition. Here, 100 µM BLT-1, 1 mM SSO and 100 µM monensin were pre-infused intraduodenally (in saline) at a rate of 1.5 mL/h during the 30-min equilibration period, and subsequently co-infused at the same concentration as part of the lipid emulsion. BLT-1 and SSO have previously been shown to inhibit lipid uptake in cell-based studies at concentrations of 1-10 µM\(^{196}\) and 400 µM\(^{195}\), respectively. Monensin has previously been shown to inhibit endocytosis in cultured cells at 10 µM\(^{205}\).

Intravenous administration studies were also conducted in control rats and monensin-treated rats to assess the effect of endocytosis inhibition on the systemic distribution and clearance of cinnarizine and cholesterol. In these studies, blank (i.e. not containing cinnarizine and \(^{14}\)C-cholesterol) lipid emulsion (with or without 100 µM monensin) was infused intraduodenally as
described above, and intravenous infusion of the cinnarizine and \(^{14}\)C-cholesterol containing intravenous formulation was commenced at the same time. The intravenous formulation (1 mL) was infused into the right jugular vein at a rate of 0.05 mL/15 sec (total infusion period < 5 min). Blood samples (0.3 mL) were collected via the carotid artery cannula at \(t = 5, 15, 30, 60, 120, 180, 240, 360, 480\) min after infusion initiation into tubes containing 3 I.U. heparin. After each blood sample was taken, the cannula was flushed with 0.3 mL of 2 I.U./mL heparinised saline. Plasma was separated by centrifugation (10,000 \(\times g\), 5 min) to enable analysis of drug and labelled cholesterol content.

### 3.3.11 *In situ single-pass rat jejunum perfusion*

After surgery, animals were equilibrated for 30-min, during which time heparinised donor rat blood was infused via the jugular vein as described above. During re-equilibration, blood from the cannulated mesenteric vein (\(~0.3\) mL/min) was collected for re-infusion. Perfusion buffer was pumped through the jejunal segment at a rate of 0.1 mL/min and outflowing buffer discarded to waste. The exposed jejunal segment was kept moist by covering with saline-soaked gauze throughout the experiment.

In all experiments, the concentration of cinnarizine in the perfusate was held at 0.1 mg/mL (\(~40\%\) saturated solubility). Therefore, in experiments where micelles or vesicles were perfused alone, cinnarizine was loaded into the perfusate at 0.1 mg/mL. In experiments where micelles or vesicles were co-perfused in a 1:1 v/v ratio with a secondary perfusate of bile/bile pH 6.30/buffer pH 6.30, cinnarizine was loaded into the primary perfusate at 0.2 mg/mL, such that 1:1 v/v dilution led to a final perfusate concentration of 0.1 mg/mL.
Perfusate flow was maintained at 0.1 mL/min in all experiments to minimize variations in the thickness of the unstirred water layer\textsuperscript{206}. For experiments where 1:1 v/v co-perfusion of the phases with bile/bile pH 6.30/buffer pH 6.30 was required, micelles/vesicles and bile/buffer were pumped at 0.05 mL/min, and mixed via a three-way “T” connector immediately prior to entry into the jejunal segment, providing a total perfusate flow of 0.1 mL/min. Perfusate was sampled at \( t = 0 \) to confirm lipid and drug concentrations. After this time, the outflowing perfusate was continuously collected at 10-min intervals, and briefly vortexed before samples were taken for analysis of drug and lipid content. For experiments where drug supersaturation was generated, perfusate samples were taken before and after centrifugation (2,200 x \( g \), 2 min), to assess the degree of drug precipitation within the jejunal segment. Blood draining the perfused jejunal segment was collected at 5-min intervals, plasma separated by centrifugation (10,000 x \( g \), 5 min), and samples taken for analysis of drug content by HPLC as described below.

### 3.3.12 Analytical procedures

#### 3.3.12.1 Sample preparation and HPLC assay conditions for cinnarizine

Samples of lipid emulsion were prepared for HPLC assay by an initial 80-fold dilution with chloroform:methanol (2:1 v/v), followed by a 10-fold dilution with mobile phase (50% v/v acetonitrile:50% v/v 20 mM NH\(_4\)H\(_2\)PO\(_4\)). Samples of IV formulation and micelles/vesicles were prepared for HPLC assay by a 900-fold, and a 400-fold dilution with mobile phase (50% v/v acetonitrile:50% v/v 20 mM NH\(_4\)H\(_2\)PO\(_4\)), respectively. Plasma samples were prepared for HPLC using a validated extraction procedure, with flunarizine as an internal standard, as reported previously\textsuperscript{43}.
Cinnarizine HPLC assay conditions were as described previously, with slight modification to the mobile phase employed for cinnarizine quantification in plasma, to 45% v/v acetonitrile:55% v/v 20 mM NH₄H₂PO₄ in this study. Replicate analysis of n = 4 quality control samples revealed acceptable accuracy and precision (± 10%, ± 15% at the limit of quantification) for concentrations between 20–1000 ng/mL for IV formulation, emulsion, micelles and vesicles, and 10–320 ng/mL for plasma.

3.3.12.2 Scintillation counting

Quantification of ¹⁴C-cholesterol and ³H-oleic acid in the plasma was performed via scintillation counting on a Packard Tri-Carb 2000CA liquid scintillation analyser (Packard, Meriden, Connecticut, USA). Plasma samples (50 µL) were added to 2 mL Irga-safe Plus scintillation fluid followed by a 10-sec vortex. Samples were corrected for background radioactivity by the inclusion of a blank plasma sample in each run.

3.3.12.3 Blood:plasma ratio determination for cinnarizine

The blood:plasma ratio for cinnarizine was determined by spiking 0.5 mL blank blood with known amounts of cinnarizine to achieve low, medium, and high concentrations (in triplicate). Plasma was separated by centrifugation (10,000 xg, 5 min) and plasma drug concentration assayed by HPLC. The blood:plasma ratio was calculated from the ratio of known concentration in spiked blood to the concentration measured in plasma separated from spiked blood. The mean blood:plasma ratio was subsequently used to convert plasma concentrations to blood concentrations in perfusion experiments, enabling quantification of total drug transport into mesenteric blood.
3.3.12.4 Calculations

In the single-pass rat jejunum perfusion model, permeability coefficients were calculated using steady state drug concentrations in perfusate and blood. Two apparent permeability coefficients \( \text{P}_{\text{app}} \) were calculated as described previously:\(^{184}\):

\[
\text{‘Disappearance’ } \text{P}_{\text{app}} = -\frac{Q}{A} \cdot \ln \frac{C_1}{C_0}
\]

*Equation 3.2*

\[
\text{‘Appearance’ } \text{P}_{\text{app}} = \frac{\Delta M_B/\Delta t}{A <C>}
\]

*Equation 3.3*

where ‘Disappearance’ \( \text{P}_{\text{app}} \) is the apparent permeability coefficient calculated from drug loss from the perfusate (cm/s); ‘Appearance’ \( \text{P}_{\text{app}} \) is the apparent permeability coefficient calculated from drug appearance in the mesenteric blood (cm/s); \( Q \) is the perfusate flow rate (mL/s); \( A \) is the surface area of the perfused jejunal segment (cm\(^2\)), which is calculated by multiplying the diameter by the length of the perfused intestinal segment as described previously:\(^{207}\); \( C_1 \) is the average steady state drug concentration exiting the perfused jejunal segment (ng/mL); \( C_0 \) is the drug concentration entering the jejunal segment (ng/mL); \( \Delta M_B/\Delta t \) is the average rate of drug mass appearance in mesenteric blood at steady state (ng/s); and \(<C>\) is the logarithmic mean drug concentration in the lumen (ng/mL), where \(<C> = (C_1 - C_0)/(\ln C_1 - \ln C_0)\).

3.3.12.5 Non-compartmental pharmacokinetic analysis

The maximum plasma concentration (\( C_{\text{max}} \)), time to reach \( C_{\text{max}} \) (\( T_{\text{max}} \)), area under the plasma concentration-time curve from time zero to the last measured concentration (AUC\(_{0-8\, \text{h}}\)), area under the plasma concentration-time curve extrapolated to infinity (AUC\(_{0-\text{inf}}\)), elimination rate constant
(K<sub>e</sub>), volume of distribution (V<sub>d</sub>), and clearance (Cl) were calculated using WINNONLIN version 5.3 (Pharsight Inc., Apex, NC, USA).

### 3.3.13 Statistical analysis

Results were analysed using Student’s t test. A <i>P</i> value of < 0.05 was considered to be a significant difference. Analyses were performed using SPSS v19 for Windows (SPSS Inc., Chicago, IL, USA).

### 3.4 RESULTS

#### 3.4.1 SR-BI, CD36, NPC1L1 and endocytosis have little impact on drug absorption from intestinal colloidal phases

Inhibition of the lipid uptake receptors SR-BI (by co-infusion of 100 µM BLT-1), CD36 (by co-infusion of 1 mM SSO), and NPC1L1 (by intravenous administration of 0.3 mg/kg ezetimibe) did not result in significant changes to the systemic plasma concentration-time profiles or systemic exposure of cinnarizine (Figure 3.2A, Table 3.2). Inhibition of endocytosis (by co-infusion of 100 µM monensin) unexpectedly led to significant increases in cinnarizine systemic plasma concentration at t = 2.5 and 4 h (Figure 3.2A), and increased the AUC<sub>0-8 h</sub> 1.5-fold (Table 3.2). However, subsequent intravenous dosing studies revealed significantly lower V<sub>d</sub> and Cl values in the monensin-treated rats when compared to control (Table 3.3), suggesting that the increase in cinnarizine systemic exposure was due to a decrease in cinnarizine systemic distribution and clearance, rather than changes to intestinal absorption. The data suggest a limited role for SR-BI, CD36, NPC1L1, and endocytosis generally, in the absorption of cinnarizine from micelles and vesicles.
In contrast, inhibition of NPC1L1 did lead to significantly lower systemic plasma concentrations of exogenously dosed cholesterol at $t = 2, 2.5, 3, 4, 6, 8$ h (Figure 3.2B). Plasma concentrations of exogenously dosed oleic acid also appeared lower in rats following inhibition of CD36 although differences were not significantly different (Figure 3.2C). While inhibition of SR-BI and CD36 did not lead to significant changes to the systemic plasma concentration-time profile of exogenously dosed cholesterol, inhibition of endocytosis significantly increased the systemic plasma concentrations of exogenously dosed cholesterol at $t = 4, 8$ h (Figure 3.2B). This increase in exposure is likely explained by a decrease in systemic distribution of $^{14}$C-cholesterol in the monensin-treated rats, as plasma $^{14}$C-cholesterol concentration at early sample time points (which reflect the distribution phase) in intravenous studies were significantly higher in the monensin-treated rats (Figure 3.3B) when compared to controls ($V_d$ and $Cl$ could not be calculated for cholesterol as a typical elimination phase was not evident in the systemic plasma concentration vs. time profiles of cholesterol - Figure 3.2B and Figure 3.3B).
Figure 3.2: Systemic plasma concentration-time profiles of (A) cinnarizine (CIN), (B) $^{14}$C-labelled cholesterol (Ch), and (C) $^3$H-labelled oleic acid (OA) following intraduodenal infusion of a 3 mL lipid emulsion consisting 1 mg cinnarizine emulsified in 59 mM oleic acid, 8 mM sodium taurocholate, 2 mM phosphatidylcholine, 2 mM cholesterol, 1 µCi $^{14}$C-cholesterol and/or 3 µCi $^3$H-oleic acid. Experiments were performed in control rats (filled circle); rats treated with 100 µM BLT-1 (open circle); rats treated with 1 mM SSO (open triangle), rats treated with 0.3 mg/kg ezetimibe (open square), and rats treated with 100 µM monensin (open diamond), to inhibit the lipid uptake receptors SR-BI, CD36, NPC1L1, and endocytosis, respectively. BLT-1, SSO, and monensin were co-infused as part of the lipid emulsion; ezetimibe was administered intravenously. Data represent mean ± SEM of n = 4 rats for (A) and (B); and n = 3 rats for (C). Statistically significant difference with respect to control rats (p < 0.05) is denoted by the symbol *.
**Table 3.2:** Pharmacokinetic parameters for cinnarizine after intraduodenal administration of a 3 mL lipid emulsion in rats. Experiments were performed in control rats; rats treated with 100 µM BLT-1; rats treated with 1 mM SSO, rats treated with 0.3 mg/kg ezetimibe, and rats treated with 100 µM monensin, to inhibit the lipid uptake receptors SR-BI, CD36, NPC1L1, and endocytosis, respectively. Values represent mean ± SEM of n = 4 rats.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Formulation type</th>
<th>CIN dose (mg/kg)</th>
<th>AUC$_{0-8\ h}$ (ng h/mL)</th>
<th>C$_{max}$ (ng/mL)</th>
<th>T$_{max}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Emulsion</td>
<td>3.33</td>
<td>978 ± 122</td>
<td>288.1 ± 26.6</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>BLT-1 treated</td>
<td>Emulsion</td>
<td>3.33</td>
<td>883 ± 21</td>
<td>219.0 ± 20.0</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>SSO-treated</td>
<td>Emulsion</td>
<td>3.33</td>
<td>924 ± 88</td>
<td>195.5 ± 33.6</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Ezetimibe-treated</td>
<td>Emulsion</td>
<td>3.33</td>
<td>1048 ± 92</td>
<td>333.9 ± 13.6</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Monensin-treated</td>
<td>Emulsion</td>
<td>3.33</td>
<td>1487 ± 103$^a$</td>
<td>432.8 ± 45.9$^a$</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$Significant difference when compared to control group
Figure 3.3: Systemic plasma concentration-time profiles of (A) cinnarizine (CIN), and (B) $^{14}$C-labelled cholesterol (Ch) following intravenous infusion over 5 min of 1 mL formulation containing 0.5 mg cinnarizine and 1 µCi $^{14}$C-Ch. Experiments were performed in control rats (filled circle) and rats treated with 100 µM monensin (open diamond). Intraduodenal infusion of blank lipid emulsion was also commenced at $t = 0$ (to match experimental conditions in Figure 3.2). Monensin, an endocytosis inhibitor, was pre-infused intraduodenally 30 min prior to $t = 0$, and subsequently co-infused as part of the lipid emulsion. Data represent mean ± SEM of $n = 4$ rats for (A); and $n = 3$ rats for (B). Statistically significant difference with respect to control rats ($p < 0.05$) is denoted by the symbol *. 

Table 3.3: Pharmacokinetic parameters for cinnarizine after intravenous administration in rats. Values represent mean ± SEM of $n = 3-4$ rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>CIN dose (mg/kg)</th>
<th>$\text{AUC}_{0-\text{inf}}$ (ng h/mL)</th>
<th>$K_e$ (1/h)</th>
<th>$V_d$ (L/kg)</th>
<th>$\text{Cl}$ (L/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.67</td>
<td>821 ± 24</td>
<td>0.25 ± 0.03</td>
<td>5.8 ± 0.2</td>
<td>2.03 ± 0.06</td>
</tr>
<tr>
<td>Monensin-treated</td>
<td>1.67</td>
<td>1077 ± 28*</td>
<td>0.28 ± 0.04</td>
<td>4.0 ± 0.4*</td>
<td>1.55 ± 0.04*</td>
</tr>
</tbody>
</table>

*Significant difference when compared to control group
3.4.2 Drug absorption from micelles and vesicles is determined by $C_{\text{free}}$ and not colloidal structure

The intestinal perfusion of colloidal media with markedly different compositional profiles (micelles and vesicles) but with comparable $C_{\text{free}}$ and thermodynamic activity did not result in significant differences in steady-state absorptive flux, disappearance $P_{\text{app}}$ or appearance $P_{\text{app}}$ of cinnarizine (Table 3.4, Figure 3.4A – filled symbols). Similarly, in spite of large differences in particle size and composition, the intraduodenal infusion of the same micellar or vesicular systems to bile-diverted rats did not result in significant differences in systemic plasma concentration-time profiles (Figure 3.5 – filled symbols) and pharmacokinetic parameters of cinnarizine (Table 3.5 - micelles vs. vesicles in bile-diverted rats). The results suggest that cinnarizine absorption from lipid colloidal phases is relatively insensitive to the physical nature of the colloidal milieu, and is instead controlled largely by $C_{\text{free}}$. 
Figure 3.4: (A) Absorptive flux of cinnarizine (CIN) into mesenteric blood (ng/5 min/10 cm²) and (B) & (C) CIN disappearance from intestinal perfusate (% drug dose passing through jejunum) when micelles/vesicles were perfused through an isolated rat jejunal segment (~ 10 cm²), with (open symbols) and without (filled symbols) 1:1 v/v co-perfusion with rat bile. Co-perfusion of micelles and vesicles with donor bile generates drug supersaturation in situ within the perfused jejunal segment. SS denotes experiments where drug is supersaturated in the perfusate. The degree of drug precipitation within the perfusate is illustrated in (B) and (C) as the difference in perfusate concentration between pre- and post-centrifugation data. Precipitation was significant in the vesicle, but not micellar groups. Experiments were performed using an in situ single-pass rat jejunum perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. Data represent mean ± SEM of n = 3-4 experiments.
Table 3.4: Cinnarizine disappearance $P_{app}$ (x $10^6$ cm/s) from the intestinal perfusate, appearance $P_{app}$ (x $10^6$ cm/s) in the mesenteric blood, and steady state absorptive flux into mesenteric blood (ng/5 min/10 cm$^2$) after 60 min of single-pass perfusion of ~ 10 cm$^2$ segments of rat jejunum with model micelles and vesicles, with and without 1:1 v/v co-perfusion with rat bile, rat bile pH 6.30 or buffer pH 6.30. Values calculated using data obtained after steady state attainment ($t = 40-60$ min). In all experiments, cinnarizine concentration in perfusate and total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. Data represent mean ± SEM of n = 3-4 experiments.

<table>
<thead>
<tr>
<th></th>
<th>SS ratio</th>
<th>Disappearance $P_{app}$ (x $10^6$ cm/s)</th>
<th>Appearance $P_{app}$ (x $10^6$ cm/s)</th>
<th>Flux into mesenteric blood (ng/5 min/10 cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelles</td>
<td>0.4</td>
<td>19.4 ± 3.0</td>
<td>1.1 ± 0.2</td>
<td>310 ± 50</td>
</tr>
<tr>
<td>Micelles + Bile $^{SS}$</td>
<td>6.1</td>
<td>29.3 ± 3.0 $^a$</td>
<td>3.7 ± 0.1 $^a$</td>
<td>979 ± 30 $^a$</td>
</tr>
<tr>
<td>Micelles + Bile pH 6.30 $^{SS}$</td>
<td>3.0</td>
<td>39.5 ± 5.6 $^a$</td>
<td>4.2 ± 1.0 $^a$</td>
<td>1099 ± 279 $^a$</td>
</tr>
<tr>
<td>Micelles + Buffer pH 6.30 $^{SS}$</td>
<td>2.1</td>
<td>38.9 ± 4.1 $^a$</td>
<td>4.4 ± 0.4 $^a$</td>
<td>1180 ± 100 $^a$</td>
</tr>
<tr>
<td>Vesicles</td>
<td>0.4</td>
<td>22.0 ± 2.2</td>
<td>1.2 ± 0.1</td>
<td>340 ± 29</td>
</tr>
<tr>
<td>Vesicles + Bile $^{SS}$</td>
<td>11.6</td>
<td>66.0 ± 18.3 $^b$</td>
<td>2.0 ± 0.7</td>
<td>499 ± 155</td>
</tr>
</tbody>
</table>

$^a$ Significant increase from micelles alone  
$^b$ Significant increase from vesicles alone  
$^{SS}$ denotes drug supersaturation in perfusate  
$^#$ SS ratio = Supersaturation ratio = (Supersaturated) concentration of drug in perfusate / Equilibrium solubility of drug in perfusate
Figure 3.5: Systemic plasma concentration-time profiles of cinnarizine (CIN) following a 2 h intraduodenal infusion of cinnarizine-loaded (0.2 mg/mL) (A) micelles and (B) vesicles to bile-intact and bile-diverted rats. Consistent with observations in rat jejunum perfusion studies, bile-induced supersaturation translated into increased \textit{in vivo} exposure during the absorption phase in the case of micelles but not vesicles. Data represent mean ± SEM of \( n = 4 \) rats. Statistical significance (\( p < 0.05 \)) is denoted by the symbol \(*\).

Table 3.5: Pharmacokinetic parameters for cinnarizine after intraduodenal administration of cinnarizine-loaded (0.2 mg/mL) micelles and vesicles to bile-intact and bile-diverted rats. Values represent mean ± SEM of \( n = 4 \) rats.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Formulation type</th>
<th>CIN dose (mg/kg)</th>
<th>AUC(_{0-8,h}) (ng h/mL)</th>
<th>( C_{\text{max}} ) (ng/mL)</th>
<th>( T_{\text{max}} ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelles (bile-intact)</td>
<td>Micelles</td>
<td>2</td>
<td>502 ± 59(^a)</td>
<td>138.8 ± 11.6(^a)</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Micelles (bile-diverted)</td>
<td>Micelles</td>
<td>2</td>
<td>362 ± 39</td>
<td>85.7 ± 14.3</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Vesicles (bile-intact)</td>
<td>Vesicles</td>
<td>2</td>
<td>411 ± 74</td>
<td>109.4 ± 10.6</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Vesicles (bile-diverted)</td>
<td>Vesicles</td>
<td>2</td>
<td>393 ± 82</td>
<td>97.6 ± 12.8</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\)Significant increase when compared to micelles (bile-diverted) group
### 3.4.3 Bile-mediated dilution of cinnarizine-loaded micelles and vesicles generates drug supersaturation

The equilibrium solubility and change in cinnarizine solubilisation capacity of model micelles and vesicles before and after 1:1 v/v dilution with bile, bile pH 6.30 or buffer pH 6.30 are tabulated in Table 3.6, and shown graphically in Figure 3.6.

**Table 3.6:** Equilibrium solubility (37 °C) and percent original solubilisation capacity values of cinnarizine in model micelles and vesicles, before and after 1:1 v/v addition of rat bile, rat bile pH 6.30 or buffer pH 6.30. Data represent mean ± SEM of n = 3-4 determinations.

<table>
<thead>
<tr>
<th></th>
<th>Equilibrium solubility (µg/mL)</th>
<th>Percent original solubilisation capacity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Micelles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micelles alone</td>
<td>236 ± 9.3</td>
<td>100</td>
</tr>
<tr>
<td>+ Bile (1:1)</td>
<td>16.5 ± 0.8</td>
<td>14.0 ± 0.7</td>
</tr>
<tr>
<td>+ Bile pH 6.30 (1:1)</td>
<td>33.1 ± 1.0</td>
<td>28.0 ± 0.9</td>
</tr>
<tr>
<td>+ Buffer pH 6.30 (1:1)</td>
<td>48.7 ± 0.8</td>
<td>41.2 ± 0.6</td>
</tr>
<tr>
<td><strong>Vesicles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vesicles alone</td>
<td>232 ± 3.3</td>
<td>100</td>
</tr>
<tr>
<td>+ Bile (1:1)</td>
<td>8.60 ± 0.5</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>+ Bile pH 6.30 (1:1)</td>
<td>17.4 ± 0.4</td>
<td>15.0 ± 0.3</td>
</tr>
<tr>
<td>+ Buffer pH 6.30 (1:1)</td>
<td>24.9 ± 0.3</td>
<td>21.4 ± 0.2</td>
</tr>
</tbody>
</table>

\[
\text{Percent original solubilisation capacity} = \frac{\text{Solubility}_{\text{final}} \times \text{Volume}_{\text{final}}}{\text{Solubility}_{\text{initial}} \times \text{Volume}_{\text{initial}}} \times 100 \%
\]
Figure 3.6: Percent original cinnarizine (CIN) solubilisation capacity of model colloids (micelles or vesicles), before and after a 1:1 v/v addition of rat bile, rat bile pH 6.30 or buffer pH 6.30. Data represent mean ± SEM of n = 3-4 determinations. Statistical significant difference (p < 0.05) to colloid only is denoted by the symbol *. 

Dilution of the micellar or vesicular systems in a 1:1 v/v ratio with bile obtained from donor animals led to significant decreases in cinnarizine solubilisation capacity. As a proportion of initial, the solubilisation capacity of the micellar system dropped significantly from 100% to 14%, and for vesicles the decrease was even greater from 100% to 7%. This was in spite of the fact that the bile salt concentration in donor bile was higher than that in the micellar or vesicular system (average total bile salt concentration in donor bile was 14.7 ± 0.9 mM; mean ± SEM, n = 13), and therefore ‘dilution’ with bile did not reduce bile salt concentrations below the critical micellar concentration (CMC) and instead increased the overall bile salt concentration.

The pH of donor bile was higher than intestinal pH (average pH of donor bile was 8.02 ± 0.02; mean ± SEM, n = 5). As such additional studies were performed to examine whether the effects on solubility reflected a pH effect. 1:1 v/v dilution of the phases with pH adjusted bile (pH of bile adjusted to 6.30 to match the pH of the micelles and vesicles) also resulted in significant decreases
in solubilisation capacity (to 28% and 15% of initial for micelles and vesicles, respectively), although the decrease was slightly attenuated when compared to non-pH adjusted bile. Finally, the micellar and vesicular systems were diluted 1:1 v/v with buffer at pH 6.30 in an attempt to uncouple simple dilution effects from pH effects and bile-mediated effects. Dilution with pH 6.30 buffer also decreased cinnarizine solubilisation capacity significantly, although to a lesser extent (41% and 21% in micelles and vesicles, respectively).

Analysis of the kinetics of drug precipitation (Figure 3.7) demonstrated that bile addition to cinnarizine-loaded micelles and vesicles (cinnarizine present in phases at 0.2 mg/mL, ~ 80% saturated solubility) did not result in immediate drug precipitation, and was preceded by a period of supersaturation. The time taken for cinnarizine precipitation to occur was variable, however supersaturation was maintained for longer periods in micelles (> 20 min in all cases) when compared to vesicles (1-20 min).

![Figure 3.7: Kinetics of cinnarizine (CIN) precipitation from model micelles (filled circles, n = 5) and model vesicles (open circles, n = 4) upon addition of rat bile (in a 1:1 v/v ratio) at t = 0. Addition of bile reduces the equilibrium cinnarizine solubilisation capacity of micelles and vesicles to 14% and 7% of initial, respectively (see Table 3.6, and shown here as the lines denoted](image-url)
equilibrium solubility). Cinnarizine supersaturation appeared to be maintained for longer in micelles than in vesicles. Cinnarizine was loaded into micelles and vesicles at 80% saturation (~0.2 mg/mL). Each line represents individual experiments.

**3.4.4 Bile-induced drug supersaturation increases jejunal absorptive flux for micelles but not vesicles**

The absorptive flux vs. time profiles for cinnarizine under supersaturated conditions (i.e. when bile was co-perfused with the phases) and under non-supersaturated conditions (when bile was not co-perfused with the phases) are shown in Figure 3.4A. Steady state-absorptive flux, disappearance \( P_{\text{app}} \), and appearance \( P_{\text{app}} \) of cinnarizine in all perfusion experiments are reported in Table 3.4.

Co-perfusion of micelles with bile in a 1:1 v/v ratio increased the absorptive flux, disappearance \( P_{\text{app}} \), and appearance \( P_{\text{app}} \) of cinnarizine from micelles 3.2-fold, 1.5-fold, and 3.4-fold, respectively. In contrast, 1:1 v/v co-perfusion of vesicles with bile did not lead to significant changes in cinnarizine absorptive flux or appearance \( P_{\text{app}} \). Disappearance \( P_{\text{app}} \) for cinnarizine did increase 3.0-fold when vesicles were co-perfused with bile, however the drop in perfusate drug concentration was largely a result of rapid drug precipitation in the perfusate (see below).

For the micellar preparation, bile-induced supersaturation was relatively stable throughout the experimental period. Thus the cinnarizine concentration in the perfusate was essentially the same before or after centrifugation (Figure 3.4B). In contrast, when vesicles were co-perfused with bile, significant drug precipitation was observed during the time required for perfusate to transit the jejunal segment (as indicated by the difference between the pre- and post-centrifugation data in Figure 3.4C). This was consistent with the in vitro dilution profiles in Figure 3.7.
To distinguish between bile-induced increases in cinnarizine absorptive flux resulting from drug supersaturation, pH increases (since an increase in pH might be expected to increase the permeability of a weak base), and non-specific effects of bile components on membrane permeability, micelles were also co-perfused with pH-adjusted bile (pH 6.30) and buffer (pH 6.30). 1:1 v/v co-perfusion of micelles with bile pH 6.30 or buffer pH 6.30 increased cinnarizine absorptive flux 3.5-fold and 3.8-fold, respectively, when compared to the perfusion of micelles alone (Figure 3.8, Table 3.4). Stable supersaturation was also generated within the perfused jejunal segment in these experiments (Figure 3.9 - cinnarizine concentration in the perfusate was essentially the same before and after centrifugation).

**Figure 3.8:** Absorptive flux-time profiles of cinnarizine (CIN) when micelles were perfused through an isolated rat jejunal segment (~ 10 cm²), with and without 1:1 v/v co-perfusion with rat bile, rat bile pH 6.30 or buffer pH 6.30. SS denotes experiments where drug is supersaturated in perfusate. Co-perfusion of rat bile, rat bile pH 6.30 or buffer pH 6.30 with micelles generates drug supersaturation *in situ* within the perfused jejunal segment in all cases, and increased cinnarizine absorptive flux by 3.2-fold, 3.5-fold and 3.8-fold, respectively. Experiments were performed using an *in situ* single-pass rat jejunum perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. This series of experiments provides further support for the suggestion that
supersaturation was responsible for the increase in absorptive flux seen in Figure 3.4A. Data represent mean ± SEM of n = 3-4 experiments.

Figure 3.9: Perfusate disappearance (% drug dose passing through jejunum) profiles of cinnarizine (CIN) when micelles were perfused through an isolated rat jejunal segment (~ 10 cm²), with and without 1:1 v/v co-perfusion with (A) rat bile pH 6.30 or (B) buffer pH 6.30. Co-perfusion of micelles with rat bile pH 6.30 or buffer pH 6.30 generates drug supersaturation in situ within the perfused jejunal segment. The degree of drug precipitation within the perfusate is represented by the difference in perfusate concentration between pre- and post-centrifugation data. Experiments were performed using an in situ single-pass rat jejunum perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. Data represent mean ± SEM of n = 3-4 experiments.

Since 1:1 v/v co-perfusion of micelles with bile, bile pH 6.30 and buffer pH 6.30 are all expected to generate cinnarizine supersaturation within the perfused jejunal segment (according to the solubility data in Table 3.6, and the lack of drug precipitation in outflow perfusate in all cases), the observation that absorptive flux enhancement was similar in all groups (Figure 3.8) suggest that the enhancement was attributable to drug supersaturation, and not an increase in system pH (by comparing micelles + bile group with micelles + bile pH 6.30 group), or non-specific effects of bile on membrane permeability (by comparing micelles + bile pH 6.30 group with micelles +
buffer pH 6.30 group). However, the degree of flux enhancement did not correlate with the degree of supersaturation, as flux enhancement was similar in all groups despite the significantly higher supersaturation ratio generated in the micelles + bile group, when compared to the micelles + bile pH 6.30 group and micelles + buffer pH 6.30 group (supersaturation ratio of 6 vs. 2-3) (Table 3.4). Previous studies have shown that for lipophilic drugs such as cinnarizine, the drug fraction extracted into octanol (and analogous to the permeable fraction) as a function of pH is shifted to lower pHs than would be expected based on the unionised fraction\textsuperscript{208, 209}. This left shift would limit pH effects on permeability over the range of pH 6-8, consistent with the observations here.

3.4.5 Bile-induced drug supersaturation increases in vivo cinnarizine exposure after intraduodenal infusion

The systemic plasma concentration-time profiles of cinnarizine following intraduodenal infusion of cinnarizine-loaded micelles and vesicles (0.2 mg/mL; ~80% saturated solubility) to bile-intact and bile-diverted rats are shown in Figure 3.5. The pharmacokinetic parameters of cinnarizine for the bioavailability studies are reported in Table 3.5. Consistent with the results from the rat jejunal permeability studies, intraduodenal administration of micelles to bile-intact rats resulted in significantly higher systemic plasma concentrations of cinnarizine at t = 1, 1.5 and 2 h (AUC\textsubscript{0-8 h} increased 1.4-fold) when compared to bile-diverted rats. In contrast, when vesicles were dosed to bile-intact rats, the systemic plasma concentration-time profiles and AUC\textsubscript{0-8 h} of cinnarizine were not different to that observed in bile-diverted rats.

3.5 DISCUSSION

After oral administration, the absorption of poorly water-soluble drugs (PWSD) is often limited by slow dissolution and low solubility in the GI tract. LBF overcome many of the dissolution limitations of PWSD (by providing a mechanism to circumvent dissolution from the solid to liquid
state), however, the solubility limitations of PWSD are seemingly unaddressed, since solubilisation in the lipid-based colloidal phases that result from the digestion of LBF does not typically enhance free drug concentrations\textsuperscript{101,143}. Nonetheless, co-administration of lipids (either formulation lipids or via co-administration with lipid-rich foods) remains a highly effective means to promote the absorption of PWSD. This suggests the potential for alternative mechanisms by which LBF enhance drug absorption. In the current communication, two possible alternatives to the traditional model of drug absorption from LBF have been explored. Firstly, that drug absorption from lipid colloidal phases may involve a collisional uptake component (i.e. drug absorption directly from the solubilised phase), and secondly that flux across the absorptive membrane may be enhanced by a transient increase in the thermodynamic activity of drug in intestinal colloidal phases due to supersaturation.

The data describing drug absorption from micellar and vesicular colloidal phases suggest that direct interactions between colloids (or at least the systems examined here) and the absorptive membrane do not play an important role in cinnarizine absorption. Thus, comparable absorptive flux (Figure 3.4A – filled symbols) and systemic plasma concentration-time profiles (Figure 3.5 – bile diverted rats) were observed following jejunal perfusion and intraduodenal infusion of micelles and vesicles. The thermodynamic activity and $C_{\text{free}}$ of both systems were held constant, but the large difference in hydrodynamic radius (9 nm of micelles \textit{vs.} 443 nm of vesicles) and higher bile salt, LPC and lipid concentrations in the micellar system (Table 3.1) suggest that the number of administered micellar particles was substantially higher than that of vesicular particles. Collision-mediated absorption is highly sensitive to increased particle number, since this increases the statistical likelihood of collisions and collisional transfer\textsuperscript{150}. As such collisional interactions did not appear to dictate the degree of drug absorption from micelles and vesicles and instead, absorption was seemingly controlled by thermodynamic activity (or $C_{\text{free}}$). Differences in particle
size might also be expected to alter colloid diffusion across the unstirred water layer (UWL). The similarity in absorption profiles from micelles and vesicles (Figure 3.4A – filled symbols) therefore suggests that diffusion across the UWL was either non-limiting, or was not affected by particle size in this experimental model. Similarly, the rates of replenishment of $C_{\text{free}}$ (i.e. the rate of re-establishment of the equilibrium between solubilised and free drug) might be expected to be different between the two different particle size colloids, however this was presumably sufficiently fast in both cases to have little impact on drug absorption in the current model.

Data obtained from the lipid uptake receptor inhibition studies further support the notion that collisional uptake mechanisms have a limited role in drug absorption from these systems. SR-BI, CD36, and NPC1L1 were examined since they have previously been shown to mediate the cellular uptake of fatty acids and/or cholesterol\cite{61, 66, 210}; the absorption of which is also facilitated by micellar solubilisation. Whether SR-BI, CD36, and NPC1L1 function as authentic transporters that directly mediate lipid absorption\cite{61, 66-68, 210}, or whether they act to facilitate intracellular lipid trafficking or to modify signalling processes that mediate lipid absorption\cite{154, 211, 212}, or both, remains contentious but nonetheless all merit examination here. Recent reports also suggest the involvement of SR-BI, CD36, and NPC1L1 in the absorption of fat-soluble nutrients (such as carotenoids\cite{66, 213}, vitamin D\cite{214}, and vitamin E\cite{215}), providing further support for a role in drug absorption. In the current study, consistent with previous reports \textit{in vivo}, inhibition of NPC1L1 reduced cholesterol absorption (Figure 3.2B)\cite{68}, and inhibition of CD36 reduced (albeit non-significantly) the absorption of oleic acid (Figure 3.2C)\cite{61}. In contrast, inhibition of SR-BI and CD36 had little impact on cholesterol absorption (Figure 3.2B). Inhibition of SR-BI and CD36 was expected to reduce cholesterol absorption based on previous \textit{in vitro} studies\cite{61, 66, 67}. However, \textit{in vivo} evidence of a role of SR-BI and CD36 in cholesterol absorption is less clear and, for example, no significant differences in intestinal cholesterol absorption were reported in SR-BI
knockout vs. wild-type mice\textsuperscript{66, 157}. The lack of effect of SR-BI and CD36 inhibition on cholesterol absorption in the current study therefore runs contrary to previous \textit{in vitro} studies but is in agreement with some previous \textit{in vivo} data. Inhibition of endocytosis pathways via administration of monensin, also failed to reduce cholesterol and cinnarizine absorption. Rather, the volume of distribution and clearance of cholesterol and cinnarizine appeared to be reduced, resulting in increases in plasma exposure. Since exogenously dosed \textsuperscript{14}C-cholesterol and cinnarizine are likely to be present within lipoproteins in the systemic circulation, the changes in systemic disposition of \textsuperscript{14}C-cholesterol and cinnarizine in the monensin-treated rats may reflect inhibition of receptor-mediated endocytosis of LDL\textsuperscript{216}.

Inhibition of SR-BI, CD36 and NPC1L1 did not result in significant changes to the systemic exposure of cinnarizine following intraduodenal infusion of a lipid emulsion formulation in rats (Figure 3.2A). The data suggest that while lipid colloidal phases may be capable of direct interaction with lipid receptors (as suggested previously), the transfer of solubilised content into absorptive cells is likely selective and more applicable to solubilised lipids and nutrients rather than drugs. Together with the data describing cinnarizine absorption from micelles and vesicles, the results indicate that cinnarizine absorption from lipid colloidal phases is largely independent of the physical nature of the infused colloid (realising that in these first experiments cinnarizine was present at concentration well below the solubilisation limit and therefore under conditions where solubility/precipitation-mediated events were avoided), is not influenced significantly by common lipid uptake receptors, and instead appears to be primarily dependent on the free drug concentration in equilibrium with the solubilised reservoir.

Subsequent studies addressed the possibility that bile secretion may enhance drug absorption via the induction of supersaturation during the intestinal processing of dietary or formulation-related
The addition of bile to cinnarizine-loaded micelles led to sustained drug supersaturation that ultimately led to increased intestinal drug absorption and systemic drug exposure. In contrast, although addition of bile to cinnarizine-loaded vesicles also led to supersaturation, the metastable supersaturated state was less stable than that generated by bile addition to micelles, resulting in more rapid precipitation of solubilised drug and therefore a lack of increase in drug absorption and systemic drug exposure. The data indicate that bile-mediated dilution of lipid colloidal phases may represent an endogenous mechanism of supersaturation generation during lipid processing in the small intestine; and that the transient increase in thermodynamic activity may lead to enhanced drug absorption. The observations also highlight both the potential for supersaturation to enhance drug absorption, and the need to achieve an optimal balance between drug supersaturation and drug precipitation.

Interaction of intestinal colloidal phases with bile leads to dilution, an increase in pH, and an increase in the concentrations of bile components (bile salts, phosphatidylcholine and cholesterol) associated with the colloidal species. For a solubilised system above the critical micellar concentration (CMC), simple 1:1 v/v dilution is expected to reduce the solubilised concentration, but to maintain total solubilisation capacity (i.e. for the drug concentration to drop by 50% but the volume to double and therefore for solubilisation capacity to remain unchanged). However, the data in Table 3.6 show that dilution of the micelles or vesicles with bile or buffer results in a drop in total solubilisation capacity to only 7-41% of initial. Greater proportional decreases were apparent for dilution of vesicles (in all cases when compared to micelles), and after dilution with bile rather than buffer (Table 3.6). The loss of solubilisation capacity on dilution suggests the likelihood of a phase transition to structures with reduced solubilisation capacity. Although a complete explanation for these phase transition is not apparent at this time, for the micellar systems, it may be related to the ability of ionised caprylic acid to self-associate and form fatty
acid micelles at high lipid concentration\textsuperscript{217}. Thus, dilution of medium-chain colloidal phases may reduce the concentration of caprylic acid below the CMC, leading to a loss of cinnarizine solubilisation capacity in the micellar phase. For vesicles, previous studies have suggested that increasing bile concentrations facilitate a vesicular to micellar transition\textsuperscript{218,219}. Since micelles are expected to have lower solubilisation capacities for lipophilic drugs than vesicles (micelles are smaller and less lipid-rich)\textsuperscript{92,194}, a reduction in cinnarizine solubilisation capacity might therefore be anticipated when bile is added to model vesicles. In the case of the micelles, therefore, it seems likely that the addition of bile disrupted the structure of swollen, mixed micelles leading to lower colloidal lipid content and lower solubilisation capacity. Unfortunately, attempts to quantify changes to particle size on bile addition were unsuccessful due to high polydispersity. However, the broad trends observed were consistent with the suggestions above and decreases in particle size were apparent for vesicles (consistent with initiation of a vesicle to micelle transition) and increases in particle size for the mixed micelles (consistent with micellar destabilisation and transformation to less dispersed structures) (data not shown). An additional complexity in these dilution studies was the realisation that the pH of bile (pH 8.02) is higher than that normally employed for simulated intestinal fluids\textsuperscript{39,220}, and therefore incubation of the colloidal phases (prepared at pH 6.30) with bile increases system pH. Cinnarizine is a weak base with a pK\textsubscript{a} of 7.47\textsuperscript{221} and is therefore expected to be less ionised, less soluble and potentially more permeable at higher pHs. The impact of pH in the current studies was therefore examined by dilution of micelles and vesicles with pH-corrected bile at pH 6.30. Comparison to the data obtained with bile at pH 8.02 suggests that the higher pH of bile provides an additional driver for drug precipitation/supersaturation since the solubility drop was greater after incubation with bile at pH 8.02 vs. bile at pH 6.30.
While dilution of micelles and vesicles with bile resulted in decreases in drug solubilisation capacity, cinnarizine did not precipitate immediately. A period of drug supersaturation was evident for both micellar and vesicular systems, although drug precipitation from vesicles was much more rapid than from micelles. This in turn translated into increases in drug absorption (Figure 3.4A) and systemic exposure (Figure 3.5A) for the micellar systems. The difference in the capacity of micelles and vesicles to maintain drug supersaturation (Figure 3.7) may be explained by the difference in the degree of drug supersaturation induced by dilution with bile. The degree of supersaturation is described by the supersaturation ratio, which is the ratio of the (supersaturated) concentration of drug in solution relative to the equilibrium solubility of the drug in the same system\textsuperscript{173}. Crystallization theory suggests that the thermodynamic drivers of precipitation from supersaturated solutions increase with increasing supersaturation ratios, as the likelihood of nucleation and crystal growth increases with increases in (metastable) drug concentration in solution\textsuperscript{173, 174}. Here, 1:1 v/v addition of bile resulted in the attainment of cinnarizine supersaturation ratios of 6 and 12, in micelles and vesicles, respectively. Therefore, the faster rate of drug precipitation from vesicles (when compared to micelles) may be explained by the higher supersaturation ratio induced by bile addition. It is also possible that micellar structures are more effective in stabilising supersaturation when compared to vesicular structures, although this has not been examined explicitly here. Notably, cinnarizine was found to precipitate in the crystalline form in these experiments (Figure 3.10), precluding the possibility that the enhanced cinnarizine absorption from micelles (when bile was co-perfused) observed in Figure 3.4A was due to accelerated cinnarizine dissolution from precipitated amorphous forms.
Chapter 3: Bile-induced supersaturation

Figure 3.10: Analysis of the cinnarizine precipitate using polarised light microscopy following precipitation kinetics experiments where fasted rat bile was added in a 1:1 v/v ratio to cinnarizine-loaded (A) micelles or (B) vesicles. Cinnarizine was loaded into micelles and vesicles at 80% saturation (~ 0.2 mg/mL). Addition of bile reduced the equilibrium cinnarizine solubilisation capacity of micelles and vesicles to 14% and 7% of initial, respectively, and triggered cinnarizine supersaturation and precipitation (see Figure 3.7). The polarised light microscopy images show that cinnarizine precipitated in the crystalline form after the addition of bile. The crystals obtained from the vesicles + bile group were significantly smaller than those obtained from the micelles + bile group, an observation that likely reflects the higher degree of supersaturation stimulated in the vesicular system (SS ratio = 12) when compared to the micellar system (SS ratio = 6).

The role of bile in enhancing drug absorption from LBF has been reported previously. In the majority of cases, bile-mediated bioavailability enhancement has been suggested to stem from the ability of bile to expand the solubilisation reservoir for PWSD in the GI tract. This is typically assumed to occur via PWSD solubilisation in simple bile micelles, or via the ability of bile to solubilise lipid digestion products and to generate more complex lipid colloidal phases with enhanced solubilisation capacities. It seems likely that the ability to solubilise lipid digestion products and to promote colloid formation remains an integral part of the role of bile in supporting drug (and lipid) absorption. However, the data described here suggest that continued dilution of lipid colloidal phases with bile in the small intestinal lumen may also lead to physical changes that
promote drug supersaturation, and ultimately promote drug absorption. In doing so, supersaturation induction may be a means by which the decrease in thermodynamic activity inherent in solubilisation is reversed, such that the free concentration of drug available for absorption is maximised. Thus, a dual role of bile in facilitating drug absorption from LBF may be conceived (see Figure 3.11). First, bile-mediated solubilisation of lipid digestion products at the interface of a digesting lipid droplet results in the generation of lipid colloidal phases such as vesicles and micelles that promote drug solubilisation during lipid digestion. Second, continued bile-mediated dilution of existing lipid colloidal phases promotes drug supersaturation, and enhances drug absorption by significantly increasing drug thermodynamic activity in colloidal phases. The combination of these two highly kinetic events likely contributes to the effective drug absorption often observed with lipid co-administration, as it affords a means to simultaneously increase solubilisation capacity and promote thermodynamic activity of co-administered PWSD in the small intestine.

Supersaturation induction via interaction with bile also provides a means of overcoming the recently described solubility-permeability interplay observed in studies where PWSD are co-administered with cosolvents, cyclodextrins or surfactant systems\textsuperscript{100, 146, 225}. In these studies the authors describe the reduction in thermodynamic activity common to most solubilisation technologies and show that this off-sets the potential increases in membrane flux that might be expected by an increase in solubilised drug concentration\textsuperscript{100, 146}. More recent studies by the same authors have shown that this solubility-permeability interplay can be addressed via the use of amorphous solid dispersion formulations that stimulate supersaturation, but do not promote solubilisation\textsuperscript{147, 226}. Here we report that essentially similar outcomes are also possible with solubilising formulations, when the solubilising formulations contain lipids and when the kinetic changes that occur in the GI lumen in the presence of bile secretion promote supersaturation. The
current data therefore suggest that endogenous lipid processing pathways provide an exquisitely sensitive and triggerable supersaturation mechanism that allows drug to remain in a solubilised state during initial lipid digestion and at high lipid:bile concentration ratios, but that on-going bile secretion subsequently provides a boost to thermodynamic activity and in doing so supports enhanced absorption. This is in contrast to other common solubilisation strategies that may not interact with the dynamic GI environment, or for which interaction with bile typically reduces thermodynamic activity by increasing solubilisation capacity. Conversely, the ability of LBF to promote drug solubilisation until drug supersaturation is triggered in the small intestine may confer an advantage over formulation approaches that only utilise supersaturating strategies, as the risk of drug precipitation in the GI tract may be reduced by solubilisation within lipid colloidal phases.

3.6 CONCLUSION

Improved understanding of the mechanism of drug absorption from lipid colloidal phases such as micelles and vesicles is required to provide a platform for more rational design of lipid-based formulations. Using medium-chain lipids, we have demonstrated that the absorption of cinnarizine (a lipophilic, poorly water-soluble drug) may be enhanced when drug supersaturation is generated during bile-mediated dilution of lipid colloidal phases. Previous studies suggest that a similar induction of supersaturation may occur as a result of initiation of digestion of some microemulsion based LBFs. These observations indicate that supersaturation, and its associated benefits in enhancing drug absorption, may occur intrinsically during LBF incorporation into endogenous lipid processing pathways in the small intestine. Future work will be directed toward assessing the impact of bile dilution on supersaturation tendency, and thus absorption, for an extended range of PWSD and in a series of different micellar and vesicular colloidal systems.
Figure 3.11: The dual role of bile during lipid digestion and dispersion. (i) Bile-mediated solubilisation of lipid digestion products at the interface of a digesting oil droplet results in the generation of lipid colloidal phases such as vesicles and micelles that maintain drug solubilisation in the small intestine. (ii) The continuing interaction of secreted bile with existing lipid colloidal phases in the lumen results in progressively less lipid-rich phases with lowered solubilisation capacity. Thus, on-going bile-mediated dilution of lipid colloidal phases promotes drug supersaturation and enhances drug absorption by increasing drug thermodynamic activity in colloidal phases. The combination of the two highly kinetic, bile-mediated events affords a means to simultaneously increase solubilisation capacity and promote thermodynamic activity of co-administered drug in the small intestine, and may contribute to the increase in drug absorption often observed with lipid co-administration. D represents the free concentration of drug available for absorption. Dss is used to signify the increase in free concentration resulting from bile-mediated supersaturation that drives increases in drug absorption.
Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

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

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Concept and design of studies, planning and execution of experimental work, data analysis and interpretation, formulation of conclusions and hypotheses resulting from the relevant studies, drafting and revision of manuscript</td>
<td>70%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Name</th>
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<th>Extent of contribution (%)</th>
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<tbody>
<tr>
<td>C.J.H. Porter</td>
<td>Project supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
<tr>
<td>N.L. Trevaskis</td>
<td>Project co-supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
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</table>

Candidate's Signature

Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate’s contribution to this work, and the nature of the contribution of each of the co-authors.

(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

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CHAPTER 4 : THE POTENTIAL FOR DRUG SUPERSATURATION DURING INTESTINAL PROCESSING OF LIPID-BASED FORMULATIONS IS ENHANCED FOR BASIC DRUGS

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Manuscript in submission.
Chapter 4: Supersaturation potential is enhanced for basic drugs

4.1 ABSTRACT

Co-administration of poorly water-soluble drugs (PWSD) with dietary or formulation lipids stimulates the formation of lipid colloidal phases such as vesicular and micellar species, and significantly expands the drug solubilisation capacity of the small intestine. The mechanism of drug absorption from the solubilising phases, however, has not been fully elucidated. Recently, we observed that drug supersaturation may be triggered during endogenous processing of lipid colloidal phases containing medium-chain lipid digestion products, and that this may represent a mechanism to enhance absorption and to reverse the reduction in thermodynamic activity inherent in drug solubilisation. The current studies expand these preliminary findings and explore the supersaturation tendency of five model PWSD during endogenous processing of intestinal colloidal phases containing long-chain lipid digestion products. Bile:lipid concentration ratios progressively increase during colloid transit through the gastrointestinal tract due to biliary dispersion of lipid digestion products and lipid absorption. Supersaturation potential was therefore evaluated under conditions of increasing bile and decreasing lipid concentrations and was found to be greater for basic drugs (cinnarizine and halofantrine) than neutral (fenofibrate and danazol) or acidic drugs (meclofenamic acid). Assessment of intestinal absorptive flux using rat jejunal perfusion experiments subsequently showed that the absorption enhancement afforded by bile dilution was greater for cinnarizine than danazol. The results confirm that bile may play a significantly greater role in the absorption of weak bases such as cinnarizine from long-chain intestinal colloids when compared to uncharged molecules such as danazol, and that this difference reflects a greater propensity for supersaturation as intestinal colloids are dispersed and diluted by bile. The data also suggest that co-administered digestible lipids may have a particular utility in enhancing the absorption of poorly water-soluble weak bases.
4.2 INTRODUCTION

The oral bioavailability of many poorly water-soluble drugs (PWSD) is low and variable due to slow dissolution and low solubility in the aqueous gastrointestinal (GI) contents. Lipid-based formulations (LBF) have proven to be an effective approach in improving the oral bioavailability of lipophilic PWSD. LBF overcome the dissolution and solubility limitations of PWSD by presenting drug to the GI tract in a molecularly dispersed form (i.e. in solution in the formulation), and by stimulating the generation of lipid colloidal phases that promote drug solubilisation and reduce the risk of drug precipitation\(^2\), \(^76\). Classically, therefore, absorption enhancement by LBF has been considered to result from mechanisms that enhance drug dissolution and expand the solubilisation capacity of the GI tract.

Recently, interest has increased in the potential for LBF to both promote solubilisation, and simultaneously to stimulate supersaturation as a means of enhancing drug absorption\(^175\), \(^191\). The dispersion and digestion of LBF typically leads to a reduction in drug solubilisation capacity\(^5\), \(^91\), \(^176\), \(^177\), \(^193\), and where precipitation is not immediate, a period of supersaturation results where solubilised drug concentrations are higher than the equilibrium drug solubility in an identical colloidal milieu\(^176\), \(^227\). Supersaturation has the potential to enhance drug absorption via transient increases in thermodynamic activity. Conversely, however, supersaturation is also a driver of drug nucleation and precipitation, processes that break the continuity of solubilisation and commonly reduce drug absorption. Balancing supersaturation and precipitation is therefore a key design element of LBF.

The digestion of triglycerides or partial glycerides in LBF, or dietary lipids, liberates amphiphilic digestion products including fatty acids and monoglycerides. Digestion products so formed combine with biliary components to generate a range of lipid colloidal phases in which co-
administered PWSD may be solubilised. At the surface of a digesting oil droplet, digestion products accumulate and ultimately slough off to form lipid-rich dispersed liquid crystalline phases such as lamellar, cubic and hexagonal phases with relatively large particle sizes and high drug solubilisation capacities\textsuperscript{39-41}. On dilution with intestinal fluids these colloidal structures become smaller and less lipid-rich and typically have lower drug solubilisation capacities\textsuperscript{39}. Drug absorption is thought to occur from these smaller, more dilute lipid colloidal phases such as unilamellar vesicles and mixed micelles, although the mechanisms involved in drug absorption from these species have not been fully elucidated\textsuperscript{2,76,101}.

Recently we described the ability of bile to promote drug absorption from lipid colloidal phases in the small intestine. Contrary to traditional solubilisation paradigms, the addition of bile to intestinal colloidal phases containing digestion products of medium-chain (MC) triglycerides enhanced drug absorption \textit{in vivo} by reducing the solubilisation capacity of the colloidal phases for a model PWSD (cinnarizine), thereby generating a transiently supersaturated state (Chapter 3). These initial findings were suggested to provide a means to reverse the reduction in drug thermodynamic activity, and therefore absorptive drug flux that is inherent in solubilisation-mediated formulation approaches\textsuperscript{100,146}.

The current study expands this preliminary evaluation of supersaturation events during the LBF processing and has examined the impact of bile on the potential for drug supersaturation in intestinal colloidal phases containing digestion products of long-chain (LC) triglycerides. Importantly the work also delineates the relative sensitivities to supersaturation for weak bases, acids and neutral compounds and shows that weak bases may be particularly suited to supersaturation-mediated absorption enhancement from LBF.
Chapter 4: Supersaturation potential is enhanced for basic drugs

4.3 METHODS

4.3.1 Materials

Cinnarizine, flunarizine dihydrochloride, fenofibrate, meclofenamic acid, progesterone, ammonium sulphate, sodium taurocholate, sodium taurodeoxycholate, sodium glycocholate, sodium glycochenodeoxycholate, cholesterol, L-α-lysophosphatidylcholine (LPC, from egg yolk), oleic acid, monoolein, sodium chloride (NaCl), sodium dodecyl sulphate (SDS), formic acid and ammonium formate were obtained from Sigma-Aldrich, Australia. Sodium taurochenodeoxycholate, sodium glycodeoxycholate, ortho-phosphoric acid 85% (H₃PO₄), sodium hydroxide pellets (NaOH), tert-butyl methyl ether (TBME), glacial acetic acid and absolute ethanol were from Merck, Australia. Disodium hydrogen orthophosphate (Na₂HPO₄), sodium dihydrogen orthophosphate (NaH₂PO₄•2H₂O) and ammonium dihydrogen orthophosphate (NH₄H₂PO₄) (Ajax Finechem, Australia), oleic acid, [9,10-³H(N)] (60 Ci/mmol) (American Radiolabeled Chemicals, MO, USA), danazol (Sterling Pharmaceuticals, Australia), halofantrine base (GlaxoSmithKline, King of Prussia, PA, USA), heparin sodium injection BP (1000 I.U./mL, Hospira, Australia), xylazine (100 mg/mL, Troy Laboratories, Australia), acepromazine (10 mg/mL, Ceva Delvet, Australia), ketamine (100 mg/mL, Provet, Australia) and pentobarbitone sodium (325 mg/mL, Virbac, Australia) were obtained from listed suppliers. Acetonitrile, methanol and chloroform used were analytical reagent grade. Water was obtained from a Millipore milliQ Gradient A10 water purification system (Millipore, MA, USA).

4.3.2 Experimental outline

Nine colloidal systems designed to mimic the colloidal phases that form in the small intestine during the digestion of LC triglycerides were prepared. The systems consisted of triolein digestion products (oleic acid and monoolein) solubilised in bile components (bile salts,
lyosphosphatidylcholine and cholesterol). The concentrations of lipid and bile components in the systems were varied systematically, as shown in Table 4.2. The equilibrium solubility of five lipophilic PWSD (two weak bases and two neutral drugs with differing triglyceride solubilities, and one weak acid) were determined in each of the colloidal systems, to assess drug solubility trends as a function of colloidal bile salt concentration [bile] and colloidal lipid concentration [lipid]. The melting points, octanol-water distribution coefficients, pK\textsubscript{a}, molecular weights, soybean oil and oleic acid solubilities of the five drugs are shown in Table 4.3.

During bile-mediated dilution of lipid colloidal phases in the small intestine, increases in the concentration of biliary components and decreases in lipid concentrations occur in tandem. Under these circumstances, drug solubilisation capacity may increase (decreasing thermodynamic activity at constant drug concentration) or decrease (invoking the potential for precipitation or supersaturation where precipitation is not immediate). The net effect is dictated by the magnitude of the change in solubility expected by increasing [bile] and decreasing [lipid]. Under conditions of lipid absorption, lipids from intestinal colloids are also removed via absorption, further decreasing lipid concentrations. In the second part of the current studies, two model drugs with markedly different solubility relationships with [bile] and [lipid] were selected for further study. For these compounds, absorption was assessed from a model long-chain colloidal system \textit{in situ} and \textit{in vivo}, to determine the potential for endogenous lipid processing pathways to generate drug supersaturation, and to enhance drug absorption, from intestinal colloidal phases containing LC lipid digestion products.
4.3.3 Preparation of model intestinal colloidal phases containing long-chain lipids

Colloidal systems containing triolein digestion products (oleic acid and monoolein) solubilised in simulated endogenous intestinal fluid (SEIF) were prepared. SEIF comprised the six most prevalent bile salts in human bile\textsuperscript{199}, lysophosphatidylcholine (LPC), and cholesterol. The total bile salt:LPC:cholesterol molar ratio was kept constant at 16:4:1, reflecting known ratios within fasted human bile\textsuperscript{200, 201}. The bile salts employed were glycocholate, glycodeoxycholate, glycochenodeoxycholate, taurocholate, taurodeoxycholate, and taurochenodeoxycholate, at a molar ratio of 4.0:2.8:4.0:2.0:1.2:2.0. The concentration ratios of the bile salts were chosen based on average concentrations of the six most prevalent bile salts found in human bile\textsuperscript{199}. The oleic acid:monoolein molar ratio was kept at 2:1, reflecting the ratio of digestion products expected from digestion of 1 mole of triolein. The concentrations of lipid and bile components in the systems were varied systematically, according to the compositions shown in Table 4.2. Bile salt concentrations of 4–16 mM were selected to span the typical concentration range seen \textit{in vivo}\textsuperscript{76} and previously used in simulated intestinal fluids\textsuperscript{220}; lipid concentrations were chosen to reflect the solubilised concentrations that might be attained in the intestine following the digestion of LC triglycerides\textsuperscript{91}. Based on physical examination and the maintenance of consistent drug solubilising capacities, colloids were stable for 5 days after preparation.

SEIF 16 (16 mM total bile salt:4 mM LPC:1 mM cholesterol) was prepared in 50 mL batches. Briefly, LPC and cholesterol were dissolved in 2 mL chloroform in a round bottom flask, followed by solvent evaporation under vacuum. The thin film formed was reconstituted with buffered bile salt solution (consisting 16 mM total bile salt, 18 mM NaH\textsubscript{2}PO\textsubscript{4}.2H\textsubscript{2}O, 12 mM Na\textsubscript{2}HPO\textsubscript{4}, 92 mM NaCl), vortexed for 1 min, and allowed to equilibrate at room temperature overnight. When SEIF 8 (8 mM total bile salt:2 mM LPC:0.5 mM cholesterol) and SEIF 4 (4 mM total bile salt:1 mM cholesterol)
LPC:0.25 mM cholesterol) were required, SEIF 16 was diluted 2-fold and 4-fold, respectively, with buffer (18 mM NaH₂PO₄·2H₂O and 12 mM Na₂HPO₄, 108 mM NaCl). The colloidal systems were prepared in 10 mL batches by adding appropriate masses of oleic acid and monoolein into SEIF, followed by a 1-min vortex. The mixture was then ultrasonicated (30 sec continuous ultrasonication followed by pulsatile, 1 sec-on/1 sec-off ultrasonication for 5 min) with a Misonix XL 2020 ultrasonic processor (Misonix, Farmingdale, NY, USA) equipped with a 3.2-mm microprobe tip running at an amplitude of 240 µm and a frequency of 20 kHz. The colloids were subsequently adjusted to pH 6.30 with H₃PO₄ solution and/or NaOH solution.

For the preparation of drug-loaded model colloids (for in situ jejunal perfusion and in vivo bioavailability studies), cinnarizine was first dissolved in oleic acid at a concentration of 61 mg/g and 115 mg/g prior to generating colloidal systems as described above, resulting in colloids containing 65 µg/mL cinnarizine (representing ~ 40% of cinnarizine saturated solubility in the colloidal system, solubility determined as described below) and 130 µg/mL (~ 80% saturated solubility), respectively. Due to the lower solubility of danazol in oleic acid, colloids containing danazol were drug-loaded by spiking 50 µL of 1.4 mg/mL or 2.8 mg/mL danazol in ethanol solution into 10 mL blank model colloids, followed by vortexing for 1 min, and equilibration for 1 h to prepare colloids containing 7 µg/mL (~ 40% saturated solubility) and 14 µg/mL (~ 80% saturated solubility) danazol, respectively. As a final step, trace amounts of ³H-oleic acid (0.25 µCi/mL in jejunal perfusion studies; 1 µCi/mL in bioavailability studies) were also added to the drug-loaded colloids, followed by a 1-min vortex. The total ethanol concentration in the colloids was ≤ 1% v/v. Drug and oleic acid concentrations in the colloidal systems were confirmed by HPLC and scintillation counting immediately prior to use.
4.3.4 Equilibrium solubility of drugs in oleic acid and soybean oil

Excess solid drug (cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN), meclofenamic acid (MFA)) was added to 2 mL glass vials containing approximately 0.5 g oleic acid or soybean oil. Vials were briefly vortexed, incubated at 37 °C, and samples taken every 24 h over a period of 120 h. During sampling, vials were centrifuged (2,200 xg, 10 min, 37 °C), and 1-2 drops of supernatant accurately weighed into 5 mL volumetric flasks using glass Pasteur pipettes. After sampling, vials were re-vortexed such that undissolved drug particles were re-suspended in the lipid slurry. The 5 mL volumetric flasks containing accurately weighed supernatant samples were made up to volume with chloroform-methanol (2:1, v/v) and briefly vortexed. 50 µL aliquots were then diluted > 10-fold with respective mobile phases (Table 4.1) and analysed for drug content via HPLC (HPLC assay conditions for CIN, HF, FF, DAN and MFA in LC colloids are reported in Table 4.1). Equilibrium solubility was defined when drug concentrations in consecutive samples varied by ≤ 5%, and was determined on three separate occasions.
Table 4.1: HPLC assay conditions for cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) in long-chain colloidal systems.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Flow rate (mL/min)</th>
<th>Detection</th>
<th>Retention time (min)</th>
<th>Validated concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN</td>
<td>Waters Symmetry® C18, 5 µm, 3.9 x 150 mm</td>
<td>50% v/v Acetonitrile : 50% v/v 20 mM NH₄H₂PO₄</td>
<td>1.0</td>
<td>Fluorescence λ = 249/311 nm</td>
<td>5.7</td>
<td>20–1000 ng/mL</td>
</tr>
<tr>
<td>HF</td>
<td>Phenomenex Luna® C8(2), 5 µm, 4.6 x 250 mm</td>
<td>75% v/v Acetonitrile : 25% v/v H₂O (0.2% w/v SDS &amp; 0.2% v/v acetic acid)</td>
<td>1.5</td>
<td>UV λ = 254 nm</td>
<td>4.2</td>
<td>0.5–25 μg/mL</td>
</tr>
<tr>
<td>FF</td>
<td>Waters Xbridge™ C18, 5 µm, 4.6 x 150 mm</td>
<td>80% v/v Acetonitrile : 20% v/v H₂O (total 0.01% v/v formic acid)</td>
<td>1.0</td>
<td>UV λ = 286 nm</td>
<td>3.9</td>
<td>0.5–25 μg/mL</td>
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<tr>
<td>DAN</td>
<td>Waters Xbridge™ C18, 5 µm, 4.6 x 150 mm</td>
<td>75% v/v Methanol : 25% v/v H₂O</td>
<td>1.0</td>
<td>UV λ = 286 nm</td>
<td>4.4</td>
<td>0.25–50 μg/mL</td>
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<tr>
<td>MFA</td>
<td>Phenomenex Luna® C8(2), 5 µm, 4.6 x 250 mm</td>
<td>80% v/v Acetonitrile : 20% v/v 10 mM H₃PO₄</td>
<td>1.0</td>
<td>UV λ = 285 nm</td>
<td>4.8</td>
<td>0.5–25 μg/mL</td>
</tr>
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</table>

^ CIN in long-chain colloidal systems was assayed via HPLC as described above; while CIN in plasma was assayed via HPLC using a validated extraction procedure (with flunarizine as an internal standard) and slightly modified HPLC conditions as reported previously^{43}.

^ DAN in long-chain colloidal systems was assayed via HPLC as described above; while DAN in plasma was assayed via LC-MS using a validated precipitation procedure (with progesterone as an internal standard) and slightly modified HPLC conditions as reported previously^{228}. 
4.3.5 Equilibrium solubility of drugs in long-chain colloids

Excess solid drug (CIN, HF, FF, DAN, MFA) was added to 2 mL of long-chain colloids (prepared as above) in glass vials. Vials were briefly vortexed, incubated at 37 °C, and samples taken every 24 h over a period of 120 h. During sampling, vials were centrifuged (2,200 x g, 10 min, 37 °C), 50 µL of supernatant removed, and vials re-vortexed. 50 µL aliquots were then diluted > 10-fold with respective mobile phases (see Table 4.1) and analysed for drug content via HPLC (HPLC assay conditions for CIN, HF, FF, DAN and MFA in LC colloids are reported in Table 4.1). Equilibrium solubility was defined when drug concentrations in consecutive samples varied by ≤ 5%, and was determined on three separate occasions. Equilibrium solubilities of CIN and DAN were also determined after 1:1 v/v addition of fasted rat bile (obtained from donor animals as described below) or buffer (18 mM NaH₂PO₄.2H₂O, 12 mM Na₂HPO₄, 108 mM NaCl; adjusted to pH 6.30) to the colloidal phases.

4.3.6 Kinetics of cinnarizine precipitation

The kinetics of cinnarizine precipitation was monitored after addition of fasted rat bile (obtained from donor animals as described below) to model colloids, to determine whether a period of drug supersaturation preceded drug precipitation. In a temperature (37 °C) and stirring rate-controlled vessel, 2.5 mL of bile was added to 2.5 mL model colloids containing 130 µg/mL cinnarizine (~ 80% saturated solubility). A 1:1 v/v addition ratio of bile to colloids was used based on the known rates of bile flow in rats in vivo (~ 1.2 ± 0.05 mL/h; mean ± SEM; n = 22) and the volume of a typical oral dose in rats (~ 1 mL/h). Samples (100 µL) were taken before the addition of bile, and at 1, 10, 20, 30, 40, 50, 60, 80, 100, 120 min after bile addition. Samples were immediately centrifuged (2,200 x g, 5 min, 37 °C) to separate precipitated drug, and 50 µL of supernatant assayed for drug content. The proportion of the initial solubilised cinnarizine concentration that remained solubilised after bile addition was assessed as the percent of the drug mass remaining in
solution (i.e. the concentration in the supernatant multiplied by the volume remaining in vessel after sampling) relative to the total drug mass in the vessel at each time point.

### 4.3.7 Solid-state analysis of the cinnarizine precipitate

Selected cinnarizine pellets from the precipitation kinetics experiments were analysed using a Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarising filters. At the end of the precipitation kinetics experiments, 1.5 mL of remaining bile + colloid mixture were centrifuged (2,200 xg, 10 min, 37 °C), the supernatant was discarded, and a small amount of pellet was carefully placed on a microscope slide. Samples were analysed under cross-polarised light, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan).

### 4.3.8 Animals

Animal care and anaesthesia have been described in Section 2.2.2.2. All animals were fasted overnight (12–18 h) prior to surgery. At the end of all experiments, rats were euthanized via an intravenous or intracardiac injection of 100 mg sodium pentobarbitone.

### 4.3.9 Surgical procedures

#### 4.3.9.1 Fasted rat bile collection

The bile duct was cannulated near the hilum of the liver (where the duct is free of pancreatic tissue) in order to facilitate the collection of bile fluid without contamination with exocrine pancreatic secretions. The bile duct and jugular vein were cannulated as described previously. Rats were rehydrated via a continuous infusion of saline (1.5 mL/h) into a cannula inserted in the right jugular vein, and bile continuously collected for 5 h. The concentration of total bile salt in
collected bile was assayed using a validated enzymatic colorimetric assay (Total Bile Acids kit #431-15001; Wako Pure Chemical Industries, Osaka, Japan) on a plate reader (Fluostar Optima plate reader, BMG Lab technologies, Germany) measuring absorbance at a wavelength of 540 nm. In all subsequent experiments, bile was stored at 4°C (and allowed to warm up to ambient temperature before use) and used within 24 h of collection.

### 4.3.9.2 Single-pass rat jejunal perfusion

The model employed to assess flux across rat jejunum involved *in situ* perfusion (single-pass) of an isolated jejunal segment and simultaneous blood collection from the corresponding mesenteric vein branch. The surgical procedures for the setup of the single-pass rat jejunum perfusion model have been described in Section 2.2.2.3.

### 4.3.9.3 Cinnarizine bioavailability studies following intraduodenal administration

The surgical procedures for the conduct of bioavailability studies included cannulations of the right carotid artery, right jugular vein, duodenum (1 cm below pylorus), and common bile duct (only for bile-diverted rats). The surgical procedures for the cannulations have been described in Section 2.3.2.

### 4.3.10 *In situ single-pass rat jejunum perfusion*

After surgery, animals were equilibrated for 30 min, during which time heparinised donor rat blood was infused via the jugular vein as described previously. During the equilibration period, blood from the cannulated mesenteric vein (~ 0.3 mL/min) was collected to enable re-infusion via the jugular vein. Perfusion buffer was pumped through the jejunal segment at a rate of
0.1 mL/min and outflowing buffer discarded to waste. The exposed jejunal segment was kept moist by covering with saline-soaked gauze throughout the experiment.

In all experiments to assess cinnarizine intestinal absorptive flux, the inflow concentration of cinnarizine was constant at 65 µg/mL. Therefore, in experiments where model colloids were perfused alone, cinnarizine was loaded into the perfusate at 65 µg/mL (~ 40% saturated solubility). In experiments where model colloids were co-perfused in a 1:1 v/v ratio with a secondary perfusate of bile, cinnarizine was loaded into the primary perfusate at 130 µg/mL (~ 80% saturated solubility), such that 1:1 v/v dilution led to a final inflow perfusate concentration of 65 µg/mL. In this way the concentration of cinnarizine on entry into the intestine was corrected for biliary dilution effects, realising that the intent of the current studies was to evaluate whether bile addition resulted in additional changes to drug solubilisation capacity and thermodynamic activity beyond simple dilution.

Similarly, in all experiments that assessed danazol intestinal absorptive flux, the concentration of danazol flowing into the perfused jejunum segment was held at 7.2 µg/mL. Therefore, in experiments where model colloids were perfused alone, danazol was loaded into the perfusate at 7.2 µg/mL (~ 40% saturated solubility). In experiments where model colloids were co-perfused in a 1:1 v/v ratio with a secondary perfusate of bile, danazol was loaded into the primary perfusate at 14.4 µg/mL (~ 80% saturated solubility), such that 1:1 v/v dilution led to a final inflow perfusate concentration of 7.2 µg/mL.

Perfusate flow was maintained at 0.1 mL/min in all experiments to minimise variations in the thickness of the unstirred water layer that may influence drug flux\textsuperscript{206}. For experiments where 1:1 v/v co-perfusion was required, model colloids and bile were individually pumped at 0.05 mL/min,
and mixed via a three-way “T” connector immediately prior to entry into the jejunal segment, providing a total perfusate flow of 0.1 mL/min. Perfusate was sampled at t = 0 to confirm drug and \( ^3 \text{H} \)-oleic acid concentrations. After this time, the outflowing perfusate was continuously collected for 10 min intervals, and briefly vortexed before samples were taken for analysis of drug and \( ^3 \text{H} \)-oleic acid content. For experiments where drug supersaturation was generated (i.e. cinnarizine in model colloids + bile experiments), perfusate samples were briefly vortexed, and samples taken before and after centrifugation (2,200 xg, 2 min) to obtain an indication of the degree of drug precipitation within the jejunal segment. Blood draining the perfused jejunal segment was collected at 5 min intervals, plasma separated by centrifugation (10,000 xg, 5 min), and samples taken for analysis of drug and \( ^3 \text{H} \)-oleic acid concentrations.

### 4.3.11 Cinnarizine bioavailability after intraduodenal infusion

A 30 min-equilibration period was allowed between the end of surgery and drug dosing. To examine the impact of bile-induced drug supersaturation on cinnarizine absorption, studies were conducted in bile-intact or bile-diverted rats. Model colloids containing 130 µg/mL cinnarizine (~80% saturated solubility) were infused into the duodenum of rats at a rate of 1.5 ml/h for 2 h. Following infusion of the model colloids, saline was infused at a rate of 1.5 mL/h for 10 min to flush remaining formulation in the tubing into the duodenum. Blood samples (0.3 mL) were collected via the carotid artery cannula up to 8 h after infusion initiation into tubes containing 3 I.U. heparin. The sampling intervals were t = 0, 1, 1.5, 2, 3, 4, 6, 8 h. After each blood sample was taken, the cannula was flushed with 0.3 mL of 2 I.U./mL heparinised saline to ensure cannula patency, and to replace the volume of blood removed. Plasma was separated by centrifugation (10,000 xg, 5 min) to enable analysis of plasma drug and \( ^3 \text{H} \)-oleic acid concentrations as below.
4.3.12 Analytical procedures

4.3.12.1 Sample preparation and HPLC assay conditions for CIN, HF, FF, DAN, MFA

The HPLC assay conditions for CIN, HF, FF, DAN and MFA in LC colloids are reported in Table 4.1. LC colloid samples were prepared for HPLC assay by a minimum of 5-fold dilution with the respective mobile phases (see Table 4.1). The injection volume for all samples was 50 µL. Replicate analysis of n = 4 quality control samples revealed acceptable accuracy and precision (± 10%, ± 15% at the limit of quantification) for the reported concentration ranges.

CIN plasma samples were prepared for HPLC using a validated extraction procedure, with flunarizine as an internal standard, as reported previously. DAN plasma samples were prepared for LC-MS using a validated precipitation procedure, with progesterone as an internal standard, as reported previously. The assay conditions for CIN and DAN in plasma are described elsewhere. Replicate analysis of n = 4 quality control samples revealed acceptable accuracy and precision (± 10%, ± 15% at the limit of quantification) for CIN plasma concentrations between 10–320 ng/mL, and DAN plasma concentrations between 5–250 ng/mL.

4.3.12.2 Scintillation counting

Quantification of $^3$H-oleic acid in the perfusate and plasma was performed via scintillation counting on a Packard Tri-Carb 2000CA liquid scintillation analyser (Packard, Meriden, CT, USA). Perfusate samples (100 µL) and plasma samples (50 µL for in vivo studies, 200 µL for in situ studies) were added to 2 mL Irga-safe Plus scintillation fluid followed by a 10 sec vortex. Samples were corrected for background radioactivity by the inclusion of a blank sample in each run.
4.3.12.3 Blood:plasma ratio determination for CIN, DAN, oleic acid

The blood:plasma ratio for cinnarizine, danazol, and oleic acid were determined by spiking 0.5 mL blank blood with known amounts of compound to achieve low, medium, and high concentrations (in triplicate). Plasma was separated by centrifugation (10,000 xg, 5 min) and plasma drug concentration assayed by HPLC (cinnarizine), LC-MS (danazol), or scintillation counting (oleic acid). The blood:plasma ratio was calculated from the ratio of known concentration in spiked blood to the concentration measured in plasma separated from spiked blood. The mean blood:plasma ratio was subsequently used to convert plasma concentrations to blood concentrations in perfusion experiments, enabling quantification of total transport into mesenteric blood.

4.3.12.4 Calculations

In the single-pass rat jejunum perfusion model, permeability coefficients were calculated from the flux data obtained after attainment of steady state drug transport into mesenteric blood. Two apparent permeability coefficients (P_{app}) were calculated as described previously:

\[
\text{′Disappearance′ } P_{\text{app}} = -\frac{Q}{A} \ln \frac{C_1}{C_0}
\]

\[
\text{′Appearance′ } P_{\text{app}} = \frac{\Delta M_B/\Delta t}{AC_{<C>}}
\]

where ‘Disappearance’ P_{app} is the apparent permeability coefficient calculated from drug loss from the perfusate (cm/sec); ‘Appearance’ P_{app} is the apparent permeability coefficient calculated from drug appearance in the mesenteric blood (cm/sec); Q is the perfusate flow rate (mL/sec); A is the surface area of the perfused jejunal segment (cm²), which is calculated by multiplying the
diameter by the length of the perfused intestinal segment as described previously; $C_1$ is the average steady state drug concentration exiting the perfused jejunal segment (ng/mL); $C_0$ is the drug concentration entering the jejunal segment (ng/mL); $\Delta M_\theta/\Delta t$ is the average rate of drug mass appearance in mesenteric blood at steady state (ng/s); and $<C>$ is the logarithmic mean drug concentration in the lumen (ng/mL), where $<C> = (C_1 - C_0) / (\ln C_1 - \ln C_0)$.

### 4.3.12.5 Pharmacokinetic analysis

The maximum plasma concentration ($C_{\text{max}}$), the time to reach $C_{\text{max}}$ ($T_{\text{max}}$) and the area under the plasma concentration-time curve from time zero to the last measured concentration ($\text{AUC}_{0-\text{h}}$) were calculated using WinNonLin version 5.3 (Pharsight Inc., Apex, NC, USA).

### 4.3.13 Statistical analysis

Results were analysed using Student’s $t$ test. A $P$ value of $< 0.05$ was considered to be a significant difference. Analyses were performed using SPSS v19 for Windows (SPSS Inc., Chicago, IL, USA).

### 4.4 RESULTS

#### 4.4.1 Trends in drug solubility in long chain lipid-based colloids as a function of bile and lipid concentration are different for basic, neutral and acidic drugs

The solubilities of cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) in the lipid colloidal systems examined are presented in Table 4.2. The solubility enhancement (or reduction) resulting from increasing bile concentrations (holding lipid
concentration constant), and solubility reduction caused by decreasing lipid concentration (holding bile concentration constant), are shown in Figure 4.1 and Figure 4.2, respectively. Percent changes in solubility were calculated relative to low bile concentration (i.e. 4 mM) and high lipid concentration (i.e. 7.08 mM) in Figure 4.1 and Figure 4.2 respectively, to assess likely changes in drug solubilisation during bile dilution (where bile concentration increases and lipid concentration decreases in tandem) and lipid absorption (where lipid concentration decreases).

Table 4.2: Equilibrium solubility (37 °C) of cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) in long-chain colloidal systems.

| [Lipid] # (mM) || [Bile] ^ (mM) || Saturated solubility (µg/mL) |
|-------------|----------------|------------------|
|             | CIN            | HF               | FF   | DAN  | MFA  |
| 1.77        | 4              | 49               | 330  | 13   | 12   | 85   |
|             | 8              | 19               | 267  | 15   | 18   | 90   |
|             | 16             | 13               | 250  | 21   | 29   | 130  |
| 3.54 *      | 4 *            | 163              | 704  | 37   | 18   | 62   |
|             | 8              | 84               | 610  | 32   | 24   | 86   |
|             | 16             | 35               | 471  | 37   | 35   | 157  |
| 7.08        | 4              | 357              | 1270 | 84   | 29   | 84   |
|             | 8              | 261              | 1276 | 71   | 36   | 107  |
|             | 16             | 144              | 1023 | 63   | 48   | 268  |

# Lipid refers to oleic acid and 1-monoolein in 2:1 molar ratio
## Concentration refers to concentration of oleic acid
^ Bile contained bile salts, lysophosphatidylcholine, cholesterol in 16:4:1 molar ratio. The bile salt mixture comprised 25 mol% sodium glycocholate, 17.5 mol% sodium glycodeoxycholate, 25 mol% sodium glycochenodeoxycholate, 12.5 mol% sodium taurocholate, 7.5 mol% sodium taurodeoxycholate and 12.5 mol% sodium taurochenodeoxycholate
^^ Concentration refers to concentration of total bile salt
* Model long-chain colloidal system used in in situ and in vivo experiments is highlighted
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Figure 4.1: Percent change in cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) solubility stimulated by increasing concentrations of bile at varying lipid concentrations. Values calculated using raw data in Table 4.2. Solubility change was calculated relative to the solubility in 4 mM bile at each lipid concentration.

The different drugs were found to have very different solubility behaviours with respect to bile concentration in the prepared colloidal systems (Table 4.2, Figure 4.1). The solubilisation of the weak bases (CIN and HF) was significantly reduced by increasing bile concentration (i.e. an inverse relationship was apparent between solubility and bile concentration – as exemplified by negative slopes in Figure 4.1); whilst the solubilisation of the neutral drugs (DAN and FF) and the weak acid (MFA) increased in most cases in the presence of increasing bile concentrations (i.e. a direct relationship between solubility and bile concentration - as exemplified by positive slopes in Figure 4.1). The rank order by which bile ‘enhanced’ the solubilisation of drugs was CIN < HF <
FF < DAN < MFA, and appeared to be inversely correlated to drug solubility in oleic acid, such that drugs that were most soluble in oleic acid (see Table 4.3) had their solubility reduced the most by increases in bile concentration, whereas those with lower oleic acid solubility had their solubilities increased by increases in bile concentration. The magnitude of the change was seemingly greater for drugs with lower triglyceride (TG) solubility, for example, CIN solubilisation was reduced to a greater extent when compared to HF; and the solubilisation of DAN was increased to a greater extent when compared to FF.

In contrast, the solubility relationship between the drugs and colloidal lipid concentrations were more predictable (Figure 4.2). With the exception of MFA at lower bile concentrations, the solubility of all the drugs in the model colloids decreased with decreasing lipid concentration (i.e. all drugs demonstrated a direct relationship between solubility and lipid concentration). The proportional decrease in drug solubility varied significantly across the drug types, however, and was greatest for CIN (which reduced to ~ 7% of the solubility value in colloids containing 7.08 mM lipid when the lipid content was dropped to 1.77 mM), followed by FF (which reduced to 15%), HF (21%), DAN (41%), and MFA (49%). The rank order by which decreasing lipid concentration reduced the solubilisation of drugs was therefore CIN > HF ≈ FF > DAN > MFA, and reflected drug solubility in oleic acid, such that drugs with the highest solubility in oleic acid (see Table 4.3) had their solubility reduced the most by decreasing lipid (oleic acid and monoolein) concentration.
Figure 4.2: Percent solubility reduction of cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA) by decreasing lipid concentration, at various bile concentrations. Values calculated using raw data in Table 4.2. Solubility reduction was relative to the solubility in colloids containing 7.08 mM oleic acid at each bile concentration. Blue solid lines (without symbols) represent theoretical changes in solubility upon simple dilution.
Table 4.3: Melting points, Log $D_{pH7.5}$ (octanol-water distribution coefficients at pH 7.5), $pK_a$, molecular weights, and lipid solubilities of cinnarizine (CIN), halofantrine (HF), fenofibrate (FF), danazol (DAN) and meclofenamic acid (MFA).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug type</th>
<th>$T_m$ * (°C)</th>
<th>Log $D_{pH7.5}$</th>
<th>$pK_a$</th>
<th>Molecular weight</th>
<th>Solubility in soybean oil (mmol/mol)</th>
<th>Solubility in oleic acid (mmol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN</td>
<td>Weak base</td>
<td>120§</td>
<td>5.48**</td>
<td>1.95</td>
<td>7.47§§</td>
<td>368.52</td>
<td>63.9**</td>
</tr>
<tr>
<td>HF</td>
<td>Weak base</td>
<td>83#</td>
<td>8.85**</td>
<td>5.58***</td>
<td>536.88</td>
<td>82.4**</td>
<td>111.9</td>
</tr>
<tr>
<td>FF</td>
<td>Neutral</td>
<td>81^</td>
<td>5.24$$</td>
<td>n/a</td>
<td>360.83</td>
<td>197.7</td>
<td>81.6</td>
</tr>
<tr>
<td>DAN</td>
<td>Neutral</td>
<td>229§</td>
<td>4.53**</td>
<td>n/a</td>
<td>337.46</td>
<td>10.1**</td>
<td>4.8</td>
</tr>
<tr>
<td>MFA</td>
<td>Weak acid</td>
<td>257-260§</td>
<td>3.61###</td>
<td>3.76$$</td>
<td>296.15</td>
<td>19.4</td>
<td>7.8</td>
</tr>
</tbody>
</table>

n/a – not applicable

* $T_m$ denotes melting point
§ Data from Bergström et al. 230
# Data from McIntosh et al. 231
^ Data from Van Speybroeck et al. 232
§ Data from Sanphui et al. 233
** Data from Kaukonen et al. 91
$$ Data quoted in Guichard et al. 234
### Calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V9.12 (1994–2006 ACD/Labs)
^^ Data quoted in Gu et al. 235
§§ Data from Peeters 236
*** Data from Khoo et al. 237
$$$ Data quoted in Marriner et al. 238
4.4.2 The addition of rat bile to drug-loaded model colloids reduces cinnarizine solubility and promotes supersaturation, but increases danazol solubilisation

Cinnarizine (CIN) and danazol (DAN) were selected as model drugs for more detailed assessment of the impact of solubilisation properties on *in situ* and *in vivo* absorption. These compounds were chosen since bile had opposing effects on their colloidal solubility properties (Figure 4.1, Table 4.2), and lipid concentration had a greater effect on the solubility of CIN when compared to DAN (Figure 4.2, Table 4.2). To explore the impact of dilution with bile on CIN and DAN solubilisation (rather than dilution with model intestinal fluids in Figure 4.1), donor bile was collected from rats, and the change in CIN and DAN solubilisation capacity in model colloids assessed before and after 1:1 v/v addition of rat bile (Figure 4.3A). 1:1 v/v addition of donor bile decreased CIN solubility from 157.7 µg/mL to 9.2 µg/mL and therefore, as a proportion of initial, reduced CIN solubilisation capacity from 100% to 12% (taking into account the 2-fold increase in volume after the addition of bile). In contrast, for DAN, combination of the model colloids with bile in a 1:1 v/v ratio increased DAN solubility from 17.6 µg/mL to 22.6 µg/mL, and therefore increased DAN solubilisation capacity from 100% to 257% (again taking into account a doubling in volume on bile addition). Dilution of the colloids with the same volume of buffer did not result in significant changes in the solubilisation capacity of either DAN or CIN (Figure 4.3A). As such, the changes in solubilisation capacity observed on addition of bile were not simply due to a volume/dilution effect (i.e. decreasing colloidal lipid concentration), but instead appear to reflect increases in bile concentration, since the average total bile salt concentration in donor bile was 15.0 ± 1.0 mM (mean ± SEM, n = 4) whereas the bile concentration in the model colloidal system was 4 mM.

The solubility of CIN therefore decreased with increasing bile concentration, whereas the solubility of DAN increased with increasing bile concentration. This trend with donor rat bile was
consistent with the \textit{in vitro} data obtained using SEIF (Figure 4.1), and suggests that CIN may supersaturate when drug-loaded model colloids interact with secreted bile \textit{in vivo}.

To assess the kinetics of drug precipitation on interaction with bile, CIN-loaded model colloids (CIN loaded at 130 µg/mL, ~80% saturated solubility) were incubated 1:1 v/v with donor rat bile in a temperature (37 °C) and stirring rate-controlled vessel, and samples taken over time to evaluate whether precipitation occurred. Figure 4.3B shows that bile addition did not result in immediate drug precipitation, and instead was preceded by a period of supersaturation that lasted for at least 80 min in all cases. Since DAN solubility increased rather than decreased on bile addition, supersaturation/precipitation did not occur and therefore precipitation kinetics were not evaluated.

\textbf{Figure 4.3:} (A) Percent original solubilisation capacity of cinnarizine (CIN) and danazol (DAN), before and after a 1:1 v/v addition of buffer or rat bile to model colloids. After the addition of rat bile, solubility of CIN decreased from 157.7 ± 3.0 µg/mL to 9.2 ± 0.7 µg/mL, while solubility of DAN increased slightly from 17.6 ± 0.5 µg/mL to 22.6 ± 2.5 µg/mL. After the 1:1 v/v addition of buffer, solubility of CIN and DAN was approximately halved, from 157.7 ± 3.0 µg/mL to 73.8 ± 1.0 µg/mL, and 17.6 ± 0.5 µg/mL to 8.8 ± 0.2 µg/mL, respectively. Percent original solubilisation capacity is the solubilisation capacity of the colloids after bile addition (taking into account...
volume increases as a result of dilution) relative to the solubilisation capacity of the colloids prior to bile addition. Data represent mean ± SEM of n = 3 determinations. Significant difference (p < 0.05) to colloid only is denoted by the symbol *. (B) Kinetics of CIN precipitation from model colloids following 1:1 v/v addition of rat bile at t = 0. CIN was loaded into colloids at 80% saturation (~130 µg/mL). Each line represents individual experiments. The addition of bile to drug-loaded colloids resulted in CIN supersaturation that was maintained for at least 80 min in all experiments.

4.4.3 Co-perfusion of bile with model colloids increases cinnarizine absorption to a greater extent than danazol

The perfusate disappearance and mesenteric blood appearance profiles of cinnarizine (CIN), danazol (DAN) and oleic acid after perfusion of drug in LC lipid-loaded model colloids through an isolated jejunal segment (~ 10 cm²), with and without 1:1 v/v co-perfusion with donor rat bile, are shown in Figure 4.4. Steady state-absorptive flux, disappearance $P_{\text{app}}$, and appearance $P_{\text{app}}$ from all perfusion experiments are reported in Table 4.4.

Co-perfusion of model colloids with bile increased the absorptive flux, disappearance $P_{\text{app}}$, and appearance $P_{\text{app}}$ of CIN 3.2-fold, 3.0-fold, and 4.2-fold, respectively. Throughout the perfusion experiments, bile-induced supersaturation appeared to be stable, consistent with the in vitro studies (Figure 4.3B), since the CIN perfusate concentration was unchanged before or after centrifugation, therefore indicating a lack of CIN precipitation during co-perfusion with bile (Figure 4.4AI).

Co-perfusion of model colloids with bile was expected to lead to an increase in DAN solubilisation capacity within the perfused jejunal segment and therefore to not stimulate DAN supersaturation or an increase in thermodynamic activity. In spite of this, however, co-perfusion of
model colloids with bile led to a moderate (1.7-fold) increase in both the absorptive flux and appearance $P_{app}$ of DAN (disappearance $P_{app}$ was unchanged) (Figure 4.4II and Table 4.4). The increase in absorptive flux in the presence of bile was, however, notably higher for CIN than DAN (3.2-fold increase in CIN vs. 1.7-fold increase in DAN).

In assessing the permeability of CIN and DAN, the concentration of the drug in the perfusate was kept constant (65 µg/mL and 7 µg/mL, respectively), allowing direct comparisons of absorptive flux in the absence and presence of bile. For oleic acid, however, the concentration in the perfusate was reduced by 50% due to bile co-perfusion. Therefore, direct comparisons of absolute oleic acid absorptive flux was not possible, and transport into the mesenteric blood is represented instead as % dose transported (Figure 4.4BIII). Nonetheless, bile co-perfusion led to an increase in the proportion of oleic acid absorbed (Figure 4.4BIII), and an increase in the appearance $P_{app}$ of oleic acid (Table 4.4).
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Figure 4.4: (A) Perfusate disappearance (% dose passing through jejunum) and (B) mesenteric blood appearance of (I) cinnarizine (CIN), (II) danazol (DAN), and (III) oleic acid (OA) when model colloids were perfused through an isolated rat jejunal segment (~ 10 cm²), with (open symbols) and without (closed symbols) 1:1 v/v co-perfusion with donor rat bile. Appearance profiles are plotted as absorptive flux (ng/5 min/10 cm²) for CIN and DAN, and % dose passing...
through jejunum for OA. Co-perfusion of model colloids with bile generates supersaturation *in situ* within the perfused jejunal segment for CIN but not DAN. It is not known if co-perfusion of colloids with bile generates OA supersaturation within the perfused jejunal segment. SS denotes experiments where drug is supersaturated in perfusate. Degree of CIN and OA (if any) precipitation within the perfusate is illustrated in (AI) and (AIII) as the difference in perfusate concentration pre- and post-centrifugation. Experiments were performed using an *in situ* single-pass rat jejunum perfusion model. In all experiments, the total perfusate flow rate was kept constant at 0.1 mL/min. Data represent mean ± SEM of n = 3-4 rats.
Table 4.4: Cinnarizine (CIN), danazol (DAN) and oleic acid (OA) disappearance $P_{\text{app}}$ (x $10^6$ cm/s) from the intestinal perfusate, appearance $P_{\text{app}}$ (x $10^6$ cm/s) in the mesenteric blood, and steady state absorptive flux into mesenteric blood (ng/5 min/10 cm$^2$) after 70 min of single-pass perfusion of ~ 10 cm$^2$ segments of rat jejunum with model colloids (composition reported in Table 4.2), with and without 1:1 v/v co-perfusion with donor rat bile. Values calculated using data obtained after steady state attainment ($t = 55-70$ min). In all experiments, total perfusate flow rate was kept constant at 0.1 mL/min. Data represent mean ± SEM of $n = 3-4$ rats.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Conc. perfused (µg/mL)</th>
<th>SS ratio $#^*$</th>
<th>Disappearance $P_{\text{app}}$ (x $10^6$ cm/s)</th>
<th>Appearance $P_{\text{app}}$ (x $10^6$ cm/s)</th>
<th>$P_{\text{app}}$</th>
<th>Flux into mesenteric blood (ng/5 min/10 cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN</td>
<td>Model colloid only</td>
<td>65.0</td>
<td>0.4</td>
<td>$12.2 \pm 2.8$</td>
<td>$0.6 \pm 0.2$</td>
<td>$138 \pm 28$</td>
</tr>
<tr>
<td></td>
<td>Model colloid + Bile $^{ss}$</td>
<td>65.0</td>
<td>7.1</td>
<td>$36.9 \pm 5.5^*$</td>
<td>$2.5 \pm 0.2^*$</td>
<td>$443 \pm 34^*$</td>
</tr>
<tr>
<td>DAN</td>
<td>Model colloid only</td>
<td>7.2</td>
<td>0.4</td>
<td>$27.7 \pm 6.8$</td>
<td>$3.8 \pm 0.1$</td>
<td>$75 \pm 2$</td>
</tr>
<tr>
<td></td>
<td>Model colloid + Bile</td>
<td>7.2</td>
<td>0.3</td>
<td>$29.4 \pm 3.0$</td>
<td>$6.6 \pm 0.8^*$</td>
<td>$131 \pm 15^*$</td>
</tr>
<tr>
<td>OA</td>
<td>Model colloid only</td>
<td>1.0</td>
<td>n/a</td>
<td>$18.0 \pm 5.9$</td>
<td>$2.0 \pm 0.3$</td>
<td>$6 \pm 1$</td>
</tr>
<tr>
<td></td>
<td>Model colloid + Bile</td>
<td>0.5</td>
<td>n/a</td>
<td>$26.1 \pm 3.8$</td>
<td>$6.1 \pm 0.8^*$</td>
<td>$9 \pm 1$</td>
</tr>
</tbody>
</table>

$^a$ Significant difference from model colloid only  
$^{ss}$ SS denotes drug supersaturation in perfusate 
$^*$ SS ratio = Supersaturation ratio = (Supersaturated) concentration of drug in perfusate / Equilibrium solubility of drug in perfusate  
$^{ss}$ SS ratio not reported for OA because equilibrium solubility of OA in perfusate was not determined
4.4.4 Supersaturation increases in vivo cinnarizine exposure after intraduodenal infusion of drug-loaded model colloids

The systemic plasma concentration-time profiles for cinnarizine (CIN) and $^3$H-oleic acid following intraduodenal infusion of CIN-loaded model colloids (130 µg/mL CIN; ~80% saturated solubility) to bile-intact and bile-diverted rats are shown in Figure 4.5, and the pharmacokinetic parameters reported in Table 4.5. Consistent with the results from the rat jejunal permeability studies, intraduodenal administration of drug-loaded model colloids to bile-intact rats resulted in significantly higher systemic plasma concentrations of CIN at $t = 1.5, 2, 3$ and $4$ h, and significantly higher $\text{AUC}_{0-8\text{ h}}$ (2.0-fold increase) when compared to bile-diverted rats. The systemic plasma concentrations of $^3$H-oleic acid were also significantly higher in bile-intact rats at all sample time points.

Bioavailability studies for danazol (DAN) are not reported here because the dose administered (0.14 mg/kg – corresponding to 80% saturated solubility) did not lead to detectable systemic plasma concentrations in both bile-intact and bile-diverted rats at all sample time points (limit of quantification for DAN plasma assay was 5 ng/mL).
Chapter 4: Supersaturation potential is enhanced for basic drugs

Figure 4.5: Systemic plasma concentration-time profiles of (A) cinnarizine (CIN) and (B) oleic acid (OA) following a 2-hour intraduodenal infusion of 3 mL model colloids containing 130 µg/mL CIN (~ 80% saturated solubility) and 1 µCi/mL OA to bile-intact and bile-diverted rats. Consistent with observations in rat jejunum perfusion studies, bile-induced supersaturation translated into increased in vivo CIN exposure. Data represent mean ± SEM of n = 3 rats. Statistical significance (p < 0.05) is denoted by the symbol *.

Table 4.5: Pharmacokinetic parameters for cinnarizine (CIN) after intraduodenal administration of model colloids (composition reported in Table 4.2) loaded with 1.3 mg/kg CIN (~ 80% saturated solubility) to bile-intact and bile-diverted rats. The interaction between the colloids and secreted bile in the duodenum is expected to generate CIN supersaturation in bile-intact animals, whilst CIN is expected to be sub-saturated in bile-diverted animals. Values represent mean ± SEM of n = 3 rats.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Experimental group</th>
<th>$\text{AUC}_{0-8\ h}$ (ng h/mL)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$T_{\text{max}}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnarizine</td>
<td>Bile-intact</td>
<td>403 ± 3 $^a$</td>
<td>107.1 ± 10.8 $^a$</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Bile-diverted</td>
<td>190 ± 17</td>
<td>53.0 ± 8.8</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ Significant difference when compared to bile-diverted group
4.5 DISCUSSION

Previously, we described the ability of bile secretion to trigger drug supersaturation and to enhance the absorption of a model PWSD (cinnarizine) from intestinal colloidal phases containing digestion products of medium-chain triglycerides (Chapter 3). In the current study, we aimed to extend these initial observations to include assessment of colloidal phases containing long-chain lipids (to evaluate the dependence on lipid chain length) and PWSD with differing physicochemical properties (to assess the importance of acid/base functionality, lipid solubility and octanol-water distribution coefficient). The supersaturation tendency of the different PWSD during endogenous processing of post-digestion lipid colloidal phases was predicted by assessing drug solubility relationships with increasing bile concentration and decreasing lipid concentration; and the absorption of drugs with different supersaturation tendencies was evaluated in situ and in vivo. The data suggest that under the conditions explored, bile dilution of, and lipid removal/absorption from, LC colloids may induce supersaturation and enhance the absorption of PWSD, and that the potential for drug supersaturation during lipid processing is more pronounced for basic drugs.

The solubility of lipophilic PWSD in lipid-containing intestinal colloids is a complex function of the solid state properties of the drug (usually captured by melting point), and drug affinity for core lipids, the colloid interface and water, and is not easily predicted de novo. For example, the lipid solubility of FF far exceeds that of the similarly lipophilic (by log P) DAN (Table 4.3), presumably due to differences in melting point. However, both drugs had comparable solubility in the colloidal systems examined here (Table 4.2). In contrast, although CIN and HF have similar solubility properties in lipids, the solubility of HF in the model colloids was up to an order of magnitude higher than CIN, suggesting specific affinity of HF for the colloidal structures rather than their component parts. MFA has low solubility in lipids but had moderate solubility in the
prepared colloids, presumably due to ionisation (and higher water solubility) at the pH of model intestinal fluids.

Examination of the impact of bile concentration on the drug solubilisation capacities of the long-chain colloidal systems revealed widely varying solubility trends for the drugs evaluated (Figure 4.1 and Table 4.2). Traditionally it has been assumed that bile secretion promotes drug solubilisation (and in this way promotes drug absorption), and indeed increases in drug solubility were evident with increasing bile concentration for the neutral (DAN, FF) and acidic (MFA) drugs. In contrast, however, and in large part counter-intuitively, the solubility of the basic drugs (CIN, HF) in the long-chain colloids decreased with increasing bile concentration. The different relationships between the colloid solubility of the basic, neutral and acidic drugs and bile concentration was not expected, but may reflect changes in the structure of the colloidal phases on bile addition, as well as differences in the preferred drug solubilisation site. Under digesting conditions in the small intestine, many amphiphilic molecules (e.g. bile salts, lyso-phospholipids, fatty acids, monoglycerides) co-exist, and the colloidal species formed display complex phase behaviour that is dependent on the type and concentration of the components present\textsuperscript{199,239,240}. For example, raising the concentration of endogenous biliary components (e.g. bile salts) relative to the concentration of lipid digestion products has been shown to stimulate phase changes from larger lipid rich species such as cubic, hexagonal and multilamellar liquid crystals to smaller, more highly dispersed species such as unilamellar vesicles and mixed micelles\textsuperscript{39,40}. Continued phase changes from unilamellar vesicles to mixed micelles on addition of higher concentrations of bile salts are also well-documented\textsuperscript{218,241,242}. In view of the dynamic nature of the structure of intestinal lipid colloidal species, drug solubilisation capacity may also change with the addition or removal of amphiphilic components, since different phases possess different solubilisation capacities for PWSD\textsuperscript{194,243}. 
Solubility changes as a function of microstructure have been described for many poorly water-soluble compounds in larger, microemulsion-based colloidal systems\textsuperscript{244-246}. The dependency of compound solubility on microstructure is thought to be related to the preferred solubilisation locus within the microstructure, and drugs that preferentially reside at an interface may have increased solubility when the interfacial area of colloidal systems is increased\textsuperscript{247}. It is thus possible that in the colloidal systems examined here, differences in the solubility behaviour of the basic/neutral/acidic drugs reflect differences in preferred solubilisation sites within the colloids. CIN and HF have higher solubilities in oleic acid (presumably due to favourable electrostatic interactions in lipid solution), and are therefore more likely to co-localise with oleic acid in the hydrophobic core of a micelle or in the lipid bilayer of a vesicle when compared to DAN and MFA, which in turn may be more highly solubilised at the amphiphilic interface. Under these circumstances, the addition of bile constituents to the colloids (which disperses the lipids, decreasing the proportional volume of the lipid core and increasing the colloid interfacial area) may lead to increased DAN and MFA solubilisation, but reduced solubilisation of CIN and HF.

It is also possible that within the lipophilic sub-compartments of the dispersed colloidal structures, molecules of oleic acid and the weak bases orientate in such a way that the hydrophobic aspects of each molecule remain dissolved in the lipid core, whereas the charged species orientate towards the interface where the cationic weak bases and the carboxylic acid head groups of oleic acid are free to favourably interact. Under these circumstances increased concentrations of bile acids may also disrupt intermolecular interactions between oleic acid and weak bases at the colloidal interface, further lowering drug solubilisation capacity.

Reduced solubilisation of the weak bases with increasing bile concentration may also occur via the formation of insoluble drug-bile salt complexes between the cationic weak bases and anionic bile
salts. To investigate the potential importance of the effects of bile on interactions between the weak bases and oleic acid vs. drug interaction or complexation with bile salt, a series of colloidal systems analogous to those in Table 4.2 (i.e. containing bile components and monoolein) but without the inclusion of oleic acid were prepared, and CIN solubility assessed. As expected, the solubility of CIN in the oleic acid-free colloidal systems (Table 4.6) was substantially lower than the corresponding oleic acid-containing colloidal systems (Table 4.2), illustrating the high dependency of the colloid solubility of the weak bases on the presence of oleic acid. More significantly, in the oleic acid-free systems, CIN solubility increased (rather than decreased) with increasing bile concentration (Table 4.6). The stark difference in CIN solubility behaviour in oleic acid-free vs. oleic acid-containing colloidal systems suggests that the effect of bile in reducing CIN solubilisation in bile salt mixed micelles is oleic acid dependent, and that the reduction in CIN solubility was not due to bile salt-drug complexation, but rather bile-mediated disruption of intermolecular interactions between oleic acid and the weak bases.
Table 4.6: Equilibrium solubility (37 °C) of cinnarizine (CIN) in colloidal systems consisting 1-monoolein solubilised in bile components. Colloidal systems presented in this table correspond to those in Table 4.2, but without the inclusion of oleic acid.

<table>
<thead>
<tr>
<th>[1-Monoolein] (mM)</th>
<th>[Bile] (∗) (mM)</th>
<th>CIN saturated solubility (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.88</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>1.77</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>3.54</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

∗ Bile refers to total bile salt, lysophosphatidylcholine, cholesterol in 16:4:1 molar ratio. Total bile salt consisted of 25 mol% sodium glycocholate, 17.5 mol% sodium glycodeoxycholate, 25 mol% sodium glycochenodeoxycholate, 12.5 mol% sodium taurocholate, 7.5 mol% sodium taurodeoxycholate, 12.5 mol% sodium taurochenodeoxycholate

^^ Concentration refers to concentration of total bile salt

Endogenous lipid processing pathways that lead to decreases in the drug solubilisation capacity of the intestinal colloidal phases may lead to drug supersaturation (provided that precipitation is not instantaneous). Since the bile:lipid concentration ratio progressively increases on passage down the GI tract, the tendency for a drug to supersaturate may be predicted by the relationship between drug solubility and increasing bile/decreasing lipid concentrations. Thus, during bile dilution of lipid colloidal phases, drugs that have an inverse solubility relationship with bile concentration are expected to supersaturate, and the higher the proportional decrease in solubilisation (caused by increasing bile concentration), the higher the supersaturation tendency. Although bile dilution also
Chapter 4: Supersaturation potential is enhanced for basic drugs

leads to a decrease in colloidal lipid concentration, the percent solubility reduction vs. [lipid] plots (Figure 4.2) suggest that drug solubility decreases no more than, and in most cases less than, the dilution factor (solubility plots mostly overlap or are above the dilution line). As such, the overall solubilisation capacity of the intestinal colloidal phases (with respect to a decrease in lipid concentration) is likely to remain unchanged or even increase. Supersaturation tendency due simply to bile dilution is therefore expected to be less dependent on changes in lipid concentration and more dependent on changes in bile concentration. During lipid absorption, however, colloidal structures are further depleted of lipid content (in the absence of volume changes). In this case solubilisation capacity is expected to drop (and therefore supersaturation tendency to increase) and this would be greatest for drugs with the highest solubility dependency on lipid concentration. Taking into consideration the proportional changes in drug solubility caused by increasing bile concentration and decreasing lipid concentration, the overall drug supersaturation tendency resulting from both bile dilution of and lipid absorption from lipid colloidal phases follows the rank order CIN > HF > FF > DAN > MFA, i.e. supersaturation tendency was greatest for basic drugs followed by neutral drugs and acidic drugs.

Indeed, when CIN-loaded colloids were mixed with endogenous rat bile (to simulate the process of bile dilution), CIN solubilisation capacity was reduced significantly, and the period of drug supersaturation that preceded precipitation translated into enhanced drug absorption (Figure 4.4BI) and enhanced systemic exposure (Figure 4.5A). Notably, cinnarizine was found to precipitate in the crystalline form in these experiments (Figure 4.6), precluding the possibility that the enhanced cinnarizine absorption from model colloids (when bile was co-perfused) observed in Figure 4.4BI was due to accelerated cinnarizine dissolution from precipitated amorphous forms. In addition, during bile co-perfusion, the proportion of oleic acid absorbed was increased (Figure 4.4BIII). Since CIN solubilisation capacity is directly related to colloidal lipid content, the increased
absorption of oleic acid (when bile was co-perfused) was expected to magnify the decrease in drug solubilisation capacity, and further increase CIN supersaturation potential and absorption.

**Figure 4.6**: Analysis of the cinnarizine precipitate using polarised light microscopy following precipitation kinetics experiments where fasted rat bile was added in a 1:1 v/v ratio to cinnarizine-loaded model colloids (cinnarizine was loaded into model colloids at 80% saturation (~130 µg/mL). Addition of bile reduced the equilibrium cinnarizine solubilisation capacity of model colloids to 12% of initial, and triggered cinnarizine supersaturation and precipitation (see Figure 4.3). The polarised light microscopy image show that cinnarizine precipitated in the crystalline form after the addition of bile.

When similar studies were repeated with DAN (which has significantly lower absolute solubility in lipid digestion products and lower supersaturation potential based on the impact of increases in bile concentration and decreases in lipid concentration on DAN solubility; Table 4.3, Figure 4.1, Figure 4.2), absorptive flux also increased when model colloids were co-perfused with bile, but to a much lower extent than CIN (1.7-fold, compared to 3.2-fold for CIN). The effect of bile in enhancing DAN absorption most likely reflected differences in oleic acid absorption in the presence of bile (Figure 4.4BIII). Thus, bile increased oleic acid absorption, and in turn decreased DAN solubilisation capacity in the lipid depleted mixed micelles, resulting in an increase in DAN
Chapter 4: Supersaturation potential is enhanced for basic drugs

thermodynamic activity at the absorptive membrane. This occurred despite a slight decrease in DAN thermodynamic activity in the bulk lumen due to bile addition (via an increase in drug solubilisation capacity due to bile). Other potential explanations for the change in DAN absorption include an increase in flux resulting from an increase in permeability due to bile-induced membrane damage\(^{248, 249}\) or bile-induced changes to pH (the average pH of donor bile used was 8.02 ± 0.02 (mean ± SEM, n = 5)). These factors were, however, excluded in previous studies using similar experimental methods and colloidal systems (Chapter 3). The presence of phospholipids in endogenous bile is also expected to reduce the toxicity (and permeability enhancement effects) of bile salts by decreasing bile salt thermodynamic activity in mixed bile salt-phospholipid micelles\(^{108}\). The permeability of DAN, a non-ionisable drug, is not expected to be affected by changes to pH.

Differences in the extent to which bile enhanced the absorption of CIN and DAN may therefore be attributed to differences in the supersaturation tendencies of the two drugs during bile dilution and lipid absorption. In the case of CIN, increases in thermodynamic activity were triggered by both bile dilution and lipid absorption; whereas in the case of DAN, the increase in thermodynamic activity was triggered only during lipid absorption. These observations suggest that bile-induced supersaturation may be a phenomenon that is specific to basic drugs; while lipid absorption-induced supersaturation is expected to apply to all lipophilic drugs, and the degree of supersaturation induced is likely to be greatest for basic drugs (which had higher solubility dependency on the concentration of lipid digestion products within bile micelles).

Thus, the \textit{in vitro} and \textit{in vivo} observations in this study suggest that drugs with high solubility in lipid digestion products (such as weak bases in fatty acid-containing colloids) may better harness the supersaturation-generating potential of endogenous lipid processing pathways. This reflects three separate, but sequential, processes (see Figure 4.7). First, strong affinity for solubilised lipid...
digestion products may enable favourable drug partitioning from digesting triglyceride droplets into post-digestion lipid colloidal phases. Subsequently, drug is preferentially solubilised in the core of lipid colloidal phases at high concentration and is therefore predisposed to bile-induced supersaturation in the event of a drop in solubilisation capacity. Finally, lipid absorption (i.e. lipid removal from colloids) exaggerates the loss of drug solubilisation capacity and therefore encourages supersaturation if precipitation is not immediate. Drug affinity for lipid digestion products may therefore be a useful indicator of supersaturation potential during LBF processing in the GI tract.

In summary, we previously described a dual role for bile in facilitating drug absorption after co-administration with medium-chain formulation lipids, where bile-mediated solubilisation of lipid digestion products and bile-mediated dilution of existing lipid colloidal phases provides a means to simultaneously increase solubilisation capacity and promote the thermodynamic activity of a co-administered drug in the small intestine (Chapter 3). The data described here further establish that endogenous processing of post-digestion lipid colloidal phases (via bile dilution and lipid absorption) may generate drug supersaturation after co-administration of long-chain lipids; and that the potential for supersaturation generation and ensuing absorption enhancement is greatest for basic drugs. The unique manner by which lipids promote both drug solubilisation and drug supersaturation likely contributes to effective drug absorption after lipid co-administration, and has implication both for LBF design and better understanding the effects of lipids in food on drug absorption. The sequential manner (see Figure 4.7) by which supersaturation is triggered during endogenous lipid processing (e.g. during lipid dispersion and digestion, as a result of ongoing bile dilution, and in response to lipid absorption), combined with the presence of drug solubilising lipid colloidal phases in the small intestine that ‘buffer’ against the generation of excessive supersaturation, dictates that the rate and extent of supersaturation generated by LBF in
vivo is highly controlled, and has the potential to maximise absorption-enhancement and reduce the risk of drug precipitation.

4.6 CONCLUSION

Supersaturation-triggered enhancements in thermodynamic activity may represent an important mechanism by which solubilised drug is made available for absorption from the lipid colloidal phases formed in the intestine following digestion of dietary or formulation lipids. In this study, we investigated drug supersaturation during bile-mediated dilution of intestinal colloid phases containing digestion products of long-chain triglycerides. The increase in colloidal concentration of biliary solubilisers upon dilution with bile was a highly effective trigger for supersaturation for basic drugs, whereas for acidic or neutral drugs, bile-mediated enhancements in lipid absorption and therefore decreases in colloidal lipid content appeared to provide the required reduction in solubilisation capacity to promote supersaturation. The data suggest that supersaturation tendency during endogenous processing of intestinal colloids is greatest for basic when compared to neutral/acidic drugs and likely reflects the high affinity of weak bases for colloidal systems containing oppositely charged fatty acids. Studies to evaluate the importance of intestinal colloids containing natural glyceride digestion products (i.e. fatty acids rather than systems based on uncharged lipids or synthetic solubilisers) in maximising the absorption of co-administered drugs are on-going.
Figure 4.7: Endogenous lipid processing pathways that facilitate both drug solubilisation and drug supersaturation in the small intestine. (i) Bile-mediated solubilisation of lipid digestion products at the interface of a digesting oil droplet results in the generation of lipid colloidal phases such as vesicles and micelles that promote drug solubilisation (ii) Digestion and dispersion of lipid based formulations (LBF) may reduce drug solubilisation capacity and trigger drug supersaturation \(^{176, 227}\) (iii) The continuing interaction of secreted bile and existing lipid colloidal phases in the lumen may reduce the solubilisation capacity of basic drugs and trigger drug supersaturation (iv) The removal of colloidal lipid components via lipid absorption reduces drug solubilisation capacity and also triggers drug supersaturation at the absorption site. Drugs that have high affinity for lipid digestion products (e.g. basic drugs such as cinnarizine and halofantrine) may be more prone to supersaturation events as they preferentially partition into post-digestion phases (instead of remaining in a digesting TG droplet), are predisposed to bile-induced supersaturation, and show exaggerated losses in drug solubilisation capacity (i.e. greater supersaturation potential) during lipid absorption. The sequential manner by which supersaturation is triggered during endogenous LBF processing (ii, iii, iv), combined with the supersaturation ‘buffering’ properties of lipid colloidal phases (via increases in intestinal solubilisation capacity), dictate that the rate and extent of supersaturation generated by LBF \textit{in vivo} is intrinsically controlled, thus maximising absorption-enhancement potential of supersaturation and reducing the risk of drug precipitation. The unique ability of LBF components to simultaneously increase solubilisation capacity and promote thermodynamic activity of co-administered drug in the small intestine may contribute to effective drug absorption often observed with LBF co-administration.
Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

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

<table>
<thead>
<tr>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concept and design of studies, planning and execution of experimental work, data analysis and interpretation, formulation of conclusions and hypotheses resulting from the relevant studies, drafting and revision of manuscript</td>
<td>70%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.J.H. Porter</td>
<td>Project supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
<tr>
<td>N.L. Trevaskis</td>
<td>Project co-supervisor, data and manuscript review</td>
<td>NA</td>
</tr>
</tbody>
</table>

Candidate’s Signature

Declaration by co-authors

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate’s contribution to this work, and the nature of the contribution of each of the co-authors.
(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(16) there are no other authors of the publication according to these criteria;
(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

| Location(s)          | Monash Institute of Pharmaceutical Sciences                |

188
<table>
<thead>
<tr>
<th>Signature 1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature 2</td>
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</table>
CHAPTER 5 : LIPID ABSORPTION TRIGGERS DRUG SUPERSATURATION AT THE INTESTINAL UNSTIRRED WATER LAYER AND PROMOTES DRUG ABSORPTION FROM MIXED MICELLES

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Manuscript in submission.
5.1 ABSTRACT

**Purpose.** To evaluate the potential for the acidic intestinal unstirred water layer (UWL) to induce drug supersaturation and enhance drug absorption from intestinal mixed micelles, via the promotion of fatty acid absorption. **Methods.** Using a single-pass rat jejunal perfusion model, the absorptive-flux of cinnarizine and $^3$H-oleic acid from oleic acid-containing intestinal mixed micelles was assessed under normal acidic microclimate conditions and conditions where the acidic microclimate was attenuated via the co-administration of amiloride. As a control, the absorptive-flux of cinnarizine from micelles of Brij® 97 (a non-ionisable, non-absorbable surfactant) was assessed in the absence and presence of amiloride. Cinnarizine solubility was evaluated under conditions of decreasing pH and decreasing micellar lipid content to assess changes in solubilisation and thermodynamic activity during micellar passage across the UWL.

**Results.** In the presence of amiloride, the absorptive-flux of cinnarizine and $^3$H-oleic acid from mixed micelles decreased 6.5-fold and 3.0-fold, respectively. In contrast, the absorptive-flux of cinnarizine from Brij® 97 micelles remained unchanged by amiloride, and was significantly lower than from the long-chain micelles. Cinnarizine solubility in long-chain micelles decreased under conditions where pH and micellar lipid content decreased simultaneously. **Conclusion.** The acidic microclimate of the intestinal UWL promotes drug absorption from intestinal mixed micelles via the promotion of fatty acid absorption which subsequently stimulates drug supersaturation. The observations suggest that formulations containing absorbable lipids (or their pre-digestive precursors) may outperform formulations that lack absorbable components due to the benefits associated with lipid absorption-induced drug supersaturation.
5.2 INTRODUCTION

Co-administration of poorly water-soluble drugs (PWSD) with lipids often leads to a significant enhancement in oral bioavailability\(^2\). In the small intestine, the digestion of formulation or dietary-derived di/triglycerides liberates fatty acids and monoglycerides that are solubilised by biliary components (bile salts, phosphatidylcholine, cholesterol) to generate a series of lipid colloidal structures including vesicles and mixed micelles. These colloidal phases in turn provide dispersed lipidic microenvironments for the solubilisation of co-administered PWSD, thereby increasing the drug solubilisation capacity of the small intestine when compared to conditions in the fasted state\(^76\).

Although solubilisation increases the apparent solubility of PWSD in the small intestine, the total concentration of drug in solution (\(C_{\text{total}}\)) exists in equilibrium between the concentration solubilised in the colloidal fraction (\(C_{\text{colloid}}\)) and the concentration in the free fraction (\(C_{\text{free}}\)):

\[
C_{\text{total}} = C_{\text{free}} + C_{\text{colloid}}
\]

**Equation 5.1**

In the absence of solid drug, solubilisation in colloidal structures such as micelles and vesicles is expected to result in a reduction in drug thermodynamic activity\(^143\). In simple micellar systems a reduction in thermodynamic activity manifests as a decrease in \(C_{\text{free}}\). Thus, solubilisation in colloids does not increase (and may reduce) free drug concentrations. Whether increases in total solubilisation capacity translate into enhancements in drug absorption is therefore difficult to predict with certainty. Indeed, recent studies suggest that in the absence of an increase in free drug concentrations, solubilisation may not result in enhanced drug absorption despite increases in total solubilised drug concentrations\(^100, 145, 146\).
Recently, however, we have observed that formulations containing lipids may provide unique absorption benefits for solubilising formulation, since drug supersaturation appears to be triggered during lipid processing in the gastrointestinal (GI) tract\textsuperscript{4, 176, 227, 251}. Under these circumstances, the induction and maintenance of supersaturation has the potential to reverse (or at least attenuate) the reduction in drug thermodynamic activity inherent in solubilisation, and may significantly enhance free drug concentrations above the aqueous solubility. For lipid-based formulations (LBF), drug supersaturation may be generated by several processes. Firstly, when the formulation loses solubilisation capacity during the dilution of water miscible co-solvents or surfactants\textsuperscript{17, 178, 191}, secondly, as a result of the digestion of triglycerides and/or surfactants within the formulation\textsuperscript{4, 91, 176, 251}, and thirdly as lipid-rich colloidal species are diluted by biliary secretions (Chapters 3 and 4).

The ability for drug supersaturation in lipid-based colloids to translate into enhanced drug absorption has been shown using systems that model the species likely to form during the digestion of glyceride lipids (i.e. micelles and vesicles containing bile salts, lysophospholipid, cholesterol, fatty acid and monoglyceride) (Chapters 3 and 4). In these studies, interaction of secreted bile with lipid colloidal phases reduced the solubilisation capacity of the colloids for poorly water-soluble weak bases, but since drug precipitation was not immediate, supersaturation was induced. The period of drug supersaturation that preceded drug precipitation coincided with significant enhancements in the absorption of cinnarizine across rat jejunum (Chapters 3 and 4).

Interestingly, in the same studies, some increase in absorption in the presence of bile was also apparent for danazol, even though interaction with bile did not reduce micellar solubilisation capacity \textit{in vitro} (and therefore did not stimulate supersaturation). Indeed, addition of bile increased drug solubility in a fashion more consistent with traditional models for micellar
solubilisation (where the addition of solubilising species such as bile typically increases solubilisation capacity). For danazol, the driver for increased drug absorption was suggested to be the potential for lipid absorption (i.e. the removal of micellar lipid content) to reduce micellar drug solubilisation capacity and to trigger drug supersaturation at the absorptive site (assuming lipid absorption was faster than drug absorption). These preliminary data provide the background for the current chapter that has examined in detail the role of lipid absorption from intestinal micellar species as a driver of drug supersaturation, and therefore increased drug absorption.

The absorption of long-chain fatty acids (LCFA) is extremely efficient and facilitated by the acidic microclimate (pH 5.3–6.2\textsuperscript{45,46,57,252}) that is present within the unstirred water layer (UWL) at the absorptive surface of the small intestine\textsuperscript{56,57}. The UWL (see Figure 5.1) separates bulk intestinal fluid from the surface of intestinal absorptive cells, and is estimated to be 500-800 µm wide\textsuperscript{45,46}. The UWL exists coincident with, and is indistinguishable from, a viscous mucus layer consisting of water (~ 95%), glycoproteins, lipids, mineral salts and free proteins\textsuperscript{45,47,48}. The acidic microclimate of the UWL is maintained by the action of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter present at the brush border membrane of enterocytes\textsuperscript{46}, as well as the mucus coating which retards H\textsuperscript{+} diffusion into bulk luminal fluid\textsuperscript{45,46}. Shiau and colleagues were the first to describe the facilitatory role of the acidic microclimate in dietary LCFA absorption from intestinal mixed micelles\textsuperscript{56}. These studies showed that LCFA absorption was higher in the presence of the low pH microclimate of the UWL. The authors postulated that the exposure of micelles to the UWL acidic microclimate led to the protonation of ionised LCFA and an increase in lipid absorption via two mechanisms (depicted in Figure 5.1(i)). Firstly, protonated LCFA were expected to preferentially partition into and across the absorptive membrane in accord with classical pH-partition theory\textsuperscript{56,57}. Secondly, the protonation of fatty acids was suggested to reduce LCFA amphiphilicity and thereby reduce LCFA solubility in bile salt micelles. The decrease in micellar LCFA solubility was subsequently
Chapter 5: Lipid absorption triggers drug supersaturation

suggested to stimulate micellar dissociation, resulting in increased LCFA thermodynamic activity and increased LCFA absorption. A decrease in pH at the UWL is therefore expected to lead to a reduction in the lipid content of intestinal mixed micelles via promotion of LCFA micellar dissociation and absorption (Figure 5.1(i)).

Since the presence of lipid digestion products within mixed micelles contributes significantly to drug solubilisation capacity\textsuperscript{39, 91}, in the current chapter we have explored the hypothesis that in promoting LCFA micellar dissociation and absorption, the acidic microclimate also promotes a reduction in the drug solubilisation capacity of LCFA-containing intestinal mixed micelles. This in turn is expected to facilitate drug absorption via the induction of drug supersaturation at the UWL.

The importance of the presence of absorbable lipids in intestinal mixed micelles as a driver of drug absorption has been studied in two ways. Firstly by examining drug absorption from LCFA-containing intestinal mixed micelles in the absence and presence of amiloride. Amiloride is an inhibitor of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter that is responsible for acidifying the UWL and is therefore an inhibitor of fatty acid absorption. Secondly, comparison has been made between drug absorption from LCFA-containing micelles and from micellar systems comprising non-ionic surfactant (Brij 97), where the micellar components are not expected to be absorbed and therefore where changes to drug solubilisation capacity are not expected.

The data suggest that lipid absorption is a significant trigger for the induction of drug supersaturation, and that the combination of fatty acid-containing solubilising species and the acidic intestinal unstirred water layer may be a particularly powerful driver for drug supersaturation and absorption. The results provide an improved mechanistic understanding of the
enhancements in drug absorption often observed from lipid-based systems containing digestible lipids, and also serve to exemplify the beneficial effects of digestible lipids in food on drug absorption.

**Figure 5.1:** Schematic of the proposed mechanisms by which the UWL acidic microclimate facilitates the absorption of micellar solubilised (i) long-chain fatty acids (LCFA) and (ii) poorly water-soluble drug (PWSD). (i) Exposure of mixed micelles to the acidic microclimate leads to protonation of LCFA, attenuating their amphiphilic character and reduces LCFA solubility in mixed micelles. Increased LCFA thermodynamic activity subsequently promotes LCFA dissociation from mixed micelles and absorption across apical membrane. (ii) At the UWL, removal of LCFA from mixed micelle via dissociation and absorption decreases the solubilisation capacity for PWSD, therefore triggering drug supersaturation in close proximity to the absorptive site, and enhances drug absorption via increases in thermodynamic potential. D represents drug. Drug is either free and available for absorption or associated with micelles. D_{ss} is used to signify acidic microclimate-induced drug supersaturation that drives increases in drug absorption.
5.3 METHODS

5.3.1 Materials

Cinnarizine, flunarizine dihydrochloride, amiloride hydrochloride hydrate, sodium taurocholate, sodium taurodeoxycholate, sodium glycocholate, sodium glycochenodeoxycholate, cholesterol, L-α-lysophosphatidylcholine (LPC, from egg yolk), oleic acid, sodium chloride (NaCl) and Brij® 97 were obtained from Sigma-Aldrich, Australia. Sodium taurochenodeoxycholate, sodium glycodeoxycholate, ortho-phosphoric acid 85% (H₃PO₄), sodium hydroxide pellets (NaOH) and tert-butyl methyl ether (TBME) were from Merck, Australia. Disodium hydrogen orthophosphate (Na₂HPO₄), sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O) and ammonium dihydrogen orthophosphate (NH₄H₂PO₄) (Ajax Finechem, Australia), Irga-Safe Plus™ (Perkin Elmer Life Sciences, MA, USA), oleic acid, [9,10-³H(N)] (60 Ci/mmol) (American Radiolabelled Chemicals, MO, USA), transcutol HP (Gattefossé, France), heparin sodium injection BP (1000 I.U./mL, Hospira, Australia), xylazine (100 mg/mL, Troy Laboratories, Australia), acepromazine (10 mg/mL, Ceva Delvet, Australia), ketamine (100 mg/mL, Provet, Australia) and pentobarbitone sodium (325 mg/mL, Virbac, Australia) were obtained from listed suppliers. Acetonitrile and chloroform used were analytical reagent grade. Water was obtained from a Millipore milliQ Gradient A10 water purification system (Millipore, MA, USA).

5.3.2 Experimental outline

In situ rat jejunal perfusion experiments were conducted to assess the role of the acidic microclimate in LCFA and drug absorption from LCFA-containing intestinal colloids. Specifically, the intestinal absorptive flux of oleic acid and cinnarizine from a model LCFA-containing colloid (“model LCFA colloids”) was assessed in the absence and presence of 2 mM amiloride, a competitive inhibitor (with respect to Na⁺) of the plasma membrane Na⁺/H⁺ exchanger253 that has
previously been shown to attenuate the acidic microclimate on the cell surface of the rat jejunum\textsuperscript{46}. As a control, the absorption of cinnarizine from “model Brij 97 colloids” (Brij 97 is a non-ionisable and non-absorbable surfactant) in the absence and presence of 2 mM amiloride was also assessed. The total cinnarizine concentration (130 µg/mL) and cinnarizine thermodynamic activity (\(\sim 80\%\) saturated solubility) were matched in both colloidal systems. Cinnarizine (a weak base) was selected as a model PWSD in this proof-of-concept study, as the solubility of cinnarizine in LCFA-containing intestinal colloids was previously found to be highly dependent on oleic acid content (Chapter 4), and therefore may be more amenable to enhancement in drug thermodynamic activity induced by fatty acid absorption. The model LCFA colloids used in this study were representative of the diluted post-digestion lipid colloidal phases likely responsible for the presentation of solubilised drug to the absorptive membrane\textsuperscript{39,76}.

\textit{In vitro} solubility studies were conducted to evaluate expected changes in cinnarizine solubilisation when model LCFA or Brij 97 colloids were exposed to the acidic microclimate \textit{in vivo}. The equilibrium solubility of cinnarizine was assessed in a series of LCFA colloids with decreasing system pH and decreasing lipid concentration (to simulate exposure to the acidic microclimate and lipid absorption); as well as in a series of Brij 97 colloids with decreasing system pH (to simulate exposure to the acidic microclimate \textit{only}, as Brij 97 is not absorbed).

The role of the acidic microclimate in the absorption of cinnarizine from supersaturated, LCFA-containing colloids was also assessed in rat jejunal perfusion studies via co-perfusion of donor bile with cinnarizine-loaded LCFA colloids (conditions previously shown to induce cinnarizine supersaturation \textit{in situ} and to promote intestinal drug absorption – Chapter 4), in the absence and presence of 2 mM amiloride.
5.3.3 Preparation of LCFA-containing intestinal colloids

Model LCFA colloids used in \textit{in situ} rat perfusion studies consisted 0.1% w/v oleic acid and 0.06% w/v monoolein solubilised in simulated endogenous intestinal fluid (SEIF)\textsuperscript{39} at pH 6.30 ± 0.01. SEIF comprised of 4 mM total bile salt (25 mol\% glycocholate, 17.5 mol\% glycdeoxycholate, 25 mol\% glycochenodeoxycholate, 12.5 mol\% taurocholate, 7.5 mol\% taurodeoxycholate, 12.5 mol\% taurochenodeoxycholate), 1 mM LPC and 0.25 mM cholesterol. The oleic acid:monoolein molar ratio was kept at 2:1, reflecting the ratio of digestion products expected from digestion of 1 mole of triolein. To model the effect of colloid interaction with the acidic microclimate and the absorption of lipid components on cinnarizine solubility, systems were prepared at decreasing pH (pH 6.3, 5.8, 5.3, 4.8) and containing decreasing quantities of lipids (0.1, 0.05, 0.025, 0% w/v oleic acid, with a proportional decrease in monoolein concentrations) for cinnarizine equilibrium solubility determination studies.

The preparation of SEIF and LCFA colloids were as described in Chapter 4. pH adjustment of colloids to 6.30, 5.80, 5.30, 4.80 was achieved by drop wise addition of H\textsubscript{3}PO\textsubscript{4} solution. For the preparation of drug-loaded LCFA colloids (for \textit{in situ} jejunal perfusion studies), cinnarizine was pre-dissolved in oleic acid and allowed to equilibrate overnight at a concentration of 61 mg/g and 115 mg/g, prior to preparation of colloids, such that the final concentration of cinnarizine in the colloids was 65 µg/mL (~ 40\% saturated solubility) and 130 µg/mL (~ 80\% saturated solubility), respectively. As a final step, trace quantities of \textsuperscript{3}H-oleic acid (to achieve 0.25 µCi/mL) were added to the drug-loaded colloids, followed by a 1-min vortex. When amiloride was included in the LCFA colloids, the appropriate mass of amiloride was dissolved in the prepared colloids at 37 °C, and used within 30 min of preparation. The total sodium strength in all the prepared colloids was kept constant at 150 mM.
5.3.4 Preparation of Brij 97 colloids

Brij 97 (a liquid at 37 °C) was weighed into a volumetric flask and made to volume with phosphate buffer (18 mM NaH₂PO₄·2H₂O, 12 mM Na₂HPO₄, 108 mM NaCl), followed by pH adjustment to 6.30 ± 0.01 with H₃PO₄ solution. From a plot of cinnarizine solubility vs. Brij 97 concentration, 3.09% w/v Brij 97 was identified as the concentration required to provide equal cinnarizine solubilisation capacity as the model LCFA colloid (i.e. 157.7 µg/mL). This concentration (3.09% w/v) was therefore used to form the model Brij 97 colloids used in jejunal perfusion experiments. Solutions of 3.09% w/v Brij 97 were also prepared at pH 5.80, 5.30, 4.80 (pH adjustment via drop wise addition of H₃PO₄ solution) for cinnarizine equilibrium solubility determination studies. For the preparation of drug-loaded Brij 97 colloids (for in situ jejunal perfusion studies), 100 µL of a 130 mg/mL cinnarizine in transcutol stock solution was spiked into 10 mL of model Brij colloids to achieve a final concentration of 130 µg/mL cinnarizine (~ 80% saturated solubility). When amiloride was included in the Brij 97 colloids, the appropriate mass of amiloride was dissolved in the prepared colloids at 37 °C, and used within 30 min of preparation. The total sodium concentration in all the prepared colloids was kept constant at 150 mM.

5.3.5 Equilibrium solubility studies of cinnarizine in colloids

To determine the equilibrium solubility of cinnarizine in LCFA colloids and Brij 97 colloids, excess solid drug was added to 2 mL of the colloids in glass vials. Vials were briefly vortexed, incubated at 37 °C, and samples taken every 24 h over a period of 120 h. During sampling, vials were centrifuged (2,200 x g, 10 min, 37 °C), 50 µL of supernatant sampled, and vials re-vortexed. Cinnarizine concentration in the supernatant was determined via HPLC. Equilibrium solubility was defined when cinnarizine concentrations in consecutive supernatant samples varied by ≤ 5% on three separate occasions. The equilibrium solubility of cinnarizine was also determined when 2 mM amiloride was included in model LCFA colloids, model Brij 97 colloids, and 1:1 v/v mixture
of model LCFA colloids and fasted rat bile, to confirm that solubilisation capacity for cinnarizine was unaltered by the inclusion of amiloride. Based on physical examination and the maintenance of consistent drug solubilisation capacity, model LCFA and Brij 97 colloids were stable for 5 days after preparation.

For some of the LCFA colloids prepared at system pHs < 5.8, phase separation into a highly dispersed aqueous phase and an undispersed oil phase was evident. Since an oil phase is not likely to be retained at the UWL \textit{in vivo} (unionised LCFA is expected to be readily absorbed in close proximity to the absorptive membrane), the drug solubilisation capacity of the aqueous micellar phase rather than the oil phase was deemed relevant in assessing expected changes to drug solubilisation and thermodynamic activity in the UWL. Therefore, to accurately determine the equilibrium solubility of cinnarizine in the aqueous phase for all LCFA colloids, solubility samples were left to equilibrate at 37 °C for 120 h (as equilibrium solubility was attained by this time), and ultracentrifuged for 30 min at 37 °C and 400,000 xg (Optima xL-100 K centrifuge, SW-60 rotor, Beckman, Palo Alto, CA, USA) to separate samples into an aqueous phase, an undispersed oil phase (if any), and a pellet phase (containing excess solid drug) as described previously\textsuperscript{254}. Samples from the aqueous phase were assayed for cinnarizine content by HPLC.

\textbf{5.3.6 Animals}

Animal care and anaesthesia have been described in Section 2.2.2.2. All animals were fasted overnight (12–18 h) prior to surgery. At the end of all experiments, rats were euthanized via an intravenous or intracardiac injection of 100 mg sodium pentobarbitone.

\textbf{5.3.7 Surgical procedures}
5.3.7.1 Single-pass rat jejunum perfusion

The model employed to assess flux across rat jejunum involved in situ perfusion (single-pass) of an isolated jejunal segment and simultaneous blood collection from the corresponding mesenteric vein branch. The surgical procedures for the setup of the single-pass rat jejunum perfusion model have been described in Section 2.2.2.3.

5.3.7.2 Fasted rat bile collection

Surgical procedures for the collection of fasted bile from donor rats have been described in Section 4.3.9.1.

5.3.8 In situ single-pass rat jejunum perfusion

After surgery, animals were equilibrated for 30-min, during which time blood was collected from the cannulated mesenteric vein (~ 0.3 mL/min) to enable re-infusion via the jugular vein. Perfusion buffer (150 mM Na⁺, 18 mM H₂PO₄⁻, 12 mM HPO₄²⁻, 108 mM Cl⁻, adjusted to pH 6.30 ± 0.01) was pumped through the jejunal segment at a rate of 0.1 mL/min and outflowing buffer discarded to waste. For experiments where amiloride was administered, perfusion buffer containing 2 mM amiloride was perfused during the equilibration period. The exposed jejunal segment was kept moist by covering with saline-soaked gauze throughout the experiment.

Perfusate flow through the jejunal segment was maintained at 0.1 mL/min in all experiments to minimise variations in the thickness of the unstirred water layer which may influence drug flux. Following the equilibration period, perfusion buffer was replaced with model colloids. The perfused colloids were sampled at t = 0 to confirm cinnarizine and/or ³H-oleic acid concentrations. After this time, the outflowing perfusate was continuously collected at 10-min intervals, and briefly vortexed before samples were taken for analysis of drug and/or ³H-oleic acid content.
Blood draining the perfused jejunal segment was also collected at 5-min intervals, plasma separated by centrifugation (10,000 xg, 5 min), and samples taken for analysis of cinnarizine and/or $^3$H-oleic acid concentrations.

The concentration of cinnarizine flowing into the perfused jejunal segment was held at 130 µg/mL in studies assessing the role of the acidic microclimate in the absorption of cinnarizine from model LCFA-containing or Brij 97 colloids. When amiloride was included in these experiments, it was pre-dissolved in the model colloids at 37 ºC at a concentration of 2 mM amiloride. In studies that assessed the role of the acidic microclimate in the absorption of cinnarizine from supersaturated LCFA-containing colloids (i.e. where co-perfusion of donor rat bile triggered drug supersaturation in the model LCFA colloids), the concentration of cinnarizine flowing into the perfused jejunal segment was held at 65 µg/mL. Therefore, in experiments where model colloids were perfused alone, cinnarizine was loaded into the perfusate at 65 µg/mL (~ 40% saturated solubility). In experiments where model colloids were co-perfused in a 1:1 v/v ratio with a secondary perfusate of bile, cinnarizine was loaded into the primary perfusate at 130 µg/mL (~ 80% saturated solubility), such that 1:1 v/v dilution led to a final perfusate concentration of 65 µg/mL cinnarizine. When amiloride was included, it was pre-dissolved in bile at 37 ºC at 4 mM, such that 1:1 v/v dilution led to a final perfusate concentration of 2 mM amiloride. Both model LCFA colloids and bile were pumped at 0.05 mL/min, and mixed via a three-way “T” connector immediately prior to entry into the jejunal segment, such that total perfusate flow was maintained at 0.1 mL/min. Since luminal cinnarizine supersaturation was generated in these experiments, outflowing perfusate samples were taken before and after centrifugation (2,200 xg, 2 min), to obtain an indication of the degree of drug precipitation within the jejunal segment.
5.3.9 Analytical procedures

5.3.9.1 Sample preparation and HPLC assay conditions for cinnarizine

Samples of LCFA colloids and Brij 97 colloids were prepared for HPLC assay by a minimum 20-fold dilution with mobile phase (50% v/v acetonitrile:50% v/v 20 mM NH₄H₂PO₄). Plasma samples were prepared for HPLC using a validated extraction procedure, with flunarizine as an internal standard, as reported previously. Replicate analysis of n = 4 quality control samples revealed acceptable accuracy and precision (± 10%, ± 15% at the limit of quantification) for cinnarizine concentrations between 20–1000 ng/mL for colloids, and 10–320 ng/mL for plasma.

5.3.9.2 Scintillation counting

Quantification of ³H-oleic acid in perfusate and collected plasma was performed via scintillation counting on a Packard Tri-Carb 2000CA liquid scintillation analyser (Packard, Meriden, Connecticut, USA). Perfusate samples (100 µL) and plasma samples (200 µL) were added to 2 mL Irga-safe Plus scintillation fluid followed by a 10-sec vortex. Samples were corrected for background radioactivity by the inclusion of a blank sample in each run.

5.3.9.3 Blood:plasma ratio determination for cinnarizine, oleic acid

The blood:plasma ratios for cinnarizine and oleic acid have been determined as described in Chapter 2. The mean blood:plasma ratio was used to convert plasma concentrations to blood concentrations in perfusion experiments, enabling quantification of total compound transport into mesenteric blood.
5.3.9.4 Calculations

In the single-pass rat jejunum perfusion model, permeability coefficients were calculated from the flux data obtained after attainment of steady state drug transport into mesenteric blood. Two apparent permeability coefficients (P\text{app}) were calculated as described previously\textsuperscript{184}:

\[
\text{′Disappearance′ } P_{\text{app}} = -\frac{Q}{A} \ln \frac{C_1}{C_0}
\]

\[
\text{′Appearance′ } P_{\text{app}} = \frac{\Delta M_B/\Delta t}{A \langle C \rangle}
\]

where ‘Disappearance’ P\text{app} is the apparent permeability coefficient calculated from drug loss from the perfusate (cm/sec); ‘Appearance’ P\text{app} is the apparent permeability coefficient calculated from drug appearance in the mesenteric blood (cm/sec); Q is the perfusate flow rate (mL/sec); A is the surface area of the perfused jejunal segment (cm\textsuperscript{2}), which is calculated by multiplying the diameter and the length of the perfused intestinal segment as described previously\textsuperscript{207}; C\textsubscript{1} is the average steady state drug concentration exiting the perfused jejunal segment (ng/mL); C\textsubscript{0} is the drug concentration entering the jejunal segment (ng/mL); ∆M\textsubscript{B}/∆t is the average rate of drug mass appearance in mesenteric blood at steady state (ng/sec); and <C> is the logarithmic mean drug concentration in the lumen (ng/mL), where <C> = (C\textsubscript{1} − C\textsubscript{0}) / (ln C\textsubscript{1} − ln C\textsubscript{0}).

5.3.10 Statistical analysis

Statistically significant differences were determined by ANOVA followed by Tukey's test for multiple comparisons at a significance level of α = 0.05 using SPSS v19 for Windows (SPSS Inc., Chicago, IL, USA).
5.4 RESULTS

5.4.1 Attenuation of the acidic microclimate using amiloride reduces oleic acid and cinnarizine absorption from model LCFA colloids; but has no effect on the absorption of cinnarizine from fatty acid-free Brij 97 colloids

Figure 5.2 shows the intestinal absorptive flux vs. time profiles of oleic acid (from LCFA containing colloids), and cinnarizine (from both LCFA and Brij 97 colloids) in the absence and presence of 2 mM amiloride during in situ rat jejunum perfusion experiments. Corresponding steady state-absorptive flux, disappearance Papp, and appearance Papp data are reported in Table 5.1. Perfusate disappearance profiles are shown in Figure 5.3.

Administration of amiloride reduced the absorption of oleic acid, resulting in a significant ($\alpha < 0.05$) 3.0-fold, 1.4-fold, and 3.0-fold reduction in absorptive flux, disappearance Papp, and appearance Papp of oleic acid, respectively (Figure 5.2A, Table 5.1). Amiloride administration also led to a significant reduction in cinnarizine absorption from LCFA colloids, resulting in 6.5-fold, 2.8-fold, and 5.7-fold reductions ($\alpha < 0.05$) in the absorptive flux, disappearance Papp, and appearance Papp of cinnarizine, respectively (Figure 5.2B, Table 5.1). In contrast, co-administration of amiloride with Brij 97 colloids did not lead to significant changes in the absorptive flux, disappearance Papp, and appearance Papp of cinnarizine (Figure 5.2C, Table 5.1).

The model colloids were chosen such that the cinnarizine solubilisation capacity in each was the same (157.7 ± 3.0 µg/mL and 153.2 ± 2.5 µg/mL for LCFA colloids and Brij 97 colloids respectively, mean ± SEM of n = 3 determinations), and drug was loaded at the same concentration (130 µg/mL). Cinnarizine thermodynamic activity was therefore also matched and cinnarizine was dissolved at ~ 80% saturated solubility in both colloidal solutions. In the absence
of amiloride (i.e. in the presence of an intact acidic microclimate), the absorptive flux of cinnarizine from model LCFA colloids was 4.9-fold higher than from Brij 97 colloids (Figure 5.2B & C, Table 5.1). This difference was abolished in the presence of amiloride (i.e. under conditions where the acidic microclimate was attenuated and lipid absorption inhibited). The data suggest that cinnarizine absorption is significantly more efficient from LCFA-containing intestinal colloids than from Brij 97 colloids, in spite of matched initial thermodynamic activity in both colloids, and that this is dependent on the presence of an acidic microclimate at the intestinal UWL. For the model LCFA colloids, the increase in cinnarizine absorption in the absence of amiloride also occurred coincidentally with an increase in oleic acid absorption (Figure 5.2A & B, Table 5.1).
Figure 5.2: Mesenteric blood appearance profiles of (A) oleic acid (OA) from model LCFA colloids, (B) cinnarizine (CIN) from model LCFA colloids, and (C) cinnarizine (CIN) from model Brij 97 colloids, after 70 min single-pass perfusion of ~ 10 cm² segments of rat jejunum with and without co-administration of 2 mM amiloride, which attenuates the acidic microclimate of the intestinal unstirred water layer. Model LCFA colloids and model Brij 97 colloids had equal solubilisation capacity for CIN (157.7 ± 3.0 µg/mL and 153.2 ± 2.5 µg/mL, respectively; average ± SEM of n = 3 determinations). CIN was loaded into both colloids at constant concentration (130 µg/mL) and thermodynamic activity (~ 80% saturated solubility). Data represent mean ± SEM of n = 3-4 experiments. Steady state-absorptive flux, disappearance Papp, and appearance Papp of CIN and OA from this series of experiments are tabulated in Table 5.1.
Table 5.1: Cinnarizine (CIN) and oleic acid (OA) disappearance $P_{app} \times 10^6 \text{cm/sec}$ from the intestinal perfusate, appearance $P_{app} \times 10^6 \text{cm/sec}$ in the mesenteric blood, and steady state absorptive flux into mesenteric blood (ng/5 min/10 cm$^2$) after 70 min of single-pass perfusion of ~10 cm$^2$ segments of rat jejunum with model long chain fatty acid (LCFA) colloids or model Brij 97 colloids, with and without co-administration of 2 mM amiloride. Values calculated using data obtained after steady state attainment ($t = 55-70$ min). Data represent mean ± SEM of $n = 3-4$ experiments.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>CIN conc. (µg/mL)</th>
<th>OA conc. (µg/mL)</th>
<th>Disappearance $P_{app} \times 10^6 \text{cm/sec}$</th>
<th>Appearance $P_{app} \times 10^6 \text{cm/sec}$</th>
<th>Mesenteric blood flux (ng/5min/10cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN</td>
<td>LCFA colloid</td>
<td>130</td>
<td>35.5 ± 2.2</td>
<td>1.7 ± 0.4</td>
<td>600 ± 129</td>
</tr>
<tr>
<td></td>
<td>+ amiloride</td>
<td>130 -</td>
<td>12.7 ± 4.1 $^a$</td>
<td>0.3 ± 0.1 $^a$</td>
<td>93 ± 25 $^a$</td>
</tr>
<tr>
<td></td>
<td>Brij 97 colloid</td>
<td>130 -</td>
<td>14.0 ± 6.7 $^a$</td>
<td>0.3 ± 0.0 $^a$</td>
<td>123 ± 14 $^a$</td>
</tr>
<tr>
<td></td>
<td>+ amiloride</td>
<td>130 -</td>
<td>5.4 ± 1.2 $^a$</td>
<td>0.2 ± 0.0 $^a$</td>
<td>90 ± 3 $^a$</td>
</tr>
<tr>
<td>OA</td>
<td>LCFA colloid</td>
<td>- 1000</td>
<td>22.1 ± 2.0</td>
<td>3.3 ± 0.4</td>
<td>9144 ± 991</td>
</tr>
<tr>
<td></td>
<td>+ amiloride</td>
<td>- 1000</td>
<td>15.3 ± 0.9 $^b$</td>
<td>1.1 ± 0.1 $^b$</td>
<td>3045 ± 304 $^b$</td>
</tr>
</tbody>
</table>

$^a$ Significant difference ($\alpha < 0.05$) from model LCFA colloid group (containing 130 µg/mL CIN) in the absence of amiloride

$^b$ Significant difference ($\alpha < 0.05$) from model LCFA colloid group (containing 1000 µg/mL OA) in the absence of amiloride
Figure 5.3: Perfusate disappearance profiles of (A) oleic acid (OA) from model LCFA colloids, (B) cinnarizine (CIN) from model LCFA colloids, and (C) cinnarizine (CIN) from model Brij 97 colloids, after 70 min single-pass perfusion of ~ 10 cm$^2$ segments of rat jejunum with and without co-administration of 2 mM amiloride, which attenuates the acidic microclimate of the intestinal unstirred water layer. Model LCFA colloids and model Brij 97 colloids had equal solubilisation capacity for cinnarizine (157.7 ± 3.0 µg/mL and 153.2 ± 2.5 µg/mL, respectively; average ± SEM of n = 3 determinations). CIN was loaded into both colloids at constant concentration (130 µg/mL) and thermodynamic activity (~ 80% saturated solubility). Data represent mean ± SEM of n = 3-4 experiments.
5.4.2 Exposure of model LCFA colloids to the acidic microclimate, and absorption of lipid components, leads to cinnarizine supersaturation and enhanced thermodynamic activity

To examine the possible mechanisms by which pH changes at the UWL (due to the acidic microclimate), and the process of lipid absorption, result in changes to cinnarizine absorption, the equilibrium solubility of cinnarizine in LCFA colloids was assessed in systems with decreasing pH and decreasing lipid concentration (to simulate exposure to the acidic microclimate and lipid absorption, Figure 5.4A). Equivalent data were also generated for Brij 97 colloids with decreasing system pH, in this case to simulate exposure to the acidic microclimate only, as Brij 97 is not absorbed (Figure 5.4B).

As system pH decreased, the LCFA colloids became increasingly turbid, ultimately leading to phase separation into an aqueous phase and an undispersed oil phase (Figure 5.5). Phase separation occurred earlier (i.e. at higher pHs) in systems containing higher concentrations of lipid. Systems that phase separated are asterisked in Figure 5.4A. Phase separation occurred at pH 4.8 for all LCFA-based colloids and at and below pH 5.3 for colloids containing the highest lipid load (i.e. 0.1% w/v oleic acid). Even where phase separation did not occur, the turbidity of the LCFA colloidal solutions increased with increasing lipid concentration. Development of turbidity was not evident in SEIF (i.e. 0% oleic acid) or the Brij 97 colloids (Figure 5.5).

Changes in turbidity of the LCFA-containing colloids appeared to correlate with changes to solubilisation capacity, such that drug solubility was higher in systems with increasing turbidity. As depicted in Figure 5.4A, cinnarizine solubility in LCFA colloids increased significantly with increasing lipid concentration and also increased (albeit more moderately) with decreasing pH. These trends continued until phase separation occurred, at which point cinnarizine solubility in the
micellar phase was reduced due to partitioning of lipids from the micellar phase into a poorly dispersed lipid phase. At 0% incorporated lipid (i.e. SEIF only), cinnarizine solubility also increased slightly with decreasing pH. However, this increase was small when compared to the increase in solubility afforded by increasing lipid concentration or decreasing pH in the LFCA-containing colloids.

*In vivo*, exposure of LCFA colloids to the intestinal acidic microclimate results in a decrease in pH and an increase in LCFA absorption. Changes to cinnarizine solubility under the same circumstances are therefore expected to be predicted by assessment of solubility changes under conditions where system pH and lipid concentration are decreased simultaneously. ‘O’ in Figure 5.4 identifies the maximum cinnarizine solubilisation capacity of the LCFA and Brij 97 colloids. Cinnarizine was loaded into model colloids at 80% saturated solubility, i.e. 130 µg/mL – blue dotted line. At the highest lipid load (0.1% w/v OA), exposure of the LCFA containing colloids to decreases in pH that are consistent with conditions in the acidic microclimate, initially increased cinnarizine solubility (at pH 5.8), but ultimately reduced cinnarizine solubility at pH 5.3 (the lowest reported microclimate pH45) (Figure 5.4). The solubility of cinnarizine was also highly dependent on the concentration of incorporated lipids, and a reduction in oleic acid content (consistent with the reductions expected on lipid absorption) significantly reduced cinnarizine solubility. Indeed, the drop in cinnarizine solubility seen at pH 5.3 most likely reflects a loss of micellar lipid content due to phase separation rather than an effect of pH alone. A theoretical line in Figure 5.4A (red broken arrow) depicts the changes in cinnarizine solubility expected under conditions where pH is reduced to pH 5.30 and lipids are fully absorbed. Under these circumstances, cinnarizine solubility is reduced dramatically and in the absence of precipitation, is expected to lead to supersaturation, enhanced thermodynamic activity and improved absorption.
Conversely, in Brij 97 colloids, a decrease in system pH led to *increased* cinnarizine solubility (Figure 5.4B), presumably due to increased cinnarizine ionisation at lower pH. Since Brij 97 is not absorbed, the concentration of micellar surfactant is expected to remain constant during passage across the UWL, and the only driver of changes to drug solubility at the UWL is the reduction in pH due to the acidic microclimate. As depicted by the red broken arrow in Figure 5.4B, cinnarizine solubilisation is therefore expected to increase during passage of Brij 97 colloids across the UWL, leading to reduced thermodynamic activity.
Chapter 5: Lipid absorption triggers drug supersaturation

Figure 5.4: (A) Three-dimensional plot of cinnarizine (CIN) solubility (37 °C) in the aqueous phase of LCFA-containing intestinal colloids as a function of oleic acid and monoolein concentration in bile micelles (concentration on y-axis refers to oleic acid concentration) and system pH (B) CIN solubility (37 °C) in 3.09% w/v Brij 97 colloids as a function of system pH. Concentration of bile components in (A) was kept constant. Black solid arrows depict the expected change in CIN solubilisation capacity when (1) colloids are exposed to the acidic microclimate of the UWL, and (2) as oleic acid and monoolein are absorbed and removed from colloids (absorption of colloidal components only applies to LCFA colloids as Brij 97 is non-absorbable). * in (A) denotes colloidal systems where phase separation into an aqueous phase and undispersed oil phase was evident during the 120 h equilibrium solubility determination study (see Figure 5.5). ‘O’ denotes the solubilisation capacity of the model LCFA and Brij 97 colloids used in Figure 5.2, and the blue dotted line shows the concentration of CIN (i.e. 130 µg/mL – 80% saturated solubility) in the model colloids used in Figure 5.2 and Table 5.1. The red broken arrows depict (A) the theoretical solubility trend when pH of system is reduced by 1 unit to pH 5.3 (lowest pH reported in the literature\(^4\)) and lipids are completely removed, and (B) when pH of system is reduced by 1 unit to pH 5.3 such as on entry into the UWL. The passage of model LCFA colloids across the UWL and subsequent absorption of lipid digestion products is therefore expected to reduce cinnarizine solubility, leading to supersaturation, enhanced thermodynamic potential and absorption; while the passage of model Brij 97 colloids across the UWL leads to a small increase in CIN solubility and reduced thermodynamic potential.
Figure 5.5: The appearance of LCFA-containing intestinal colloids (panel A-D) and 3.09% w/v Brij 97 colloids (panel E) 120 h into cinnarizine equilibrium solubility determination studies, after vials were centrifuged at 2,200 x g for 10 min at 37 °C. LCFA-containing colloids consisted oleic acid (OA) and monoolein (MO) (concentrations as labelled) solubilised in simulated endogenous intestinal fluid (SEIF) containing 4 mM total bile salt, 1 mM lysophosphatidylcholine and 0.25 mM cholesterol. In each panel, the system pH of colloids was, from left to right, 6.30, 5.80, 5.30. 
In LCFA-containing systems, the turbidity of samples was observed to increase when system pH was decreased, up to a point where colloids were destabilised and underwent phase separation into an aqueous phase and an undispersed oil phase (undispersed oil phase floats on top of aqueous phase and may not be seen clearly in pictures), by which point the samples appeared clear again. * denotes colloidal systems where phase separation into an aqueous phase and undispersed oil phase was evident. Since the concentration of bile components was held constant, phase separation of colloidal system with 0.10% w/v OA (Panel D) occurred at a higher pH than systems containing 0.05 and 0.025% w/v OA (Panel B and C). Turbidity did not develop in SEIF containing 0% OA (Panel A) or 3.09% w/v Brij 97 colloids (Panel E). White mass at the bottom of vials is excess solid cinnarizine added in the solubility determination studies. The pictures illustrate the impact of the acidic microclimate on the microstructure of LCFA-containing intestinal colloids, where exposure of LCFA colloids to the microclimate pH leads to protonation of LCFA, increasing the ratio of unionised LCFA:ionised LCFA and resulting in the formation of larger colloids with reduced thermodynamic stability \textit{in vitro}. \textit{In vivo}, the increase in LCFA thermodynamic activity is expected to lead to enhanced micellar dissociation and absorption.
5.4.3 Attenuation of the acidic microclimate using amiloride abolishes bile-induced, supersaturation-enhanced, cinnarizine absorption from model LCFA colloids

In Chapter 4, co-perfusion of fasted rat bile with cinnarizine-loaded model LCFA colloids triggered cinnarizine supersaturation in the GI lumen and enhanced cinnarizine absorptive flux by 3.2-fold in an in situ rat jejunal perfusion model (Figure 5.6B, Table 5.2). In the current chapter, inclusion of 2 mM amiloride in the same perfusate (i.e. 1:1 v/v mixture of model LCFA colloids and fasted rat bile) reduced the absorptive flux and appearance $P_{app}$ of oleic acid by 4.6-fold and 4.4-fold, respectively (Table 5.2, Figure 5.6A). A coincident decrease in the absorptive flux and appearance $P_{app}$ of cinnarizine (a decrease of 5.2-fold and 5.0-fold, respectively) was also observed. Cinnarizine appearance permeability in the presence of bile and amiloride was therefore not significantly different to that in the absence of bile (and therefore in the absence of supersaturation) (Figure 5.6B, Table 5.2). Analysis of the outflowing perfusate in the permeability experiment suggested that cinnarizine supersaturation was maintained in the bulk GI fluids, in both the absence and presence of amiloride, (i.e. precipitation did not occur, Figure 5.7B). Amiloride co-administration therefore negated the increase in cinnarizine absorption stimulated by bile-mediated supersaturation. Amiloride did not directly influence cinnarizine solubility in model LC colloids in the presence or absence of bile (cinnarizine solubility in LC colloids + amiloride was 151.6 ± 17.0 µg/mL, cinnarizine solubility in LC colloids + bile + amiloride was 10.3 ± 0.3 µg/mL respectively; mean ± SEM; n =3).
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Figure 5.6: Absorptive flux-time profiles of (A) oleic acid (OA) and (B) cinnarizine (CIN) when model LCFA colloids were perfused via single-pass through an isolated rat jejunal segment (~ 10 cm²), with and without 1:1 v/v co-perfusion with donor rat bile, in the absence and presence of 2 mM amiloride. * Data in the absence of amiloride are reproduced from Chapter 4. Co-perfusion of rat bile generates CIN supersaturation in situ within the perfused jejunal segment, and increased cinnarizine absorptive flux by 3.2-fold. Inclusion of 2 mM amiloride in the perfusate, however, abolished the absorption-enhancing effects of bile-induced supersaturation. SS denotes drug supersaturation in perfusate. Data represent mean ± SEM of n = 3-4 rats. Steady state-absorptive flux, disappearance $P_{app}$, and appearance $P_{app}$ of CIN and OA from this series of experiment are tabulated in Table 5.2.
Table 5.2: Cinnarizine (CIN) and oleic acid (OA) disappearance $P_{app}$ ($x 10^6$ cm/sec) from the intestinal perfusate, appearance $P_{app}$ ($x 10^6$ cm/sec) in the mesenteric blood, and steady state absorptive flux into mesenteric blood (ng/5 min/10 cm²) after 70 min of single-pass perfusion of ~10 cm² segments of rat jejunum with model LCFA colloids, with and without 1:1 v/v co-perfusion with donor rat bile, in the absence and presence of 2 mM amiloride. Data in the absence of amiloride are obtained from Chapter 4. Values calculated using data obtained after steady state attainment ($t = 55-70$ min). Data represent mean ± SEM of $n = 3-4$ experiments.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>CIN conc. (µg/mL)</th>
<th>OA conc. (µg/mL)</th>
<th>Disappearance $P_{app}$ ($x 10^6$ cm/sec)</th>
<th>Appearance $P_{app}$ ($x 10^6$ cm/sec)</th>
<th>Mesenteric blood flux (ng/5min/10cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN LCFA colloid *</td>
<td>65</td>
<td>-</td>
<td>12.2 ± 2.8</td>
<td>0.6 ± 0.2</td>
<td>138 ± 28</td>
</tr>
<tr>
<td>LCFA colloid + Bile $SS$ *</td>
<td>65</td>
<td>-</td>
<td>36.9 ± 5.5 $^a$</td>
<td>2.5 ± 0.2 $^a$</td>
<td>443 ± 34 $^a$</td>
</tr>
<tr>
<td>LCFA colloid + Bile + amiloride $SS$</td>
<td>65</td>
<td>-</td>
<td>52.9 ± 6.3 $^a$</td>
<td>0.5 ± 0.0 $^b$</td>
<td>85 ± 4 $^b$</td>
</tr>
<tr>
<td>OA LCFA colloid *</td>
<td>-</td>
<td>1000</td>
<td>18.0 ± 5.9</td>
<td>2.0 ± 0.3</td>
<td>5607 ± 863</td>
</tr>
<tr>
<td>LCFA colloid + Bile *</td>
<td>-</td>
<td>500</td>
<td>26.1 ± 3.8</td>
<td>6.2 ± 0.8 $^a$</td>
<td>8557 ± 1205</td>
</tr>
<tr>
<td>LCFA colloid + Bile + amiloride</td>
<td>-</td>
<td>500</td>
<td>35.0 ± 4.1 $^a$</td>
<td>1.4 ± 0.4 $^b$</td>
<td>1853 ± 441 $^b$</td>
</tr>
</tbody>
</table>

$^a$ Significant difference ($\alpha<0.05$) from model LCFA colloid group in the absence of amiloride

$^b$ Significant difference ($\alpha<0.05$) from model LCFA colloid + Bile group in the absence of amiloride

* $P_{app}$ and flux data reproduced from Chapter 4

$SS$ denotes drug supersaturation in perfusate, induced by bile dilution of model LCFA colloids
Figure 5.7: Perfusate disappearance (% drug dose passing through jejunum) profiles of (A) oleic acid (OA) and (B) cinnarizine (CIN) when model LCFA colloids were perfused via single-pass through an isolated rat jejunal segment (~ 10 cm²), with and without 1:1 v/v co-perfusion with donor rat bile, in the absence and presence of 2 mM amiloride. * Data in the absence of amiloride are reproduced from Chapter 4. Co-perfusion of model LCFA colloids with rat bile generates drug supersaturation in situ within the perfused jejunal segment. The degree of drug precipitation within the perfusate is represented by the difference in CIN perfusate concentration pre- and post-centrifugation. SS denotes drug supersaturation in perfusate. Data represent mean ± SEM of n = 3-4 rats.
5.5 DISCUSSION

The mechanism of absorption of long-chain fatty acid (LCFA) from intestinal mixed micelles is well described in the literature. Westergaard and Dietschy’s seminal studies initially proposed that solubilisation of LCFA within bile salt micelles increased LCFA diffusion across the intestinal unstirred water layer (UWL), and increased the concentration of LCFA presented to the absorptive membrane. Shiau and colleagues later suggested that the acidic microclimate within the UWL further enhanced LCFA absorption by protonating ionised LCFA, leading to enhanced micellar dissociation and absorption. These initial studies have also been followed by several studies describing the role of active transport systems such as CD36, FATP and SR-BI in lipid uptake across the apical absorptive membrane, although the quantitative importance of active vs. passive transport as a means of lipid absorption under differing lipid loads remains contentious.

In contrast to lipid absorption, the mechanism of absorption of poorly water-soluble drugs (PWSD) from intestinal mixed micelles has been less clearly defined. We recently described the potential for drug supersaturation to be induced during the interactions of lipid colloidal phases (mixed micelles and vesicles) with secreted bile (Chapter 3 and 4), and suggested that supersaturation-enhanced absorption may be an endogenous mechanism to reverse the reduction in thermodynamic activity inherent in drug solubilisation within colloidal phases. During these studies, however, it became apparent that multiple mechanisms may underpin the generation of drug supersaturation in intestinal mixed micelles. For weak bases with high solubility in LCFA containing micelles, interaction with bile appeared to decrease micellar drug solubility with increasing bile concentration. This in turn resulted in transient supersaturation and absorption promotion. However, interaction with bile also promoted (albeit to a lesser extent) the absorption of a non-ionic drug (danazol), where micellar solubility increased rather than decreased with
increasing bile concentrations. This occurred simultaneously with an increase in lipid absorption. These previous studies suggest that lipid absorption and drug absorption may be linked, and stimulated the current hypothesis, i.e. that lipid absorption from intestinal mixed micelles reduces drug solubility in micellar structures, thereby inducing supersaturation and promoting drug absorption.

The possibility that protonation and absorption of LCFA from intestinal mixed micelles during passage across the UWL can trigger drug supersaturation and enhance drug absorption has been studied in two ways. Firstly, via an examination of changes to cinnarizine solubility in mixed micelles as a function of changes to pH and lipid concentration; and secondly by modifying the conditions within the UWL such that LCFA protonation and absorption are suppressed, and examining the effects of suppressed lipid absorption on cinnarizine absorption. The latter was achieved by inhibiting the action of the Na⁺/H⁺ antiporter at the enterocyte brush border, thereby reversing the acidity of the microclimate adjacent to the absorptive surface. The acidic microclimate has long been suggested to be important in the absorption of LCFA. The pKa of oleic acid, a common LCFA component (or digestion product of dietary or formulation-derived lipids), is 9.85. When solubilised in bile salt micelles, however, the pKa of oleic acid decreases to 6.3–6.5. As such pH changes from 6.5 in the GI lumen, to 5.3 in the UWL, significantly increase the unionised:ionised fraction for LCFA. A decrease in ionisation is expected to increase LCFA absorption by 1) increasing cellular partitioning due to the pH-partition effect and 2) increasing thermodynamic activity due to a reduction in amphiphilicity and micellar solubility, and stimulation of micellar dissociation (as described by Shiau and coworkers). In contrast, conjugated bile salts are not absorbed in the upper GI tract. The pKa values of taurine and glycine conjugated bile salts are <1 and ~3.8, respectively. Bile salts therefore remain ionised during passage across the UWL, and in the absence of absorption, bile salt micellar structures are
expected to persist. The lipid content of the micelles however, is expected to reduce (due to enhanced LCFA micellar dissociation and absorption), reducing drug solubilisation capacity and generating the conditions required for drug supersaturation. In the current studies cinnarizine was employed as a model PWSD to examine the impact of the acidic UWL on drug absorption from mixed micelles. Cinnarizine was chosen for two reasons. Firstly, previous studies have shown that cinnarizine is highly soluble in bile salt-fatty acid mixed micelles; and solubility is highly dependent on micellar oleic acid content (Chapter 4). Thus, cinnarizine is likely to be amenable to enhancements in drug thermodynamic activity induced by oleic acid absorption. Secondly, as a base, exposure to the UWL microenvironment acidity is expected to increase drug ionisation, and therefore decrease drug absorption based on pH partition relationships. As such, any increase in drug absorption that occurs due to microenvironment acidity is expected to reflect mechanisms unrelated to drug ionisation/partitioning.

Under pH conditions reflective of the acidic UWL, the LCFA containing colloids examined here became increasingly turbid, suggesting increases in colloid particle size consistent with the observations of Shiau et al.\textsuperscript{57}. The increase in turbidity with decreasing pH only occurred in systems containing LCFA, and developed instantly (see Figure 5.8 for the appearance of model LCFA colloids after the addition of one drop of 20\% v/v H\textsubscript{3}PO\textsubscript{4}). Reduced LCFA solubility in bile salt micelles manifests as an increase in system turbidity or phase separation \textit{in vitro}. However, \textit{in vivo} where an absorptive sink is present directly adjacent to the UWL, increased LCFA thermodynamic activity and an increase in unionised:ionised LCFA is expected to contribute to enhanced absorption\textsuperscript{56, 57}. Consistent with this suggestion, the absorptive flux of oleic acid was significantly reduced when the acidic microclimate of the UWL was attenuated by co-administration with amiloride (Figure 5.2A). This is consistent with previous data that have shown reduced oleic acid uptake into rat jejunal brush border vesicles in the presence of amiloride\textsuperscript{259}.
Figure 5.8: Appearance of cinnarizine-loaded (A) model LCFA colloids and (B) model Brij 97 colloids immediately after the addition of one drop of 20% v/v H₃PO₄. Turbidity instantly developed in model LCFA colloids while model Brij 97 colloids remained optically clear. The addition of H₃PO₄ reduced the system pH of model LCFA colloids and Brij 97 colloids from 6.30 to 5.92, and 6.30 to 6.01, respectively. Magnetic stirrers are present at the bottom of vials. This figure visually depicts the effect of protonation of oleic acid (OA) when model LCFA colloids are exposed to acidic conditions. An increase in the ratio of unionised OA:ionised OA is thought to enhance the thermodynamic potential of OA, and induce a micelle to emulsion transition in the colloids.

In light of the significant reduction in lipid absorption in the presence of amiloride, this experimental system was utilised to explore the potential link between lipid absorption and drug absorption. The data in Figure 5.2B show a very clear reduction in cinnarizine absorption in the presence of amiloride. The reduction in drug absorption occurred coincidently with the reduction in LCFA absorption (Figure 5.2A). In contrast, perfusion of Brij 97 colloids containing the same concentrations of cinnarizine at the same thermodynamic activity resulted in drug flux that was significantly lower than that from LCFA-containing colloids in the absence of amiloride, and was independent of the co-administration of amiloride. The data suggest that drug absorption from micellar systems containing LCFA is inherently more effective than from solubilising systems that
lack fatty acids, and that fatty acid absorption is critical in mobilising the solubilised fraction to maximise drug absorption. These findings are significant in the context of the design of lipid-based formulations, and suggest that formulations containing absorbable lipids may have inherent advantages over systems containing non-absorbable surfactants and co-solvents. Consistent with this suggestion, previous studies comparing danazol absorption from LBF comprising surfactants and cosolvents alone (i.e. LFCS Type IV formulations) and formulations where the same surfactants and cosolvents were combined with glyceride lipids (i.e. a more traditional LFCS Type III self-emulsifying formulation), suggest increased drug absorption from the lipid containing formulations\textsuperscript{260}. Thus, despite similar solubilisation properties in in vitro dispersion and digestion tests, a glyceride lipid-containing formulation (55% w/w Cremophor RH 40, 7.5% w/w ethanol, 37.5% w/w Soybean oil:Maisine (1:1 w/w)) outperformed (~ 2-fold difference in in vivo exposure of danazol) a similar surfactant/cosolvent formulation (55% w/w Cremophor RH 40, 7.5% w/w ethanol, 37.5% w/w Pluronic L121). We were previously unable to explain these observations, but the current studies provide some justification for the apparent advantage of formulations containing absorbable lipids.

To better understand the impact of microclimate pH and lipid absorption on drug absorption from LCFA-containing intestinal colloids, a series of studies was undertaken to detail changes in drug solubility under conditions of both reduced pH and decreased lipid content. As a weak base with a pKa of 7.47, cinnarizine is increasingly ionised at lower pH, and this was reflected in increases in solubility when the pH of blank SEIF (i.e. bile micelles with 0% solubilised lipids) and model Brij 97 colloids was decreased from 6.3 to 4.8 (Figure 5.4). In LCFA-containing colloids, decreasing pH initially resulted in the formation of swollen colloidal particles with increased turbidity and higher cinnarizine solubilisation capacity (Figure 5.4), consistent with previous studies that suggest increases in drug solubility in larger colloidal particles\textsuperscript{39}. The expected changes to
cinnarizine solubility resulting from pH changes in the acidic UWL alone (i.e. an increase in ionisation and solubility, leading to a decrease in thermodynamic activity), are therefore unlikely to promote drug absorption and are unable to explain the in vivo data in Figure 5.2B.

However, a second major change to colloidal structure was expected on entry into the UWL in vivo, namely a significant reduction in micellar lipid content due to the promotion of LCFA micellar dissociation and absorption. At lower pHs in vitro, decreases in micellar lipid content were also stimulated by phase separation out of the micellar phase (as indicated by asterisks in Figure 5.4A, Figure 5.5). Thus, during passage of LCFA colloids across the acidic UWL in vivo, the lipid content of the micellar phase is expected to drop due to lipid absorption and potentially due to lipid phase separation, although in vivo, in the presence of a large absorption sink, phase separation may be less prevalent.

Since cinnarizine solubility in intestinal colloids is highly dependent on the concentration of incorporated lipids (cinnarizine solubility in LCFA colloids decreased dramatically with decreasing lipid load at each pH studied - Figure 5.4A), decreasing lipid concentration is likely to be the predominant factor in determining cinnarizine solubilisation at the UWL. Therefore, in predicting the impact of both a decrease in pH and a coincident decrease in micellar lipid concentration in the UWL on drug solubilisation (Figure 5.4A), the net change to the solubility of cinnarizine in the aqueous phase is likely to be a reduction due to the loss in lipid content. In the absence of precipitation, this will drive supersaturation and enhance drug thermodynamic activity, consistent with the significant increase in drug flux seen in vivo under conditions where lipid absorption was enhanced (Figure 5.2A & B).
The ability of the LCFA colloids to harness acidic microclimate-induced drug supersaturation therefore stems from the ability of LCFA to respond to a decrease in pH at the UWL, leading to enhanced LCFA absorption, subsequently decreasing colloid solubilisation capacity for PWSD. That is, the presence of oleic acid confers pH sensitivity to LCFA-containing colloids and serves as a trigger for acidic microclimate-induced drug supersaturation, ultimately enabling the absorption of solubilised drug via enhancements in thermodynamic activity. Similar events might also be expected for non-ionisable, but absorbable micellar components such as monoglycerides, although in this case, lipid absorption is not stimulated by changes in pH and therefore the effects of acidic microclimate attenuation on drug absorption may not be as significant. Conversely, for colloids that lack an absorbable (and/or pH-responsive) component, potential increases in drug thermodynamic activity due to entry into the acidic microclimate are unlikely, and thermodynamic activity (and absorption) of drug is expected to remain constant (or to decrease as drug is absorbed) during colloidal passage across the UWL. The data obtained here for cinnarizine absorption from Brij 97 colloids (a non-ionisable, non-absorbable surfactant) is consistent with this suggestion (Figure 5.2C). Indeed, when the acidic microclimate was attenuated, and when lipid absorption was effectively inhibited, cinnarizine absorption from LCFA and Brij 97 micelles was comparable and low (Figure 5.2B & C).

The intercalation of lipid digestion products into bile-derived intestinal colloids has previously been shown to increase solubilisation capacity for a range of PWSD\textsuperscript{39, 91, 99}. Therefore, although cinnarizine was used as a model PWSD in the current study, acidic microclimate-induced drug supersaturation is likely to be a common endogenous mechanism of absorption enhancement for lipophilic PWSD after co-administration with absorbable lipids. The degree of supersaturation that is induced by entry into the acidic microclimate will be dictated by the sensitivity of drug solubility to micellar lipid content, and the efficiency of lipid absorption. Under these
circumstances the combination of weak bases, with LBF comprising fatty acids or glyceride that generate fatty acids *in situ*, may be particularly beneficial since the colloid solubility of weak bases in structures containing fatty acids is high (refer Chapter 4), and the efficiency of fatty acid absorption is also high.

Previously, we have shown that the interaction of biliary components (i.e. bile salts, phospholipid, cholesterol) with similar lipid colloidal phases to those examined here leads to changes in colloid microstructure that dramatically reduce cinnarizine solubilisation capacity (Chapters 3 and 4). Similar to the events described in the UWL here, precipitation in the previous studies was delayed sufficiently that supersaturation occurred in the bulk luminal fluids and ultimately enhanced the intestinal absorptive flux of cinnarizine. In an attempt to link the current studies at the UWL, with these previous studies that describe luminal supersaturation, a final experiment was performed to explore the importance of a functioning acidic microclimate on increases in drug absorption resulting from supersaturation in the bulk luminal fluids. From the data in Figure 5.6B it is apparent that the increase in cinnarizine absorptive flux that occurred due to interaction of LCFA-containing colloids with bile (and the period of drug supersaturation in the luminal fluids that ensued), were abolished when the acidic microclimate was inhibited with amiloride. It seems likely therefore that even where supersaturation is generated in the intestinal lumen by, for example, initiation of lipid digestion\(^4, 91, 176\) or interaction with bile, micellar structures are still required to promote transport across the UWL, and the acidity of the UWL is critical to allowing efficient absorption of LCFA and PWSD from these micellar structures. Thus, on approach to the absorptive surface, LFCA protonation and absorption is required to translate luminal supersaturation into enhanced drug absorption, presumably via enhancements in thermodynamic activity that enable drug absorption from the supersaturated solubilised reservoir.
As a final caveat, the generation of drug supersaturation during the processing of LBF is not always expected to be beneficial. Indeed, supersaturation is also a precursor to crystal nucleation and drug precipitation, events that are likely to reduce drug absorption for PWSD with dissolution-rate limited absorption. The benefits of supersaturation generation must therefore be weighed against the potential for precipitation. However, supersaturation at the UWL may be particularly effective in enhancing drug absorption from intestinal colloids, as proximity to the absorptive membrane is likely to provide an effective sink for effective removal (i.e. absorption) of supersaturated drug, thereby minimising the risk of drug precipitation. Similar concepts in non-lipid based systems have recently been elegantly exemplified by the Augustijns group where the likelihood of drug precipitation from supersaturated solutions (formed in this case by solvent shift) was dramatically reduced by the presence of an absorptive membrane\textsuperscript{261}. The viscous, mucus layer that is present at the UWL may also play a role in stabilising drug supersaturation, as increased viscosity has been suggested to delay nucleation and crystal growth of solutes from supersaturated solutions\textsuperscript{262, 263}.

\section*{5.6 CONCLUSION}

Lipid absorption is an endogenous mechanism that triggers drug supersaturation and facilitates effective drug absorption from intestinal mixed micelles. Formulations containing absorbable lipids or the pre-digestive precursors to absorbable lipids may therefore be more effective in enhancing PWSD absorption when compared to formulations that lack absorbable components. The degree of drug supersaturation (and therefore absorption enhancement potential) generated by lipid absorption is likely maximised under conditions where the dependency of drug solubility on micellar lipid content is high, and where lipid absorption is highly efficient. Combinations of drug and excipients that are formulated according to the above paradigm (e.g. weak bases and lipid-
based formulations comprising precursors of fatty acids) may therefore maximise the absorptive benefits associated with lipid absorption-induced drug supersaturation. These data reinforce previous suggestions that lipid-based formulations are able to interact with the dynamic GI environment, and in doing so promote both drug solubilisation and thermodynamic activity. This provides unique benefits for drug absorption when compared to formulations that promote solubilisation or supersaturation alone.
CHAPTER 6 : SUMMARY AND PERSPECTIVES
The ability of lipids in food or lipid-based formulations (LBF) to enhance the oral bioavailability of poorly water-soluble drugs (PWSD) has long been recognised. However, confident application of LBF, and accurate simulation or prediction of oral drug absorption from LBF, is limited by the lack of a holistic understanding of the mechanisms by which lipids enhance the absorption of PWSD. The studies described in this thesis aimed to address this gap in understanding, and focussed on elucidating the mechanism of drug absorption from the colloidal micellar and vesicular species that are formed in the gastrointestinal (GI) tract during lipid digestion.

Co-administration with lipids promotes drug solubilisation within intestinal lipid colloidal phases and increases the apparent solubility of PWSD in the small intestine (SI). The mechanism of drug absorption from these solubilising species, however, remains poorly-defined. Current understanding suggests that drug flux across absorptive membrane is the product of the free drug concentration and drug permeability across the membrane. For solubilised systems, therefore, drug solubilised within colloidal phases serves to rapidly replenish the free drug fraction via re-establishment of the equilibrium between solubilised and free drug. This results in a significant increase in dissolution rate when compared to dissolution from a typical solid dose form. Since solubilisation does not directly enhance the inter-micellar free drug concentration, however, lipid co-administration may not increase membrane flux for drugs where absorption is solubility-limited (rather than dissolution-limited). Indeed, it has recently been suggested that solubilising formulation approaches result in a ‘solubility-permeability interplay’ where potential enhancements in drug absorption due to solubilisation are compromised by decreases in free drug fraction\[100,146\]. This suggestion, however, runs counter to a body of evidence that demonstrates that solubilising formulations such as LBF commonly increase the oral bioavailability of PWSD\[87\].

Whilst the advantages of LBF may stem from increases in dissolution rate alone, the available data also suggest that drug flux in excess of that predicted by the free drug concentration is likely. This
apparent contradiction between robust drug absorption in the presence of LBF or food, and the realisation that this occurs even though the free drug concentration is often extremely low, stimulated the work described in this thesis. The principle hypothesis that underpins the work is that drug absorption from intestinal colloidal species is not simply a function of the free drug concentration in equilibrium with the solubilised reservoir. The alternative hypotheses that have been explored include the possibility that drug absorption occurs directly from the solubilised fraction and/or that transient changes to colloidal structure in situ may lead to colloidal supersaturation and an increase in the free drug concentration that subsequently increases drug absorption.

In Chapter 3 the prospect for the solubilised fraction to contribute directly to drug absorption has been explored via examination of the potential role of collisional drug absorption. This was investigated by comparing the intestinal absorption of a model PWSD, cinnarizine (CIN), from micelles and vesicles that had equal CIN solubilisation capacities and were loaded with drug at the same concentration and therefore that had the same thermodynamic activity/free concentration. The micelles and vesicles employed were assembled using differing quantities of medium-chain fatty acid and monoglyceride solubilised in simulated intestinal fluid. Whilst both species had identical solubilising properties, the structure of the two colloids was notably different and the particle size of the vesicles was significantly larger than that of the micelles (443 nm vs. 9 nm respectively). Since the rate of collisional transfer is directly related to the number of particles, collisional absorption of CIN from micelles was expected to be higher since the number of micellar particles was substantially higher than that of vesicular particles. In contrast, the data in Chapter 3 show that intestinal absorption of CIN from vesicles and micelles was the same when the free concentration was matched, suggesting limited collisional transfer and instead reliance on the free concentration.
Receptor-mediated collisional absorption was also investigated by assessing CIN bioavailability in the absence and presence of inhibitors of common lipid uptake transporters (e.g. SR-BI, CD36, NPC1L1). These transporters were hypothesised to interact directly with intestinal colloids to mediate the absorption of solubilised lipid and drug since they have similar interactions with other colloidal particles (e.g. HDL) in the blood\textsuperscript{161}. Consistent with the micelle/vesicle data, however, no significant differences in CIN absorption were seen in the presence and absence of the inhibitors, providing further support for the suggestion that collision-mediated uptake was not a significant driver for drug absorption from intestinal lipid colloidal phases, and that drug absorption occurred largely from the free fraction.

Subsequently, attention turned to the possibility that drug absorption from intestinal colloidal phases may be promoted, not by direct absorption from the solubilised phase, but by boosting thermodynamic activity, thereby rendering solubilised drug more available for absorption via the free fraction. In the first instance, the potential for increases in thermodynamic activity based on the generation of supersaturation due to interaction with biliary fluids was explored. Bile dilution was hypothesised to stimulate drug supersaturation since the addition of bile to intestinal lipid colloidal phases has previously been shown to generate colloids that are less lipid-rich with lower solubilisation capacities\textsuperscript{39,194}. To investigate the impact of biliary dilution on drug absorption from lipid colloidal phases, fasted bile was collected from donor rats and added to CIN-loaded (CIN was loaded at sub-saturated concentrations) micelles and vesicles in a 1:1 v/v ratio. Bile addition resulted in CIN supersaturation, and supersaturation was maintained for longer time periods in micellar systems when compared to vesicles. The ability of bile-induced supersaturation to enhance drug absorption was subsequently evaluated \textit{in situ} and \textit{in vivo} via the assessment of CIN absorptive flux from colloids with and without the co-perfusion of donor bile in rat jejunal perfusion studies, and the assessment of CIN bioavailability in bile diverted \textit{vs.} bile-intact rats.
The results showed that bile-induced supersaturation led to enhanced intestinal absorption and systemic exposure of CIN from micelles, but failed to enhance the absorption of CIN from vesicles due to rapid precipitation. Thus, bile-induced supersaturation was identified as a novel mechanism to increase drug thermodynamic activity and to better mobilise the solubilised drug fraction for absorption.

The ability of bile to enhance the absorption of PWSD is well-described in the literature, however the mechanism by which this occurs, has previously been assumed to reflect increases in drug solubilisation. The novelty of the data in chapter 3 therefore lies in showing that bile may also enhance drug absorption via promotion of drug supersaturation. The difference in the absorption enhancement of CIN from micelles and vesicles also highlights the need to achieve an optimal balance between drug supersaturation and precipitation.

Chapter 4 extended the findings in Chapter 3 to include micellar systems containing long-chain lipids (which have the added advantage of increasing relevance beyond LBF to also include typical food-related lipids), and to a range of drugs with differing physicochemical properties. Initially, the sensitivity of the solubility of five PWSD (2 basic drugs, 2 neutral drugs, 1 acidic drug) to colloidal bile or long-chain lipid content was evaluated. Consistent with the data obtained with CIN and the medium-chain lipid containing colloids in Chapter 3, the solubility of the basic drugs in long-chain lipid containing micelles was found to decrease with increasing bile concentration. In contrast, and more consistent with typical solubilisation behaviour, the solubility of the neutral and acidic drugs increased with increasing bile (surfactant) concentration. The data suggest that the potential for drug supersaturation to be triggered during bile dilution is highest for basic drugs. The basis for supersaturation stimulation was suggested to be related to drug affinity for the core lipids within the colloids. Thus, high affinity for colloidal lipids resulted in high initial
solubility, and the addition of bile served to disrupt intermolecular interactions between drug and lipid, leading to reductions in solubilisation capacity. Consistent with this suggestion, *in situ* evaluation of the absorption enhancement afforded by 1:1 v/v co-perfusion of bile with drug-containing colloids showed that bile enhanced the absorption of cinnarizine (a basic drug) to a greater extent than danazol (a neutral drug).

Interestingly, however, bile did stimulate some increase in danazol absorption, in spite of a reduction in thermodynamic activity (bile addition increased danazol solubility in colloidal species thereby decreasing thermodynamic activity). Since oleic acid absorption was also increased by bile co-perfusion, the increase in danazol absorption was suggested to be due to the increase in lipid absorption in the presence of bile. Thus lipid absorption was hypothesised to reduce micellar drug solubilisation capacity, and therefore to increase thermodynamic activity and absorption (assuming that absorption enhancement precedes drug precipitation).

In Chapter 5, the potential for lipid absorption (i.e. a reduction in colloidal lipid content) to stimulate drug supersaturation and absorption was examined directly. The acidic microclimate of the intestinal unstirred water layer (UWL) facilitates the absorption of long-chain fatty acids (LCFA) by protonating LCFA and increasing thermodynamic activity and membrane partitioning \(^{56, 57}\). In this series of experiments, therefore, the acidity of the UWL was attenuated using amiloride in order to inhibit oleic acid absorption. This enabled a comparison of CIN absorption from oleic acid-containing colloids under conditions of normal lipid absorption vs. conditions of inhibited lipid absorption. In the rat jejunal perfusion model the absorptive flux of CIN was dramatically attenuated (6.5-fold) when oleic acid absorption was suppressed. *In vitro* assessment of CIN solubilisation behaviour under conditions that simulate lipid absorption at the UWL subsequently indicated that supersaturation was likely to be responsible for the enhancement
in CIN absorption during normal lipid absorption. Importantly, in this chapter, it was shown that the intrinsic solubilisation capacity of colloids did not dictate the efficiency of drug absorption. Instead, the ability of colloids to lose solubilisation capacity and to generate drug supersaturation at the UWL was seemingly more important. Thus, head-to-head comparison of CIN absorption from two colloidal systems with equal solubilisation capacity (colloids were loaded with CIN at the same concentration such that total, solubilised, and free concentrations were equal) showed that drug absorption was dramatically more efficient when micelles comprised bile salt and oleic acid, when compared to micelles comprising Brij 97 (a non-ionisable and non-absorbable surfactant). These differences were abolished when oleic acid absorption was inhibited. The data support the importance of lipid absorption-induced supersaturation as a means to mobilise the solubilised drug fraction and to enable effective drug absorption from intestinal mixed micellar species.

The major findings from this thesis therefore support a consensus view that LBF inherently generate drug supersaturation when incorporated into endogenous lipid processing pathways. Specifically, supersaturation has previously been shown to be generated when solubilisation capacity is lost during LBF dispersion and digestion\textsuperscript{5, 91, 176, 177, 193}. The current studies further suggest that supersaturation is generated during biliary dispersion of lipid digestion products, and during the absorption of lipid digestion products. The role of bile and the acidic UWL in promoting drug supersaturation and absorption has not been described previously, and represent interesting new avenues for further research. Thus, LBF have traditionally been regarded as formulations that promote the absorption of PWSD by increasing solubilisation. The current data, however, suggest that \textit{in vivo}, LBF are capable of promoting both drug solubilisation and drug supersaturation.
The unique ability of LBF to simultaneously increase the solubilised reservoir and to also enhance the thermodynamic activity of co-administered drug may distinguish LBF from other enabling formulations and help explain why lipids are often very effective in enhancing the oral absorption of a range of lipophilic PWSD. Firstly, the formation of intestinal mixed micelles and vesicles during lipid digestion increases the solubilisation capacity of the SI, and allows for higher amounts of drug to be maintained in solution. Secondly, although drug thermodynamic activity is initially lowered by solubilisation, the ensuing interaction between drug-containing colloids and endogenous lipid processing pathways triggers drug supersaturation, and provides a means to reverse the reduction in thermodynamic activity and to mobilise the solubilised drug fraction for absorption. Lastly, the multi-step, sequential manner by which drug supersaturation is triggered in the SI, coupled with the increased drug solubilisation capacity of the SI after lipid co-administration, ensures that the supersaturation generated during LBF processing is rate and extent-controlled, therefore providing a balance between solubilisation and supersaturation that maximises the potential for drug absorption while minimising the risk of drug precipitation. In this way, LBF possess inherent advantages when compared to other enabling formulations that either promote drug solubilisation only (e.g. cyclodextrins) or drug supersaturation only (e.g. solid dispersions), and may provide a means to finely balance the need to solubilise the drug dose, and to maintain or increase drug thermodynamic activity.

The findings from this thesis provide significant new insights into the fundamental mechanisms by which lipids enhance the oral absorption of PWSD. The studies show that supersaturation is an important driving force for drug absorption after co-administration with lipids, and identify two novel mechanisms (i.e. bile dilution and lipid absorption) by which drug supersaturation may be triggered during intestinal lipid processing. Better understanding of the mechanisms of supersaturation generation is expected to contribute to an increasingly rational basis for the
selection of lipid excipients. Although more work is needed, practical applications related to the findings of this thesis are conceivable, and relate to the opportunity to generate colloidal assemblies with built-in sensitivity to bile dilution and lipid absorption mediated supersaturation. For example, the studies in Chapter 4 suggest that the use of lipid-drug combinations where drug possesses high affinity for lipid digestion products may predispose post-digestion colloids to lose solubilisation capacity during bile dilution via the disruption of drug-lipid intermolecular interactions. Studies in Chapter 5 further suggest that the utilisation of absorbable lipids (or precursors of absorbable lipids) may lead to potent stimulation of drug supersaturation at the UWL, especially where the drug has high affinity for the lipids and where lipid absorption is efficient. Collectively, these studies suggest that combinations of drug and excipients that are formulated according to the above principles (e.g. weak bases with LBF comprising precursors of fatty acids) may maximally harness the absorptive benefits associated with bile and lipid absorption-induced drug supersaturation. The in vitro studies in Chapter 4 that delineate drug solubility relationships with bile components or lipid digestion products also serve to demonstrate that the likelihood of supersaturation generation in vivo may be predicted in vitro, and may be amenable to high throughput screening.

Finally, the data in Chapter 5 demonstrate a crucial role of the UWL acidic microclimate in the translation of luminal supersaturation into enhanced absorption (inhibition of UWL acidity blocked increases in drug absorption due to bile-mediated promotion of luminal supersaturation). This suggests that supersaturated luminal colloids alone are not sufficient to promote drug absorption and that micellar dissociation via a decrease in UWL pH is an important mechanism to mobilise supersaturated solubilised drug for absorption. This may be an important consideration for solubilising formulations that lack pH-sensitivity.
In conclusion, this thesis has demonstrated for the first time that effective drug absorption from intestinal colloidal species is a result of dynamic interactions between the solubilised drug reservoir and the GI environment that lead to multiple opportunities for supersaturation-enhanced absorption. This improved understanding of the mechanism of drug absorption is ultimately expected to inform rational design criteria for LBF and to improve oral absorption simulation models. Future work might be usefully directed towards further characterising the parameters that dictate the propensity of LBF to generate drug supersaturation during formulation processing, and in particular an assessment of the balance between drug supersaturation and drug precipitation.
REFERENCES


APPENDIX 1

Intestinal Bile Secretion Promotes Drug Absorption from Lipid Colloidal Phases via Induction of Supersaturation

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ABSTRACT: The oral bioavailability of poorly water-soluble drugs (PWSD) is often significantly enhanced by coadministration with lipids in food or lipid-based oral formulations. Coadministration with lipids promotes drug solubilization in intestinal mixed micelles and vesicles, however, the mechanism(s) by which PWSD are absorbed from these dispersed phases remain poorly understood. Classicallly, drug absorption is believed to be a product of the drug concentration in free solution and the apparent permeability across the absorptive membrane. Solubilization in colloidal phases such as mixed micelles increases dissolution rate and total solubilized drug concentrations, but does not directly enhance (and may reduce) the free drug concentration. In the absence of changes to cellular permeability (which is often high for lipophilic, PWSD), significant changes to membrane flux are therefore unexpected. Realizing that increases in effective dissolution rate may be a significant driver of increases in drug absorption for PWSD, we explore here two alternate mechanisms by which membrane flux might also be enhanced: (1) collisional drug absorption where drug is directly transferred from lipid colloidal phases to the absorptive membrane, and (2) supersaturation-enhanced drug absorption where bile mediated dilution of lipid colloidal phases leads to a transient increase in supersaturation, thermodynamic activity and absorption. In the current study, collisional uptake mechanisms did not play a significant role in the absorption of a model PWSD, cinnarizine, from lipid colloidal phases. In contrast, bile-mediated dilution of model intestinal mixed micelles and vesicles led to drug supersaturation. For colloids that were principally micellar, supersaturation was maintained for a period sufficient to promote absorption. In contrast, for primarily vesicular systems, supersaturation resulted in rapid drug precipitation and no increase in drug absorption. This work suggests that ongoing dilution by bile in the gastrointestinal tract may invoke supersaturation in intestinal colloids and promote absorption, and thus presents a new mechanism by which lipids may enhance the oral absorption of PWSD.

KEYWORDS: absorption, poorly water-soluble drug, lipid-based formulation, supersaturation, bile, micelles, food effect, membrane permeability, cinnarizine

INTRODUCTION

The potential for lipid-based formulations (LBF) to enhance the oral bioavailability of poorly water-soluble drugs (PWSD) has been recognized for over 40 years.1 Lipid coadministration is thought to enhance the oral absorption of PWSD by providing mechanisms to overcome both slow dissolution and low solubility of PWSD in the aqueous gastrointestinal (GI) milieu. First, LBF present drug to the GI tract in a molecularly dispersed form (i.e., in solution in the formulation), thereby circumventing the need for dissolution from the solid to the liquid state. Subsequently, the intercalation of formulation lipids into endogenous lipid digestion pathways results in the generation of intestinal lipid colloidal phases (such as vesicular and micellar species) that enhance the solubilization capacity of the small intestine, promote drug solubilization and reduce the risk of drug precipitation.

Solubilization within lipid colloidal phases therefore increases the apparent solubility of PWSD in the intestinal fluids and promotes dissolution. However, in the absence of solid drug solubilization also results in a reduction in thermodynamic activity.2 In simple micellar systems this reduction in thermodynamic activity is manifest in a decrease in the free concentration of drug. Where solubilized drug exists in equilibrium between the free concentration (C_{free}) and the...
Classical models of passive drug absorption suggest that drug flux across an absorptive membrane is the product of the free drug concentration and the drug permeability across the membrane. Therefore, where solubilization reduces $C_{\text{free}}$ (but does not alter permeability), absorption is expected to be reduced. Indeed, in solubilized systems, even at saturation, $C_{\text{free}}$ does not exceed the equilibrium solubility of drug in (nonmicellar) aqueous solution. Solubilization in intestinal colloidal phases therefore provides no practical advantage in free drug concentration and, in the absence of changes to permeability, is unlikely to lead to appreciable increases in membrane flux when compared to an aqueous solution containing drug at close to saturated solubility. In support of this suggestion, many authors have shown that increasing the total concentration of a PWSD by solubilization does not necessarily result in proportional increases in absorptive flux.

Dahan et al. and Miller et al. recently proposed a model to quantify this phenomenon and referred to the existence of a “solubility-permeability interplay” where potential increases in membrane flux due to increases in solubilized drug concentration were offset by a reduction in the apparent permeability, the latter being, in large part, a function of decreases in free fraction.

The dispersion and digestion of LBF therefore creates a solubilized reservoir that is in rapid equilibrium with drug in free solution and provides significant advantage in the effective rate of dissolution of a PWSD when compared to traditional dissolution from a solid dose form. In contrast, the inherent solubility limitations to flux (rather than dissolution-rate limitations) are seemingly unchanged when viewed from the perspective of the free concentration or may be made worse. This appears at odds with the wealth of experimental and practical observations that suggest the ability of lipids (either formulation- or dietary-derived) to enhance the oral absorption of a range of PWSD. A possible explanation for this anomaly is that the traditional view of drug absorption from colloidal dispersions may not adequately describe the dynamic manner in which LBF exert absorption-enhancing effects in the GI tract.

Two alternative potential mechanisms of drug absorption are therefore examined here (Figure 1).

The first mechanism evaluated was the potential for drug absorption to occur via direct collisional transfer from lipid colloidal phases to the absorptive membrane, and thus to be mediated not only by $C_{\text{free}}$ but also by the solubilized fraction, $C_{\text{colloid}}$. Previously, studies by Storch and colleagues have shown that the transfer of poorly water-soluble fatty acids between model cell membranes and proteins may occur via collisional transfer. More recently, the possibility of drug absorption via collisional uptake has been suggested by Yano et al. and Gao et al. Collisional uptake may or may not be receptor-mediated, however, lipid uptake receptors such as CD36, SR-BI, and NPC1L1 have been suggested to facilitate the absorption of cholesterol and fatty acids, poorly water-soluble molecules that are also solubilized in intestinal colloidal phases.

In the case of SR-BI and CD36, direct interaction of the receptor with colloidal structures such as HDL (high-density lipoprotein), bile salt micelles and phospholipid vesicles has also been suggested, raising the possibility that lipid uptake receptors may interact directly with intestinal lipid colloidal phases to facilitate collisional absorption of solubilized contents, including PWSD.

The second mechanism evaluated was the potential for/endogenous lipid processing pathways to lead to drug supersaturation in lipid colloidal phases. Supersaturation increases the thermodynamic activity of solubilized drug and, in the solubilization model described by eq 1, will increase $C_{\text{free}}$ above the equilibrium aqueous drug solubility. This in turn is expected to enhance drug flux. The potential for supersaturation to enhance the oral bioavailability of PWSD has received increasing recent interest. For LBF, supersaturation may be generated by the loss of drug solubilization capacity resulting from the digestion of triglycerides and/or surfactants, and the dilution of cosolvents during GI processing. In contrast, the possibility that supersaturation may result directly from interactions between lipid colloidal phases and biliary fluids has been almost entirely ignored. Although traditional micellar solubilization models suggest that increases in bile salt concentrations increase drug solubilization, previous studies have also shown that dilution of lipid colloidal phases with model intestinal fluids (containing bile salts, phospholipid and cholesterol) may lead to the generation of less lipid-rich colloidal phases with lowered drug solubilization capacity. This provides a plausible mechanism for supersaturation generation in the small intestine and has been examined in detail here.
The data suggest that under the conditions explored, collisional drug absorption has little impact on drug absorption from intestinal colloidal species. In contrast, supersaturation-enhanced drug absorption, mediated by the interaction between secreted bile and lipid colloidal phases, may provide an endogenous mechanism to promote supersaturation and to facilitate drug absorption from lipid colloidal phases.

### EXPERIMENTAL SECTION

**Materials.** Cinnarizine, flunarizine dihydrochloride, monensin sodium, sodium taurocholate, sodium taurodeoxycholate, sodium glycocholate, sodium glycochenodeoxycholate, cholesterol, 1-α-lysophosphatidylcholine (LPC, from egg yolk), 1-α-phosphatidylcholine (PC, from dried egg yolk), oleic acid, caprylic acid, monocaprylin, N-hydroxysulfosuccinimide sodium, dicyclohexylcarbodiimide solution (60% w/v in xylene), N,N-dimethylformamide, Tween 80, potassium dihydrogen phosphate (KH₂PO₄) and sodium chloride (NaCl) were obtained from Sigma-Aldrich, Australia. Sodium taurocholate, sodium glycocholate, sodium glycochenodeoxycholate, ortho-phosphoric acid 85% (H₃PO₄), sodium hydroxide pellets (NaOH), tert-butyl methyl ether (TBME), dimethyl sulfoxide (DMSO), glacial acetic acid and absolute ethanol were from Merck, Australia. Disodium hydrogen orthophosphate (Na₂HPO₄), sodium hydrogen orthophosphate (NH₄H₂PO₄) (Ajax Finechem, Australia), cholesterol, [4,14C] (49.8 mCi/mmol), and Irga-Safe Plus (Perkin-Elmer Life Sciences, Waltham, MA), oleic acid, [9,10-3H(N)] (60 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO), Block Lipid Transport-1 (BLT-1, i.e., 2-hexyl-1-cyclopentanone thiosemicarbazone) (Chembridge, San Diego, CA), ezetimibe (Jai Radhe Sales, AMD, India), heparin sodium injection BP (1000 IU/mL, Hospira, Australia), xylazine (100 mg/mL, Troy Laboratories, Australia), acepromazine (10 mg/mL, Ceva Delvet, Australia), ketamine (100 mg/mL, Provet, Australia) and pentobarbital sodium (325 mg/mL, Virbac, Australia) were obtained from listed suppliers. Acetonitrile, methanol and chloroform used were analytical reagent grade. Water was obtained from a Millipore Milli-Q Gradient A10 water purification system (Millipore, Billerica, MA).

**Sulfo-N-succinimidyl Oleate (SSO) Synthesis.** SSO synthesis was adapted from the procedure of Harmon et al. Briefly, dicyclohexylcarbodiimide (1.26 mmol) and N-hydroxysulfosuccinimide (sodium salt, 1.20 mmol) were added to a solution of oleic acid (1.20 mmol) dissolved in N,N-dimethylformamide (2 mL), and the reaction was stirred at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and ethyl acetate (2 mL) added to the filtrate, which was left to stand at 4 °C overnight. SSO (precipitate) was then collected by filtration and dried under vacuum (1 mmHg). SSO identity was confirmed by NMR and mass spectrometry.

**Experimental outline.** To assess the role of receptor-mediated collisional drug absorption, cinnarizine bioavailability was assessed after administration of a lipid emulsion formulation in the absence and presence of BLT-1, SSO and ezetimibe. BLT-1, SSO and ezetimibe are chemical inhibitors of SR-BI, CD36 and NPC1L1, respectively. The possibility of endocytosis-mediated uptake was also investigated by the use of a general endocytosis inhibitor, monensin.

The role of collisional drug absorption was assessed more generically using an in situ rat jejunum perfusion model to compare the absorptive flux of cinnarizine from two distinctly different lipid colloidal phases (micelles vs vesicles) with matched drug solubilization capacities. Colloidal systems with the same total solubilization capacity, loaded with drug at the same concentration, have the same thermodynamic activity and therefore C<sub>free</sub> is the same in both cases. Under these circumstances, comparison of the flux profiles obtained from two structurally different colloids, but with identical C<sub>free</sub> provides a means of determining whether the nature of the colloid, or C<sub>free</sub> is the principal determinant of absorption. Generation of identical flux profiles from both systems would therefore confirm the dependence of flux on thermodynamic activity and free concentration, whereas a significant difference in flux would indicate a role for factors beyond C<sub>free</sub> in determining flux. These include the potential for collisional drug absorption mechanisms since collision rates are a statistical function of particle number and are expected to be markedly higher for micelles (where the smaller particle size results in higher particle numbers) when compared to vesicles.

To assess the potential for intestinal fluids to enhance drug absorption from lipid colloidal phases via the induction of drug supersaturation, whole bile was collected from fasted rats and mixed with model micelles and vesicles to simulate the process of interaction with bile in vivo. The potential for bile to generate drug supersaturation was evaluated in vitro by assessing changes in cinnarizine solubility, and by monitoring the kinetics of cinnarizine solubilization and precipitation, following bile addition to cinnarizine-loaded micelles and vesicles. Subsequently, the impact of drug supersaturation on the intestinal absorptive flux of cinnarizine from micelles and vesicles was assessed in an in situ rat jejunum perfusion model, with and without coperfusion of donor bile. Finally, the relevance of bile-induced drug supersaturation in vivo was assessed via examination of changes to cinnarizine bioavailability after administration of drug-loaded micelles and vesicles (with matched thermodynamic activity) in bile-intact vs bile-diverted rats.

**Formulation Preparation.** Lipid Emulsion. The lipid emulsion (3 mL per dose) consisted of 1 mg of cinnarizine and 49 mg of oleic acid solubilized in 8 mM sodium taurocholate, 2 mM phosphatidylcholine, 2 mM cholesteryl stearate and trace amounts of 13C-cholesterol (1 μCi/3 mL) and/or 3H-oleic acid (3 μCi/3 mL). The emulsion was prepared in 7.5 mL batches by weighing appropriate masses of cinnarizine in oleic acid stock solution (20 mg/g), phosphatidylcholine and cholesterol into a glass vial, and the mixture made up to 260 volume with a buffered sodium taurocholate solution (buffer consisted 18 mM Na₂HPO₄ 2H₂O and 12 mM Na₂HPO₄). Appropriate volumes of 13C-cholesterol, 3H-oleic acid, 5 mg/ml BLT-1 in ethanol (for SR-BI inhibition experiments only), 25 mg/mL SSO in DMSO (for CD36 inhibition experiments only) and 10 mg/mL monensin in ethanol (for endocytosis inhibition experiments only) were spiked into the vial and vortexed for 1 min. The formulation was emulsified by ultrasonification with a Misonix XL 2020 ultrasonic processor (Misonix, Farmingdale, NY) equipped with a 3.2 mm microprobe tip running at an amplitude of 240 μm and a frequency of 20 kHz for 1.5 min. The total solvent concentration in the emulsion was ≤2.5% v/v. The emulsion was used within 4 h of preparation, and the concentration of drug and labeled cholesterol and/or oleic acid was assayed before dosing (in duplicate) to confirm compound content in the emulsion and to allow for dose normalization between rats.

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**Intravenous Formulation.** The formulation (1 mL per dose) used for intravenous administration of cinnarizine and

14C-cholesterol comprised 0.5% w/v Tween 80 in buffer (36 mM Na2HPO4 and 22 mM KH2PO4, adjusted to pH 4 with

acetic acid). Cinnarizine and cholesterol were added to the

formulation by spiking 5% v/v DMSO (containing 10 mg/mL
cinnarizine) and 5% v/v ethanol (containing 5 mg/mL
caprylic acid and 40 μg/mL 14C-cholesterol) into the micellar
solution. The formulation was mixed by vortexing, and the
concentration of drug and labeled cholesterol was assayed
before dosing to confirm compound content in the formulation
and to allow for dose normalization between rats. The
formulation was used within 1 h of preparation.

**Model Micelles and Vesicles.** The preparation of model
micelles and vesicles was guided by the methods and phase
diagram published by Kossena et al.17 Medium-chain lipid
containing colloids were chosen over long-chain systems since
the former have previously been shown to generate mono-
phasic micellar and vesicular systems.22 Relatively high lipid
concentrations were chosen to reflect the species that are
expected to initially form during the digestion of medium-chain
triglycerides.18 The model colloids consisted of tricaprylin
digestion products (caprylic acid and monocaprylin) solubilized
in simulated endogenous intestinal fluid (SEIF). SEIF
comprised the six most prevalent bile salts in human bile,28
lysocephatidylcholine (LPC) and cholesterol. The total bile
salt:LPC:cholesterol molar ratio was maintained at 16:4:1,
reflecting known ratios within fasted human bile.29,30 The
combination of bile salts used here comprised 25 mol % sodium
glycocholate, 17.5 mol % sodium glycodeoxycholate, 25 mol %
sodium glycochenodeoxycholate, 12.5 mol % sodium tauro-
cholate, 7.5 mol % sodium taurodeoxycholate and 12.5 mol %
sodium taurochenodeoxycholate. The concentration ratios of
the bile salts were chosen based on average concentrations of
the six most prevalent bile salts found in human bile.28 The
caprylic:monocaprylin molar ratio was kept at 2:1, re-
reflecting the ratio of digestion products expected on digestion
of 1 mol of triglyceride. The concentration of micellar and
vesicular components was varied by trial and error (but
maintaining the ratios described above) to identify systems with
similar drug solubilization capacities. It has previously been
shown that the thermodynamic activity (i.e., free concen-
tration) of drug in a solubilized system may be estimated via
assessment of solubility behavior, such that having different
colloidal solutions containing drug at a fixed proportion of the
saturated solubility results in matched free concentrations.31
Thus, drug was loaded into either micellar or vesicular systems
at the same concentration (and the same proportion of
saturated solubility) and was therefore present at the same
thermodynamic activity (i.e., Ctotal, Ccoll and Cchol were the
same in both micellar and vesicular systems). The compositions
of the identified micellar and vesicular systems are shown in
Table 1.

**Addition of Bile to Model Micelles and Vesicles.** The
colloidal solutions containing drug at a saturated concentration in
both micellar and vesicular systems.27 Relatively high lipid
concentrations were chosen for the former have previously been shown to generate mono-
phasic micellar and vesicular systems.27 Relatively high lipid
concentrations were chosen to reflect the species that are
expected to initially form during the digestion of medium-chain
triglycerides.18 The model colloids consisted of tricaprylin
digestion products (caprylic acid and monocaprylin) solubilized
in simulated endogenous intestinal fluid (SEIF). SEIF
comprised the six most prevalent bile salts in human bile,28
lysocephatidylcholine (LPC) and cholesterol. The total bile
salt:LPC:cholesterol molar ratio was maintained at 16:4:1,
reflecting known ratios within fasted human bile.29,30 The
combination of bile salts used here comprised 25 mol % sodium
glycocholate, 17.5 mol % sodium glycodeoxycholate, 25 mol %
sodium glycochenodeoxycholate, 12.5 mol % sodium tauro-
cholate, 7.5 mol % sodium taurodeoxycholate and 12.5 mol %
sodium taurochenodeoxycholate. The concentration ratios of
the bile salts were chosen based on average concentrations of
the six most prevalent bile salts found in human bile.28 The
caprylic:monocaprylin molar ratio was kept at 2:1, re-
reflecting the ratio of digestion products expected on digestion
of 1 mol of triglyceride. The concentration of micellar and
vesicular components was varied by trial and error (but
maintaining the ratios described above) to identify systems with
similar drug solubilization capacities. It has previously been
shown that the thermodynamic activity (i.e., free concen-
tration) of drug in a solubilized system may be estimated via
assessment of solubility behavior, such that having different
colloidal solutions containing drug at a fixed proportion of the
saturated solubility results in matched free concentrations.31
Thus, drug was loaded into either micellar or vesicular systems
at the same concentration (and the same proportion of
saturated solubility) and was therefore present at the same
thermodynamic activity (i.e., Ctotal, Ccoll and Cchol were the
same in both micellar and vesicular systems). The compositions
of the identified micellar and vesicular systems are shown in
Table 1.

**Table 1. Composition of Model Micelles and Vesicles**

<table>
<thead>
<tr>
<th></th>
<th>total bile salt</th>
<th>LPC</th>
<th>cholesterol</th>
<th>caprylic acid</th>
<th>monocaprylin</th>
</tr>
</thead>
<tbody>
<tr>
<td>micelles</td>
<td>8</td>
<td>2</td>
<td>0.5</td>
<td>69.3</td>
<td>34.7</td>
</tr>
<tr>
<td>vesicles</td>
<td>2</td>
<td>0.5</td>
<td>0.125</td>
<td>52.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

*Micelles and vesicles also consist of 18 mM NaH2PO4, 2H2O and 12 mM Na2HPO4. Sodium strength was adjusted to 150 mM with NaCl. Final pH of phases was adjusted to 6.30 ± 0.01. Total bile salt consist of 25 mol % sodium glycocholate, 17.5 mol % sodium glycodeoxycholate, 25 mol % sodium glycochenodeoxycholate, 12.5 mol % sodium taurocholate, 7.5 mol % sodium taurodeoxycholate, 12.5 mol % sodium taurochenodeoxycholate. LPC is lysosphatidylcholine.

**Intravenous Formulation.** The formulation (1 mL per dose) used for intravenous administration of cinnarizine and

14C-cholesterol comprised 0.5% w/v Tween 80 in buffer (36 mM Na2HPO4 and 22 mM KH2PO4, adjusted to pH 4 with

acetic acid). Cinnarizine and cholesterol were added to the

formulation by spiking 5% v/v DMSO (containing 10 mg/mL
cinnarizine) and 5% v/v ethanol (containing 5 mg/mL
caprylic acid and 40 μg/mL 14C-cholesterol) into the micellar
solution. The formulation was mixed by vortexing, and the
concentration of drug and labeled cholesterol was assayed
before dosing to confirm compound content in the formulation
and to allow for dose normalization between rats. The
formulation was used within 1 h of preparation.

**Model Micelles and Vesicles.** The preparation of model
micelles and vesicles was guided by the methods and phase
diagram published by Kossena et al.17 Medium-chain lipid
containing colloids were chosen over long-chain systems since
the former have previously been shown to generate mono-
phasic micellar and vesicular systems.22 Relatively high lipid
concentrations were chosen to reflect the species that are
expected to initially form during the digestion of medium-chain
triglycerides.18 The model colloids consisted of tricaprylin
digestion products (caprylic acid and monocaprylin) solubilized
in simulated endogenous intestinal fluid (SEIF). SEIF
comprised the six most prevalent bile salts in human bile,28
lysocephatidylcholine (LPC) and cholesterol. The total bile
salt:LPC:cholesterol molar ratio was maintained at 16:4:1,
reflecting known ratios within fasted human bile.29,30 The
combination of bile salts used here comprised 25 mol % sodium
glycocholate, 17.5 mol % sodium glycodeoxycholate, 25 mol %
sodium glycochenodeoxycholate, 12.5 mol % sodium tauro-
cholate, 7.5 mol % sodium taurodeoxycholate and 12.5 mol %
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Solid-State Analysis of the Cinnarizine Precipitate Using Polarized Light Microscopy. Selected cinnarizine pellets from the precipitation kinetics experiments were analyzed using a Zeiss AxioLab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters. At the end of the precipitation kinetics experiments, 1.5 mL of remaining bile + colloid mixture was centrifuged (2200g, 10 min, 37 °C), the supernatant was discarded, and a small amount of pellet was carefully placed on a microscope slide. Samples were analyzed using under crossed-polarized light, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan).

Animals. All rat studies were approved by the institutional animal ethics committee and were conducted in accordance with the guidelines of the Australian and New Zealand Council for the Care of Animals in Research and Teaching. Male Sprague–Dawley rats (280–330 g) were used in all experiments and were allowed to acclimatize in the institutional animal housing facility for at least 7 days with free access to standard chow and water. All animals were fasted overnight (12–18 h) prior to surgery.

Surgical Procedures. Anesthesia was induced in rats by subcutaneous injection of 1.0 mL/kg of “Cocktail I” (37.3 mg/mL ketamine, 9.8 mg/mL xylazine, 0.4 mg/mL acepromazine in saline), and maintained throughout the study with subcutaneous doses of 0.44 mL/kg of “Cocktail II” (90.9 mg/mL ketamine, 0.9 mg/mL acepromazine) when required. Rats were maintained on a 37 °C heated pad throughout surgery and experiments. At the end of all experiments, rats were euthanized via an intravenous or intracardiac injection of 100 mg of sodium pentobarbitone.

Cinnarizine Bioavailability Studies following Intraduodenal Administration. The surgical procedures for the conduct of bioavailability studies included cannulations of the right carotid artery, right jugular vein, duodenum (1 cm below pylorus) and common bile duct (only for bile-diverted rats). The surgical procedures for the cannulations were described previously.32,33

Fasted Rat Bile Collection. The bile duct was cannulated near the hilum of the liver (where the duct is free of pancreatic tissue) in order to facilitate the collection of bile fluid without contamination by exocrine pancreatic secretions.34 Rats were rehydrated via saline infusion (1.5 mL/h) into a cannula inserted into the right jugular vein, and bile was continuously collected for 5 h. The concentration of total bile salt in collected bile was assayed using a validated enzymatic colorimetric assay (Total Bile Acids kit #431-15001; Wako Pure Chemical Industries, Osaka, Japan) on a plate reader (FluoStar Optima plate reader, BMG Labtechnologies, Germany) measuring absorbance at a wavelength of 540 nm. In all subsequent experiments, bile was used within 24 h of collection.

Single-Pass Rat Jejunum Perfusion. The model employed to assess flux across rat jejunum involved in situ perfusion (single-pass) of an isolated jejunal segment and simultaneous blood collection from the corresponding mesenteric vein branch. The surgical procedures for the perfusion studies are similar to those described elsewhere, with slight modifications.35 Briefly, the right jugular vein was cannulated to enable infusion of donor blood. A piece of jejunal segment (~10 cm²) was isolated and cannulated at the proximal and distal ends with sections of Teflon tubing (0.03 in. i.d. proximal/inlet, Upchurch Scientific, Oak Harbor, WA; 0.0625 in. i.d. distal/outlet, Shimadzu, Kyoto, Japan). Jejunal contents were initially flushed with warm perfusion buffer (150 mM Na⁺, 18 mM H₂PO₄⁻, 12 mM HPO₄²⁻, 108 mM Cl⁻), adjusted to pH 6.30 ± 0.01. The mesenteric vein draining the jejunal segment was then isolated, the rat heparinized (90 IU/kg) via the jugular vein and the mesenteric vein immediately catheterized. A drop of superglue was placed over the site of catheterization, and silicone tubing (0.025 in. i.d., Helix Medical, CA) attached to the catheter tip for the collection of venous blood. Immediately following catheterization of the mesenteric vein and for the remainder of the experiment, rats were infused with heparinized donor rat blood (5 IU/mL) via the jugular vein, and the rate of (0.3 mL/min) was adjusted based on the outflow from the mesenteric blood.

Cinnarizine Bioavailability after Intraduodenal Infusion in Anesthetized Rats. A 30 min equilibration period was allowed between the end of surgery and drug dosing. To examine the impact of lipid uptake receptors on drug absorption, studies were conducted in the presence or absence of lipid uptake inhibitors. To examine the impact of bile-induced drug supersaturation on drug absorption, studies were conducted in bile-intact or bile-diverted rats. Colloidal systems (lipid emulsion, micelles, vesicles) containing cinnarizine were infused into the duodenum of rats at a rate of 1.5 mL/h for 2 h. When the dose infusion was complete, saline was infused at a rate of 1.5 mL/h for 10 min to flush any remaining formulation in the tubing into the duodenum. Blood samples (0.3 mL) were collected via the carotid artery cannula up to 8 h after infusion initiation into tubes containing 3 IU heparin. The sampling intervals were as follows: t = 0, 1, 2, 2.5, 3, 4, 5, 6, 7, 8 h for the receptor inhibition studies; and t = 0, 1, 1.5, 2, 3, 4, 5, 6, 7, 8 h for the bile-induced drug supersaturation studies. After each blood sample was taken, the cannula was flushed with 0.3 mL of 2 IU/mL heparinized saline to ensure cannula patency, and to replace the volume of blood removed. Plasma was separated by centrifugation (10000g, 5 min) to enable analysis of drug and labeled lipid content.

Administration of Lipid Uptake Inhibitors (BLT-1, SSO, Ezetimibe) and Endocytosis Inhibitor (Monensin). Ezetimibe has previously been dosed at 0.3 mg/kg intravenously into rats, and has been shown to inhibit cholesterol absorption (from an intraduodenally dosed lipid emulsion) without reports of toxicity.36 Therefore, in our study, intravenous administration of ezetimibe (0.3 mg/kg via the jugular vein) was selected as the route to administer the inhibitor at the beginning of the 30 min equilibration period. An appropriate volume of 5 mg/mL ezetimibe in ethanol was spiked into blank rat plasma, and 0.8 mL of resultant plasma was dosed into rats as an intravenous bolus. The total ethanol concentration was less than 2.5% v/v. In the case of BLT-1 and SSO, the inhibitors had not been previously administered intravenously, therefore local (i.e., intestinal) administration was selected to limit the systemic effects of the inhibitors. Monensin was also administered directly into the intestine to minimize systemic endocytosis inhibition. Here, 100 μM BLT-1, 1 mM SSO and 100 μM Monensin were preinfused intraduodenally (in saline) at a rate of 1.5 mL/h during the 30 min equilibration period, and subsequently confounded at the same concentration as part of the lipid emulsion. BLT-1 and SSO have previously shown to inhibit lipid uptake in cell-based studies at concentrations of 1–
10 μM$^{24}$ and 400 μM$^{25}$ respectively. Monensin has previously been shown to inhibit endocytosis in cultured cells at 10 μM. $^{37}$

Intravenous administration studies were also conducted in control rats and monensin-treated rats to assess the effect of endocytosis inhibition on the systemic distribution and clearance of cinnarizine and cholesterol. In these studies, blank (i.e., not containing cinnarizine and 14C-cholesterol) lipid emulsion (with or without 100 μM monensin) was infused intraduodenally as described above, and intravenous infusion of the cinnarizine and 14C-cholesterol containing intravenous (iv) formulation was commenced at the same time. The iv formulation (1 mL) was infused into the right jugular vein at a rate of 0.05 mL/15 s (total infusion period <5 min). Blood samples (0.3 mL) were collected via the carotid artery cannula at t = 5, 15, 30, 60, 120, 180, 240, 360, 480 min after infusion. At 10 min intervals and briefly vortexed before samples were taken for analysis of drug and lipid content. For experiments where micelles or vesicles were coperfused in a 1:1 v/v ratio with a secondary perfusate of bile/bile pH 6.30, the perfusate was held at 0.1 mg/mL (±10%, ±15% at the limit of quantification) for concentrations between 20 and 1000 ng/mL for iv formulation, emulsion, micelles and vesicles, and 10 and 320 ng/mL for plasma.

**Calculations.** In the single-pass rat jejunal perfusion model, permeability coefficients were calculated using steady-state drug concentrations in perfusate and blood. Two apparent permeability coefficients ($P_{app}$) were calculated as described previously: $^{35}

\[
\text{disappearance } P_{app} = -\frac{Q}{A} \ln \frac{C_1}{C_0} \quad (2)
\]

\[
\text{appearance } P_{app} = \frac{\Delta M}{\Delta t} \left(\frac{A}{C}\right) \quad (3)
\]

where “disappearance $P_{app}$” is the apparent permeability coefficient calculated from drug loss from the perfusate (cm$^2$/s); “appearance $P_{app}$” is the apparent permeability coefficient calculated from drug appearance in the mesenteric blood (cm$^2$/s); $Q$ is the perfusate flow rate (mL/s); $A$ is the surface area of the perfused jejunal segment (cm$^2$), which is calculated by multiplying the diameter by the length of the perfused intestinal segment as described previously; $C_i$ is the average steady-state drug concentration exiting the perfused jejunal segment (ng/mL); $C_0$ is the drug concentration entering the jejunal segment ($\text{ng/mL}$).
Noncompartmental Pharmacokinetic Analysis. The maximum plasma concentration (Cmax), time to reach Cmax (Tmax), area under the plasma concentration–time curve from time zero to the last measured concentration (AUC0–t), area under the plasma concentration–time curve extrapolated to infinity (AUC0–∞), elimination rate constant (keli), volume of distribution (Vd) and clearance (Cl) were calculated using WINNONLIN version 5.3 (Pharsight Inc., Apex, NC, USA).

Statistical Analysis. Results were analyzed using Student’s t test. A P value of <0.05 was considered to be a significant difference. Analyses were performed using SPSS v19 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

SR-BI, CD36, NPC1L1 and Endocytosis Have Little Impact on Drug Absorption from Intestinal Colloidal Phases. Inhibition of the lipid uptake receptors SR-BI (by coinfusion of 100 μM BLT-1), CD36 (by coinfusion of 1 mM SSO) and NPC1L1 (by intravenous administration of 0.3 mg/kg ezetimibe) did not result in significant changes to the systemic plasma concentration–time profiles or systemic exposure of cinnarizine (Figure 2A, Table 2). Inhibition of endocytosis (by coinfusion of 100 μM monensin) unexpectedly led to significant increases in cinnarizine systemic plasma concentration at t = 2.5 and 4 h (Figure 2A), and increased the AUC0–t, 1.5-fold (Table 2). However, subsequent intravenous dosing studies revealed significantly lower Vd and Cl values in the monensin-treated rats when compared to control (Table S1 in the Supporting Information), suggesting that the increase in cinnarizine systemic exposure was due to a decrease in cinnarizine systemic distribution and clearance, rather than changes to intestinal absorption. The data suggest a limited role for SR-BI, CD36, NPC1L1 and endocytosis generally, in the absorption of cinnarizine from micelles and vesicles.

In contrast, inhibition of NPC1L1 did lead to significantly lower systemic plasma concentrations of exogenously dosed cholesterol at t = 2, 2.5, 3, 4, 6, 8 h (Figure 2B). Plasma concentrations of exogenously dosed oleic acid also appeared lower in rats following inhibition of CD36 although differences were not significantly different (Figure 2C). While inhibition of SR-BI and CD36 did not lead to significant changes to the systemic plasma concentration–time profile of exogenously dosed cholesterol, inhibition of endocytosis significantly increased the systemic plasma concentrations of exogenously dosed cholesterol at t = 4, 8 h (Figure 2B). This increase in exposure is likely explained by a decrease in systemic distribution of 14C-cholesterol in the monensin-treated rats, as plasma 14C-cholesterol concentrations at early sample time points (which reflect the distribution phase) in intravenous studies were significantly higher in the monensin-treated rats (Figure S1B in the Supporting Information) when compared to controls (Vd and Cl could not be calculated for cholesterol as a typical elimination phase was not evident in the systemic plasma concentration vs time profiles of cholesterol: Figure 2B and Figure S1B in the Supporting Information).

Drug Absorption from Micelles and Vesicles Is Determined by Cfree and Not Colloidal Structure. The intestinal perfusion of colloidal media with markedly different compositional profiles (micelles and vesicles) but with comparable Cfree and thermodynamic activity did not result in significant differences in steady-state absorptive flux, disappearance Papp or appearance Papp of cinnarizine (Table 3, Figure 3A: filled symbols). Similarly, in spite of large differences in particle size and composition, the intraduodenal infusion of the same micellar or vesicular systems to bile-diverted rats did not result in significant differences in systemic plasma concentration–time profiles (Figure 4: filled symbols) and pharmacokinetic parameters of cinnarizine (Table 5: micelles vs vesicles in bile-...

Table 2. Pharmacokinetic Parameters for Cinnarizine after Intraduodenal Administration of a 3 mL Lipid Emulsion in Rats

<table>
<thead>
<tr>
<th>expl group</th>
<th>AUC0–t (ng h/mL)</th>
<th>Cmax (ng/mL)</th>
<th>Tmax (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>978 ± 122</td>
<td>288.1 ± 26.6</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>BLT-1-treated</td>
<td>883 ± 21</td>
<td>2190.9 ± 20.0</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>SSO-treated</td>
<td>924 ± 88</td>
<td>1953.5 ± 33.6</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>ezetimibe-treated</td>
<td>1048 ± 92</td>
<td>333.9 ± 13.6</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>monensin-treated</td>
<td>1487 ± 103</td>
<td>432.8 ± 45.0</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

*In each case, the formulation type was emulsion and the CIN dose was 3.33 mg/kg. Experiments were performed in control rats; rats treated with 100 μM BLT-1; rats treated with 1 mM SSO; rats treated with 0.3 mg/kg ezetimibe; and rats treated with 100 μM monensin, to inhibit the lipid uptake receptors SR-BI, CD36, NPC1L1, and endocytosis, respectively. Values represent mean ± SEM of n = 4 rats. Significant differences when compared to control group.

Figure 2. Systemic plasma concentration–time profiles of (A) cinnarizine (CIN), (B) 14C-labeled cholesterol (Ch) and (C) 3H-labeled oleic acid (OA) following intraduodenal infusion of a 3 mL lipid emulsion consisting of 1 mg of cinnarizine emulsified in 59 mM oleic acid, 1 mM cholesterol, 1 μCi of 14C-cholesterol and/or 3 μCi of 3H-oleic acid. Experiments were performed in control rats (filled circle); rats treated with 100 μM BLT-1 (open circle); rats treated with 1 mM SSO (open square); rats treated with 0.3 mg/kg ezetimibe (open square); and rats treated with 100 μM monensin (open diamond), to inhibit the lipid uptake receptors SR-BI, CD36, NPC1L1 and endocytosis, respectively. BLT-1, SSO, and monensin were coinfused as part of the lipid emulsion; ezetimibe was administered intravenously. Data represent mean ± SEM of n = 4 rats for A and B; and n = 3 rats for C. Statistically significant difference with respect to control rats (p < 0.05) is denoted by the symbol *.
Bile-Mediated Dilution of Cinnarizine-Loaded Micelles and Vesicles Generates Drug Supersaturation.

The pH of donor bile was higher than intestinal pH (average mean ± SEM of n = 3–4 experiments). SS ratio = supersaturation ratio = (supersaturated) concentration of drug in perfusate/equilibrium solubility of drug in perfusate. SS denotes drug supersaturation in perfusate. Significant increase from micelles alone. Significant increase from vesicles alone.

Table 3. Cinnarizine Disappearance $P_{\text{app}}$ ($\times 10^6$ cm/s) from the Intestinal Perfusate, Appearance $P_{\text{app}}$ ($\times 10^6$ cm/s) in the Mesenteric Blood and Steady-State Absorptive Flux into Mesenteric Blood (ng/5 min/10 cm$^2$) after 60 min of Single-Pass Perfusion of ~10 cm$^2$ Segments of Rat Jejunum with Model Micelles and Vesicles, with and without 1:1 v/v Coperfusion with Rat Bile, Rat Bile pH 6.30 or Buffer pH 6.30$^a$

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{app}}$ ($\times 10^6$ cm/s)</th>
<th>Appearance $P_{\text{app}}$ ($\times 10^6$ cm/s)</th>
<th>flux into mesenteric blood (ng/5 min/10 cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>micelles</td>
<td>0.4 19.4 ± 3.0</td>
<td>1.1 ± 0.2</td>
<td>310 ± 50</td>
</tr>
<tr>
<td>micelles + bile $^b$</td>
<td>6.1 29.3 ± 3.0</td>
<td>3.7 ± 0.1</td>
<td>979 ± 30 $^d$</td>
</tr>
<tr>
<td>micelles + bile pH 6.30$^b$</td>
<td>3.0 39.5 ± 5.6$^d$</td>
<td>4.2 ± 1.0</td>
<td>1099 ± 279$^d$</td>
</tr>
<tr>
<td>micelles + buffer pH 6.30$^b$</td>
<td>2.1 38.9 ± 4.1$^d$</td>
<td>4.4 ± 0.4$^d$</td>
<td>1180 ± 100$^d$</td>
</tr>
<tr>
<td>vesicles</td>
<td>0.4 22.0 ± 2.2</td>
<td>1.2 ± 0.1</td>
<td>340 ± 29</td>
</tr>
<tr>
<td>vesicles + bile $^b$</td>
<td>11.6 66.0 ± 18.3$^f$</td>
<td>2.0 ± 0.7</td>
<td>499 ± 155</td>
</tr>
</tbody>
</table>

Values calculated using data obtained after steady-state attainment (t = 40–60 min). In all experiments, cinnarizine concentration in perfusate and total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. Data represent mean ± SEM of n = 3–4 experiments. $^b$SS ratio = supersaturation ratio = (supersaturated) concentration of drug in perfusate/equilibrium solubility of drug in perfusate. $^f$Significant increase from micelles alone. $^g$Significant increase from vesicles alone.

The pH of donor bile was higher than intestinal pH (average mean ± SEM of n = 3–4 experiments). SS ratio = supersaturation ratio = (supersaturated) concentration of drug in perfusate/equilibrium solubility of drug in perfusate. SS denotes drug supersaturation in perfusate. Significant increase from micelles alone. Significant increase from vesicles alone.

Figure 3. (A) Absorbptive flux of cinnarizine (CIN) into mesenteric blood (ng/5 min/10 cm$^2$) and (B, C) CIN disappearance from intestinal perfusate (% drug dose passing through jejunum) when micelles/vesicles were perfused through an isolated rat jejunal segment (~10 cm$^2$), with (open symbols) and without (filled symbols) 1:1 v/v coperfusion with rat bile. Coperoxution of micelles and vesicles with donor bile generates drug supersaturation in situ within the perfused jejunal segment. SS denotes experiments where drug is supersaturated in the perfusate. The degree of drug precipitation within the perfusate is illustrated in B and C as the difference in perfusate concentration between pre- and postcentrifugation data. Precipitation was significant in the vesicle, but not micellar groups. Experiments were performed using an in situ single-pass rat jejunal perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. Data represent mean ± SEM of n = 3–4 experiments.

Figure 4. Systemic plasma concentration–time profiles of cinnarizine (CIN) following a 2 h intraduodenal infusion of cinnarizine-loaded (0.2 mg/mL) micelles (left panel) and vesicles (right panel) to bile-intact and bile-diverted rats. Consistent with observations in rat jejunal perfusion studies, bile-induced supersaturation translated into increased in vivo exposure during the absorption phase in the case of micelles but not vesicles. Data represent mean ± SEM of n = 4 rats. Statistical significance (p < 0.05) is denoted by the symbol *.

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variable, however supersaturation was maintained for longer periods in micelles (>20 min in all cases) when compared to vesicles (1–20 min).

**Bile-Induced Drug Supersaturation Increases Jejunal Absorptive Flux for Micelles but Not Vesicles.** The absorptive flux vs time profiles for cinnarizine under supersaturated conditions (i.e., when bile was coperfused with the phases) and under nonsupersaturated conditions (when bile was not coperfused with the phases) are shown in Figure 3A. Steady-state absorptive flux, disappearance $P_{\text{app}}$ and appearance $P_{\text{app}}$ of cinnarizine in all perfusion experiments are reported in Table 3.

Coperfusion of micelles with bile in a 1:1 v/v ratio increased the absorptive flux, disappearance $P_{\text{app}}$ and appearance $P_{\text{app}}$ of cinnarizine from micelles 3.2-fold, 1.5-fold and 3.4-fold, respectively. In contrast, 1:1 v/v coperfusion of vesicles with bile did not lead to significant changes in cinnarizine absorptive flux or appearance $P_{\text{app}}$. Disappearance $P_{\text{app}}$ for cinnarizine did increase 3.0-fold when vesicles were coperfused with bile, however the drop in perfusate drug concentration was largely a result of rapid drug precipitation in the perfusate (see below). For the micellar preparation, bile-induced supersaturation was relatively stable throughout the experimental period. Thus the cinnarizine concentration in the perfusate was essentially the same before or after centrifugation (Figure 3B). In contrast, when vesicles were coperfused with bile, significant drug precipitation was observed during the time required for perfusate to transit the jejunal segment (as indicated by the difference between the pre- and postcentrifugation data in Figure 3C). This was consistent with the in vitro dilution profiles in Figure 6.

To distinguish between bile-induced increases in cinnarizine absorptive flux resulting from drug supersaturation, pH increases (since an increase in pH might be expected to increase the permeability of a weak base) and nonspecific effects of bile components on membrane permeability, micelles were also coperfused with pH-adjusted bile (pH 6.30) and buffer (pH 6.30). 1:1 v/v coperfusion of micelles with bile pH 6.30 or buffer pH 6.30 increased cinnarizine absorptive flux 3.5-fold and 3.8-fold, respectively, when compared to the perfusion of micelles alone (Figure 7, Table 3). Stable supersaturation was not coperfused with the phases) are shown in Figure 3A.

**Table 4. Equilibrium Solubility (37°C) and Percent Original Solubilization Capacity Values of Cinnarizine in Model Micelles and Vesicles, before and after 1:1 v/v Addition of Rat Bile, Rat Bile pH 6.30 or Buffer pH 6.30**

<table>
<thead>
<tr>
<th></th>
<th>equilibrium solubility (μg/mL)</th>
<th>percent original solubilization capacity (%)</th>
<th>micelles</th>
<th>vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>bina</td>
<td>236 ± 9.3</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ bile (1:1)</td>
<td>16.5 ± 0.8</td>
<td>14.0 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ bile pH</td>
<td>33.1 ± 1.0</td>
<td>28.0 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.30 (1:1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ buffer pH</td>
<td>48.7 ± 0.8</td>
<td>41.2 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.30 (1:1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data represent mean ± SEM of n = 3–4 determinations. *Percent original solubilization capacity = (solubility$_{\text{final}}$ × volume$_{\text{final}}$ / solubility$_{\text{initial}}$ × volume$_{\text{initial}}$) × 100%.

**Table 5. Pharmacokinetic Parameters for Cinnarizine after Intraduodenal Administration of Cinnarizine-Loaded (0.2 mg/mL) Micelles and Vesicles to Bile-Intact and Bile-Diverted Rats**

<table>
<thead>
<tr>
<th>expl group</th>
<th>formulation type</th>
<th>CIN dose (mg/kg)</th>
<th>AUC$_{2→\text{sh}}$ (ng h/mL)</th>
<th>C$_{\text{max}}$ (ng/mL)</th>
<th>$T_{\text{max}}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>micelles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bile-intact</td>
<td>micelles</td>
<td>2</td>
<td>502 ± 59$^b$</td>
<td>138.8 ± 11.6$^b$</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>bile-diverted</td>
<td>micelles</td>
<td>2</td>
<td>362 ± 39</td>
<td>85.7 ± 14.3</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bile-intact</td>
<td>vesicles</td>
<td>2</td>
<td>411 ± 74</td>
<td>109.4 ± 10.6</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>bile-diverted</td>
<td>vesicles</td>
<td>2</td>
<td>393 ± 82</td>
<td>97.6 ± 12.8</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

*Values represent mean ± SEM of n = 4 rats. *Significant increase when compared to micelles (bile-diverted) group.

Figure 5. Percent original cinnarizine (CIN) solubilization capacity of model colloids (micelles or vesicles), before and after a 1:1 v/v addition of rat bile, rat bile pH 6.30 or buffer pH 6.30. Data represent mean ± SEM of n = 3–4 determinations. Statistical significant difference (p < 0.05) to colloid only is denoted by the symbol *.

Figure 6. Kinetics of cinnarizine (CIN) precipitation from model micelles (filled circles, n = 5) and model vesicles (open circles, n = 4) upon addition of rat bile (in a 1:1 v/v ratio) at t = 0. Addition of bile reduces the equilibrium cinnarizine solubilization capacity of micelles and vesicles to 14% and 7% of initial, respectively (see Table 4, and shown here as the lines denoted equilibrium solubility). Cinnarizine supersaturation appeared to be maintained for longer in micelles than in vesicles. Cinnarizine was loaded into micelles and vesicles at 80% saturation (~0.2 mg/mL). Each line represents individual experiments.
829 administration of micelles to bile-intact rats resulted in
827 ability studies are reported in Table 5. Consistent with the
824 and vesicles (0.2 mg/mL; 823 following intraduodenal infusion of cinnarizine-loaded micelles
821 820 Bile-Induced Drug Supersaturation Increases
817 be expected based on the un-ionized fraction.41,42 This left shift
816 fraction) as a function of pH is shifted to lower pHs than would
814 shown that, for lipophilic drugs such as cinnarizine, the drug
812 6.30 group and micelles + bu
811 micelles + bile group, when compared to the micelles + bile pH
808 enhancement did not correlate with the degree of super-
806 (by comparing micelles + bile pH 6.30 group with micelles +
804 comparing micelles + bile group with micelles + bile pH 6.30
803 drug supersaturation, and not an increase in system pH (by
802 since 1:1 v/v coperfusion of micelles with bile, bile pH 6.30
799 to the solubility data in Table 4, and the lack of drug
798 supersaturated in perfusate. Coperfusion of rat bile, rat bile pH 6.30 or
797 and bu
796 cinnarizine concentration in the perfusate was essentially the
794 cinnarizine absorptive
793 experiments. (Figure S2 in the Supporting Information:
792 was also generated within the perfused jejunal segment in these:
791 cinnarizine concentration in the perfusate was essentially the
790 same before and after centrifugation).
789 Since 1:1 v/v coperfusion of micelles with bile, bile pH 6.30
787 and buffer pH 6.30 are all expected to generate cinnarizine
786 supersaturation within the perfused jejunal segment (according
784 to the solubility data in Table 4, and the lack of drug
783 precipitation in outflow perfusate in all cases), the observation
781 that absorptive flux enhancement was similar in all groups
780 (Figure 7) suggests that the enhancement was attributable to
779 drug supersaturation, and not an increase in system pH (by
777 comparing micelles + bile group with micelles + bile pH 6.30
775 group), or nonspecific effects of bile on membrane permeability
773 by comparing micelles + bile pH 6.30 group with micelles +
771 buffer pH 6.30 group). However, the degree of flux
770 enhancement did not correlate with the degree of super-
768 saturation, as flux enhancement was similar in all groups despite
766 the significantly higher supersaturation ratio generated in the
764 micelles + bile group, when compared to the micelles + bile pH
763 6.30 group and micelles + buffer pH 6.30 group (super-
761 saturation ratio of 6 vs 2 ≈ 3) (Table 3). Previous studies have
759 shown that, for lipophilic drugs such as cinnarizine, the drug
758 suspension extracted into octanol (and analogous to the permeable
756 function) as a function of pH is shifted to lower pHs than would
754 be expected based on the un-ionized fraction.43,44 This left shift
752 would limit pH effects on permeability over the range of pH 6–
750 8, consistent with the observations here.

807 was higher systemic plasma concentrations of cinnar-
806 zine at t = 1, 1.5 and 2 h (AUC<sub>0–8h</sub> increased 1.4-fold) when
805 compared to bile-diverted rats. In contrast, when vesicles were
803 dosed to bile-intact rats, the systemic plasma concentration–
801 time profiles and AUC<sub>0–8h</sub> of cinnarizine were not different
800 from that observed in bile-diverted rats.

**DISCUSSION**

After oral administration, the absorption of poorly water-
837 soluble drugs (PWSD) is often limited by slow dissolution and
835 low solubility in the GI tract. LBF overcome many of the
833 dissolution limitations of PWSD (by providing a mechanism to
831 circumvent dissolution from the solid to liquid state), however,
830 the solubility limitations of PWSD are seemingly unaddressed,
828 since solubilization in the lipid-based colloidal phases that result
826 from the digestion of LBF does not typically enhance free drug
824 concentrations.2,43 Nonetheless, coadministration of lipids
822 (either formulation lipids or via coadministration with lipid-
820 rich foods) remains a highly effective means to promote the
820 absorption of PWSD. This suggests the potential for alternative
818 mechanisms by which LBF enhance drug absorption. In the
816 current communication, two possible alternatives to the
814 traditional model of drug absorption from LBF have been
812 explored: first, that drug absorption from lipid colloidal phases
810 may involve a collisional uptake component (i.e., drug
808 absorption directly from the solubilized phase); and, second,
805 that flux across the absorptive membrane may be enhanced by a
803 transient increase in the thermodynamic activity of drug in
801 intestinal colloidal phases due to supersaturation.

852 The data describing drug absorption from micellar and
850 vesicular colloidal phases suggest that direct interactions
848 between colloids (or at least the systems examined here) and
846 the absorptive membrane do not play an important role in
844 cinnarizine absorption. Thus, comparable absorptive flux
842 (Figure 3A: filled symbols) and systemic plasma concen-
840 tration–time profiles (Figure 4: bile-diverted rats) were observed
838 following jejunal perfusion and intraduodenal infusion
836 of micelles and vesicles. The thermodynamic activity and C<sub>free</sub>
834 of both systems were held constant, but the large difference in
832 hydrodynamic radius (9 nm of micelles vs 443 nm of vesicles)
830 and higher bile salt, LPC and lipid concentrations in the
828 micellar system (Table 1) suggest that the number of administered micellar particles was substantially higher than
826 that of vesicular particles. Collision-mediated absorption
824 is highly sensitive to increased particle number, since this
822 increases the statistical likelihood of collisions and collisional
820 transfer.7 As such collisional interactions did not appear to
818 dictate the degree of drug absorption from micelles and vesicles
816 and, instead, absorption was seemingly controlled by
814 thermodynamic activity (or C<sub>free</sub>). Differences in particle size
812 might also be expected to alter colloid diffusion across the
810 unstirred water layer (UWL). The similarity in absorption
808 profiles from micelles and vesicles (Figure 3A: filled symbols) suggests therefore that diffusion across the UWL either was
806 nonlimiting or was not affected by particle size in this
804 experimental model. Similarly, the rates of replenishment of
802 C<sub>free</sub> (i.e., the rate of re-establishment of the equilibrium
800 between solubilized and free drug) might be expected to be
706 different between the two different particle size colloids, however this was presumably sufficiently fast in both cases to
704 have little impact on drug absorption in the current model.

Data obtained from the lipid uptake receptor inhibition
800 studies further support the notion that collisional uptake
800 studies further support the notion that collisional uptake
800 of cinnarizine within the perfused jejunal segment in all cases, and increased
cinnarizine absorptive flux by 3.2-fold, 3.5-fold and 3.8-fold, respectively. Experiments were performed using an in situ single-pass rat jejunum perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. This series of experiments provides further support for the suggestion that supersaturation was responsible for the increase in absorptive flux seen in Figure 3A. Data represent mean ± SEM of n = 3–4 experiments.

![Figure 7. Absorptive flux–time profiles of cinnarizine (CIN) when micelles were perfused through an isolated rat jejunal segment (~10 cm²), with and without 1:1 v/v coperoxification with rat bile, rat bile pH 6.30 or buffer pH 6.30. SS denotes experiments where drug is supersaturated in perfusate. Coperfusion of rat bile, rat bile pH 6.30 or buffer pH 6.30 with micelles generates drug supersaturation in situ within the perfused jejunal segment in all cases, and increased cinnarizine absorptive flux by 3.2-fold, 3.5-fold and 3.8-fold, respectively. Experiments were performed using an in situ single-pass rat jejunum perfusion model. In all experiments, the concentration of cinnarizine in perfusate and the total perfusate flow rate were kept constant at 0.1 mg/mL and 0.1 mL/min, respectively. This series of experiments provides further support for the suggestion that supersaturation was responsible for the increase in absorptive flux seen in Figure 3A. Data represent mean ± SEM of n = 3–4 experiments.](image-url)
mechanisms have a limited role in drug absorption from these systems. SR-BI, CD36 and NPC1L1 were examined since they have previously been shown to mediate the cellular uptake of fatty acids and/or cholesterol, the absorption of which is also facilitated by micellar solubilization. Whether SR-BI, CD36 and NPC1L1 function as authentic transporters that directly mediate lipid absorption, or whether they act to facilitate intracellular lipid trafficking or to modify signaling processes that mediate lipid absorption, or both, remains contentious, but nonetheless all merit examination here. Recent reports also suggest the involvement of SR-BI, CD36 and NPC1L1 in the absorption of fat-soluble nutrients (such as carotenoids, vitamin D and vitamin E), providing further support for a role in drug absorption. In the current study, consistent with previous reports in vivo, inhibition of NPC1L1 reduced cholesterol absorption (Figure 2B), and inhibition of CD36 reduced (albeit nonsignificantly) the absorption of oleic acid (Figure 2C). In contrast, inhibition of SR-BI and CD36 had little impact on cholesterol absorption (Figure 2B). Inhibition of SR-BI and CD36 was expected to reduce cholesterol absorption based on previous in vitro studies. However, in vivo evidence of a role of SR-BI and CD36 in cholesterol absorption is less clear and, for example, no significant differences in intestinal cholesterol absorption were reported in SR-BI knockout vs wild-type mice. The lack of effect of SR-BI and CD36 inhibition on cholesterol absorption in the current study therefore runs contrary to previous in vitro studies but is in agreement with some previous in vivo data. Inhibition of endocytosis pathways via administration of monensin also failed to reduce cholesterol and cinnarizine absorption. Rather, the volume of distribution and clearance of cholesterol and cinnarizine appeared to be reduced, resulting in increases in plasma exposure. Since exogenously dosed 14C-cholesterol and cinnarizine are likely to be present within lipoproteins in the systemic circulation, the changes in systemic disposition of 14C-cholesterol and cinnarizine in the monensin-treated rats may reflect inhibition of receptor-mediated endocytosis of LDL.

Inhibition of SR-BI, CD36 and NPC1L1 did not result in significant changes to the systemic exposure of cinnarizine following intraduodenal infusion of a lipid emulsion formulation in rats (Figure 2A). The data suggest that while lipid colloidal phases may be capable of direct interaction with lipid receptors (as suggested previously), the transfer of solubilized content into absorptive cells is likely selective and more applicable to solubilized lipids and nutrients rather than drugs. Together with the data describing cinnarizine absorption from micelles and vesicles, the results indicate that cinnarizine absorption from lipid colloidal phases is largely independent of the physical nature of the infused colloid (realizing that in these first experiments cinnarizine was present at concentration well below the solubilization limit and therefore under conditions where solubility/precipitation-mediated events were avoided), is not influenced significantly by common lipid uptake receptors and, instead, appears to be primarily dependent on the free drug concentration in equilibrium with the solubilized reservoir.

Subsequent studies addressed the possibility that bile secretion may enhance drug absorption via the induction of supersaturation during the intestinal processing of dietary or formulation-related lipids. The addition of bile to cinnarizine-loaded micelles led to sustained drug supersaturation that ultimately led to increased intestinal drug absorption and systemic drug exposure. In contrast, although addition of bile to cinnarizine-loaded vesicles also led to supersaturation, the metastable supersaturated state was less stable than that generated by bile addition to micelles, resulting in more rapid precipitation of solubilized drug and therefore a lack of increase in drug absorption and systemic drug exposure. The data indicate that bile-mediated dilution of lipid colloidal phases may represent an endogenous mechanism of supersaturation generation during lipid processing in the small intestine; and that the transient increase in thermodynamic activity may lead to enhanced drug absorption. The observations also highlight both the potential for supersaturation to enhance drug absorption and the need to achieve an optimal balance between drug supersaturation and drug precipitation.

Interaction of intestinal colloidal phases with bile leads to dilution, an increase in pH and an increase in the concentrations of bile components (bile salts, phosphatidylcholine and cholesterol) associated with the colloidal species. For a solubilized system above the critical micellar concentration (CMC), simple 1:1 v/v dilution is expected to reduce the solubilized concentration, but to maintain total solubilization capacity (i.e., for the drug concentration to drop by 50% but the volume to double and therefore for solubilization capacity to remain unchanged). However, the data in Table 4 show that dilution of the micelles or vesicles with bile or buffer results in a drop in total solubilization capacity to only 7–41% of initial. Greater proportional decreases were apparent for dilution of vesicles (in all cases when compared to micelles), and after dilution with bile rather than buffer (Table 4). The loss of solubilization capacity on dilution suggests the likelihood of a phase transition to structures with reduced solubilization capacity. Although a complete explanation for these phase transitions is not apparent at this time, for the micellar systems, it may be related to the ability of ionized caprylic acid to self-associate and form fatty acid micelles at high lipid saturation. Thus, dilution of medium-chain colloidal phases may reduce the concentration of caprylic acid below the CMC, leading to a loss of cinnarizine solubilization capacity in the micellar phase. For vesicles, previous studies have suggested that increasing bile concentrations facilitate a vesicular to micellar transition. Since micelles are expected to have lower solubilization capacities for lipophilic drugs than vesicles (micelles are smaller and less lipid-rich), a 90% reduction in cinnarizine solubilization capacity might therefore be anticipated when bile is added to model vesicles. In the case of the micelles, therefore, it seems likely that the addition of bile disrupted the structure of swollen, mixed micelles leading to lower colloidal lipid content and lower solubilization capacity. Unfortunately, attempts to quantify changes to particle size on bile addition were unsuccessful due to high polydispersity. However, the broad trends observed were consistent with the suggestions above and decreases in particle size were apparent for vesicles (consistent with initiation of a vesicle to micelle transition) and increases in particle size for the mixed micelles (consistent with micellar destabilization and transformation to less dispersed structures) (data not shown). An additional complexity in these dilution studies was the realization that the pH of bile (pH 8.02) is higher than that normally employed for simulated intestinal fluids, and therefore incubation of the colloidal phases (prepared at pH 6.30) with bile increases system pH. Cinnarizine is a weak base with a pKa of 7.47 and is therefore expected to be less ionized, less soluble and potentially more permeable at higher pHs. The
The dual role of bile during lipid digestion and dispersion. (i) Bile-mediated solubilization of lipid digestion products at the interface of a digesting oil droplet results in the generation of lipid colloidal phases such as vesicles and micelles that maintain drug solubilization in the small intestine. (ii) The continuing interaction of secreted bile with existing lipid colloidal phases in the lumen results in progressively less lipid-rich phases with lowered solubilization capacity. Thus, ongoing bile-mediated dilution of lipid colloidal phases promotes drug supersaturation and enhances drug absorption by increasing drug thermodynamic activity in colloidal phases. The combination of the two highly kinetic, bile-mediated events affords a means to simultaneously increase solubilization capacity and promote thermodynamic activity of coadministered drug in the small intestine, and may contribute to the increase in drug absorption often observed with lipid coadministration. 

While dilution of micelles and vesicles with bile resulted in decreases in drug solubilization capacity, cinnarizine did not precipitate immediately. A period of drug supersaturation was evident for both micellar and vesicular systems, although drug precipitation from vesicles was much more rapid than from micelles. This in turn translated into increases in drug absorption (Figure 3A) and systemic exposure (Figure 4A) for the micellar systems. The difference in the capacity of micelles and vesicles to maintain drug supersaturation (Figure 6) may be explained by the difference in the degree of drug supersaturation induced by dilution with bile. The degree of supersaturation is described by the supersaturation ratio, which is the ratio of the (supersaturated) concentration of drug in solution relative to the equilibrium solubility of the drug in the same system. Crystallization theory suggests that the thermodynamic drivers of precipitation from supersaturated solutions increase with increasing supersaturation ratios, as the likelihood of nucleation and crystal growth increases with increases in (metastable) drug concentration in solution. Here, 1:1 v/v addition of bile resulted in the attainment of cinnarizine supersaturation ratios of 6 and 12, in micelles and vesicles, respectively. Therefore, the faster rate of drug precipitation from vesicles (when compared to micelles) may be explained by the higher supersaturation ratio induced by bile addition. It is also possible that micellar structures are more effective in stabilizing supersaturation when compared to vesicular structures, although this has not been examined explicitly here. Notably, cinnarizine was found to precipitate in the crystalline form in these experiments (Figure S3 in the Supporting Information), precluding the possibility that the enhanced cinnarizine absorption from micelles (when bile was copерfused) observed in Figure 3A was due to accelerated cinnarizine dissolution from precipitated amorphous forms.

The role of bile in enhancing drug absorption from LBF has been reported previously. In the majority of cases, bile-mediated bioavailability enhancement has been suggested to stem from the ability of bile to expand the solubilization reservoir for PWS in the GI tract. This is typically assumed to occur via PWS solubilization in simple bile micelles, or via the ability of bile to solubilize lipid digestion products and to generate more complex lipid colloidal phases with enhanced solubilization capacities. It seems likely that the ability to solubilize lipid digestion products and to promote colloidal formation remains an integral part of the role of bile in supporting drug (and lipid) absorption. However, the data described here suggest that continued dilution of lipid colloidal phases with bile in the small intestinal lumen may also lead to physical changes that promote drug supersaturation, and ultimately promote drug absorption. In doing so, supersaturation induction may be a means by which the decrease in thermodynamic activity inherent in solubilization is reversed, such that the free concentration of drug available for absorption is maximized. Thus, a dual role of bile in facilitating drug absorption from LBF may be conceived (see Figure 8). First, bile-mediated solubilization of lipid digestion products at the interface of a digesting lipid droplet results in the generation of lipid colloidal phases such as vesicles and micelles that promote drug solubilization during lipid digestion. Second, continued bile-mediated dilution of existing lipid colloidal phases promotes drug supersaturation, and enhances drug absorption by significantly increasing drug thermodynamic activity in colloidal phases. The combination of these two highly kinetic events likely contributes to the effective drug absorption observed with lipid coadministration, as it affords a means to simultaneously increase solubilization capacity and promote thermodynamic activity of coadministered PWS in the small intestine.

Supersaturation induction via interaction with bile also provides a means of overcoming the recently described solubility—permeability interplay observed in studies where...
PWSĐ are coadministered with cosolvents, cyclodextrins or surfactant systems. In these studies the authors describe the reduction in thermodynamic activity common to most solubilization technologies and show that this offsets the potential increases in membrane flux that might be expected by an increase in solubilized drug concentration. More recent studies by the same authors have shown that this solubility–permeability interplay can be addressed via the use of amorphous solid dispersion formulations that stimulate supersaturation, but do not promote solubilization. Here we report that essentially similar outcomes are also possible with solubilizing formulations, when the solubilizing formulations contain lipids and when the kinetic changes that occur in the GI lumen in the presence of bile secretion promote supersaturation. The current data therefore suggest that endogenous lipid processing pathways provide an exquisitely sensitive and triggerable supersaturation mechanism that allows drug to remain in a solubilized state during initial lipid digestion and at high lipid:bile concentration ratios, but that ongoing bile secretion subsequently provides a boost to thermodynamic activity and in doing so supports enhanced absorption. This is in contrast to other common solubilization strategies that may not interact with the dynamic GI environment, or for which interaction with bile typically reduces thermodynamic activity by increasing solubilization capacity. Conversely, the ability of LBF to promote drug solubilization until drug supersaturation is triggered in the small intestine may confer an advantage over formulation approaches that only utilize supersaturating strategies, as the risk of drug precipitation in the GI tract may be reduced by solubilization within lipid colloidal phases.

**CONCLUSION**

Improved understanding of the mechanism of drug absorption from lipid colloidal phases such as micelles and vesicles is required to provide a platform for more rational design of lipid-based formulations. Using medium-chain lipids, we have demonstrated that the absorption of cinnarizine (a lipophilic, poorly water-soluble drug) may be enhanced when drug supersaturation is generated during bile-mediated dilution of lipid colloidal phases. Previous studies suggest that a similar induction of supersaturation may occur as a result of initiation of digestion of some microemulsion-based LBFs. These observations indicate that supersaturation and its associated benefits in enhancing drug absorption may occur intrinsically during LBF incorporation into endogenous lipid processing pathways in the small intestine. Future work will be directed toward assessing the impact of bile dilution on supersaturation tendency, and thus absorption, for an extended range of PWSĐ and in a series of different micellar and vesicular colloidal systems.

**ASSOCIATED CONTENT**

5 Supporting Information

Table S1 showing pharmacokinetic parameters for cinnarizine after intravenous administration in rats. Figure S1 showing systemic plasma concentration–time profiles of cinnarizine and 14C-labeled cholesterol following intravenous infusion in rats. Figure S2 showing perfusate disappearance profiles of cinnarizine when micelles were perfused through an isolated rat jejunal segment, with and without 1:1 v/v coperfusion with rat bile pH 6.30 or buffer pH 6.30. Figure S3 showing polarized light microscopy images of the crystalline cinnarizine precipitate following precipitation kinetics experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ACKNOWLEDGMENTS**

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**ABBREVIATIONS USED**

LBF, lipid-based formulations; PWSĐ, poorly water-soluble drugs; GI, gastrointestinal; LPC, L-α-lysophosphatidylcholine; CD36, cluster of differentiation 36; SR-BI, scavenger receptor class B type 1; NPC1L1, Niemann-Pick C1 like 1; BLT-1, block lipid transport-1; SSO, sulfo-N-succinimidyl oleate; iv, intravenous; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; TBME, tert-butyl methyl ether; SEIF, simulated endogenous intestinal fluid; SEM, standard error of the mean; CMC, critical micellar concentration; UWL, unstirred water layer; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CIN, cinnarizine; Ch, cholesterol; OA, oleic acid; SS ratio, supersaturation ratio.
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