In-vitro evaluation of factors that affect the performance of lipid-based drug delivery systems

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by

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Notice 1

Dedicated to my –

Amma, Amamma, and Thathayya

Wife, *Vidhu*, Daughter, *Vyshu*, and Son, *Vybhu*

I. Table of Contents

II. Abstract

Development of formulations containing poorly water-soluble drugs (PWSDS) incorporated in lipid-based drug delivery systems (LBDDS) poses a great challenge to scientists (both, at academia and industry) across the globe. To date there are no standard in vitro protocols for formulation scientists which predict their performance in-vivo. This thesis addresses various key issues that are important in development of LBDDS. Chapter 1 sets the work in context by reviewing the published literature. Work carried out in Chapter 2: addressed the issue of "non-completion of lipolysis" as this has been attributed by many authors to be one factor that limits the use of in vitro digestion tests, and limits the degree of *in vitro- in vivo* correlation (IVIVC) that can be achieved. This study has investigated the effect of increasing calcium and bile salt (BS) concentration on the *in vitro* digestion of a long-chain triglyceride (soybean oil) in order to understand how these factors will affect the solubility of poorly water-soluble drugs delivered in lipid vehicles. The solubility of two model poorly-water soluble drugs (fenofibrate and Danazol) in the aqueous phase digests obtained via digestion of a long-chain triglyceride, LCT (soybean oil), increased significantly in each of the conditions (fasting and fed), by comparison with respective controls, irrespective of the molar concentration of calcium employed in the media. Systems containing 40 mM calcium concentration (high levels of calcium used in the study) when compared to that containing 5 mM calcium, had a lower capacity for solubilization of either drug in aqueous phases after digestion, in both fed and fasting conditions. This was thought to be attributed to the formation of large amount of insoluble calcium soaps which were observed (as a precipitate) during our experiments. Formation of calcium soaps has been reported elsewhere (MacGregor et al., 1997, Hu et al., 2010, Zangenberg et al., 2001a). Soap formation may

occur upon an interaction of calcium with the bile salt component of the solubilized species (Fatouros et al., 2009) when calcium is in excess. In conclusion, from our data, although high calcium concentration may prove beneficial with respect to bringing the lipolysis to completion, the addition of calcium ions should be conducted with caution because it interferes with the solubilisation of poorly water soluble drugs. Therefore, it can be anticpated that high concentrations of calcium in the system during in vitro lipolysis will result in a poor model for correlation in vivo.

A second focus of this thesis is discussed in Chapter 3: describes an investigation of a series of closely related SEDDS *viz.* Type II and Type IIIA as defined by the Lipid Formulation Classification System (LFCS) (Pouton, 2006b, Pouton, 2000b), all of which contained fenofibrate as model drug. A variety of factors influencing the performance of these systems during *in vitro* dispersion and digestion tests were studied. The results were interpreted based on the level/extent of supersaturation attained during these *in vitro* processes to gain an insight into formulation performance and to establish guidelines for formulators. Emphasis was placed on the effects of *lipid composition (long-chain vs. medium-chain)* and the *surfactant type* (*hydrophilic vs. lipophilic)* on the solubilization properties of these formulations during dispersion and digestion.

Despite generating diverse formulations by altering the nature of oils and blends of oils which made up the lipid component, the dispersion results showed that Type II formulations (containing Tween 85, a lipophilic surfactant) always supported drug in solubilized form (100%) for at least 4 days (in the absence of digestion), Type III formulations on the other hand were unable to maintain all of the drug in solubilized form on dispersion, though they maintained greater than 70% of drug in solubilized form for 4 days. Most of the loss of drug

in the form of precipitate occurred after the initial 4 hours.. The degree of supersaturation generated during dispersion was estimated by determining the solubility of fenofibrate in dispersed formulations. Type III formulations were supersaturated and drug was maintained in this meta-stable state for up to 4 hours and after which drug was lost to some extent in the form of precipitate. Type II systems were not supersaturated. Considering the transit time of all the formulations in the intestine was expected to be 3-4 hours, clearly Type II and Type IIIA formulations, prior to digestion, met the primary performance requirement for drugs meant to be administered orally. After dynamic digestion studies, the ability of each of these formulations (Type II and Type IIIA) to maintain drug in a solubilized state was highly dependent on both, the lipid composition and the choice of surfactant. For example, medium-chain lipids exhibited very good solubilizing properties in the dispersed state, but resulted in a higher degree of supersaturation on digestion, leading to higher susceptibility to drug precipitation. Results from the digestion studies showed that replacing long-chain lipids with medium-chain lipids in Type II and IIIA LBDDS is likely to promote supersaturation on digestion. Utilization of long-chain instead of medium-chain triglycerides in LBDDS prevents the development of sudden and higher degrees of supersaturation and consequently reduces the risk of precipitation (Kossena et al., 2003a). The present digestion studies in Chapter 3: have indicated that this approach alone will not work for all drugs. For fenofibrate, various other strategies needs to be explored to prevent drug precipitation from formulations, such as lowering the drug load (Williams et al., 2012a), employing polymer-based precipitation inhibitors (Anby et al., 2012c), and/or by the careful selection of surfactants (Cuine et al., 2008a). Without careful consideration of drug loading and choice of surfactant in Type II/IIIA medium-chain lipid formulations, there is a high risk of precipitation of drug in the intestine.

Critical to the utility of self-emulsifying drug delivery systems (SEDDS) in oral bioavailability enhancement is a capacity to both generate and maintain supersaturation following dispersion and digestion processes in the gastro-intestinal tract. Studies carried out in Chapter 4: investigated the effect of drug-type and drug loading on supersaturation in digested SEDDS consisting of long-chain lipids and a range of chemically diverse nonionic surfactants. Supersaturation is described in terms of the maximum supersaturation ratio (SR^M) attained on initiation of digestion. Calculated from the maximum attainable concentration in the test (a function of drug loading) and the drug solubility in the colloidal phases formed by digestion of the SEDDS, SR^M defines the maximum supersaturation pressure in the digestion experiment and proves to be a remarkable indicator of performance across a range of formulations. SEDDS containing danazol showed little evidence of precipitation on digestion, even at drug loads approaching saturation in the formulation. In contrast, fenofibrate extensively crystallized on digestion of the same SEDDS. The performance differential of danazol and fenofibrate-containing SEDDS however could be rationalized by the much higher SR^M values generated by fenofibrate. And on further analysis of formulations containing various fenofibrate loads, a threshold SR^M of ~2.6 was identified in 6 of the 7 SEDDS above which supersaturation could not be maintained. Near this threshold, performance became increasingly variable and most sensitive to surfactant-type, though overall, the SR^M attained on digestion was most predictive of performance.

III. Statement of originality

In accordance with Doctorate Regulation 17 of Monash University, the following declaration is made:

I hereby declare that the work presented in this thesis is a genuine work carried out by me at Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia and not previously submitted for acceptance for a degree or diploma at this or any other University. Also, this thesis contains work which was not carried out previously by any person and any such relevant work was duly acknowledged.

Ravi Devraj

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V. Publications

This thesis is a compilation of the following manuscripts:

Chapter 2: : DEVRAJ, R., WILLIAMS, H. D., WARREN, D. B., MULLERTZ, A., PORTER, C. J. & POUTON, C. W. (2013). In vitro digestion testing of lipid-based delivery systems: calcium ions combine with fatty acids liberated from triglyceride rich lipid solutions to form soaps and reduce the solubilization capacity of colloidal digestion products. *International Journal of Pharmaceutics,* 441(1-2)**,** 323-33.

Chapter 3: : DEVRAJ, R., WILLIAMS, H. D., WARREN, D. B., MOHSIN, K., PORTER, C. J. & POUTON, C. W. (2013). In vitro assessment of drug-free and fenofibrate-containing lipid formulations using dispersion and digestion testing gives detailed insights into the likely fate of formulations in the intestine. *European Journal of Pharmaceutical Sciences,* 49(4): 748-760.

Chapter 4: : DEVRAJ, R.,WILLIAMS, H. D., WARREN, D. B., PORTER, C. J. & POUTON, C. W. (2014). Choice of nonionic surfactant used to formulate Type IIIA self-emulsifying drug delivery systems and the physicochemical properties of the drug have a pronounced influence on the degree of drug supersaturation that develops during *in vitro* digestion

Journal of Pharmaceutical Sciences, 103(4):1050-63.

VI. List of abbreviations

AP	Aqueous phase
BS	Bile salt
DG(s)	Diglyceride(s)
FA	Fatty acid
FaSSIF	Fasted state simulated intestinal fluid
FeSSIF	Fed state simulated intestinal fluid
GI	Gastrointestinal
GIT	Gastrointestinal tract
IMW988	Imwitor 988
LCM	Long chain monoglyceride
LCT	Long chain triglyceride
LFCS	Lipid formulation classification system
LBDDS	Lipid-based drug delivery systems
MSE	Maisine 35-1
MCM	Medium chain monoglyceride
MCT	Medium chain triglyceride

MG(s)	Monoglyceride(s)
MIG812	Miglyol 812
NaTDC	Sodium taurodeoxycholate
PC	Phosphatidylcholine
PL	Phospholipid
PWSDs	Poorly water-soluble drugs
SEDDS	Self-emulsifying drug delivery systems
SMEDDS	Self-microemulsifying drug delivery systems
SR	Supersaturation ratio
SR^M	Maximum supersaturation ratio
TBU	Tributyrin units
T85	Tween 85
T80	Tween 80
TG(s)	Triglyceride(s)

Chapter 1: General Introduction

1.1 Background

Administration of drugs via the oral route is the preferred delivery route as it results in a high degree of patient compliance. Despite this major advantage, there are several potential limitations that adversely affect the drug's bioavailability including; appropriate stability and solubility in the gastro-intestinal (GI) fluids, poor intestinal permeability, and metabolism within the enterocyte and liver. A significant proportion of new pharmacologically active chemical entities (40-70%) emerging from contemporary drug discovery campaigns are poorly water-soluble, often with a high degree of lipophilicity (Robinson, 1996). According to recent estimates, the prevalence of poorly water soluble drugs is not expected to change in the near future (Stegemann et al., 2007). This low solubility presents a major barrier to oral drug delivery due to high variability in the rate and extent of GI absorption, and hence the therapeutic efficacy (Bowtle, 2007, Gursoy and Benita, 2004). Additionally, poor solubility is associated with high intra- and inter-subject variability, potential reduced clinical efficacy and lack of dose proportionality are all the potential problems associated with this poor aqueous solubility (Tang et al., 2007).

The strategies available to overcome problems caused by the low solubility and slow dissolution rate of poorly water soluble drugs (PWSDs) are modification of the drug molecule or use of specialized formulations. Modifications often aim to produce structural analogues or alternative salts (Merisko-Liversidge and Liversidge, 2008) with higher aqueous solubility. Traditional formulation strategies for solid dosage forms attempt to increase the surface area, solubility and wettability of the powder particles. These powder methods typically focus on particle size reduction, classically performed by milling, or generation of amorphous states (Hancock and Zografi, 1997, Grau et al., 2000). The increase in bioavailability after

micronization of drugs is well known and the technique has been applied to a variety of PWSDs , for examples see the following references (Liversidge and Cundy, 1995, Munoz et al., 1994, Hargrove et al., 1989, Englund and Johansson, 1981, Jounela et al., 1975, Atkinson et al., 1962, Kraml et al., 1962). Another popular strategy to enhance the oral bioavailability of these compounds has been the utilization of lipid-based formulations (Constantinides, 1995, Dahan and Hoffman, 2006b, Hauss, 2007b, Gursoy and Benita, 2004). Several individual groups previously noted that the co-administration with a diet rich in fat resulted in improved absorption and bioavailability (Crounse, 1961, Charman et al., 1993, Charman et al., 1997, Humberstone et al., 1996, Welling, 1996, Sunesen et al., 2005).

Lipids have the capability to improve absorption of co-administered PWSDs by enhancing drug solubility resulting from an increased solubilization and dissolution in the GIT fluids (Humberstone and Charman, 1997, Gershanik and Benita, 2000, Pouton, 2006b, Porter et al., 2007a).

Several authors have discussed and reviewed these concepts previously (Armstrong and James, 1980, Constantinides, 1995, Humberstone and Charman, 1997, Pouton, 1997), focusing on the use of lipid formulations to enhance the oral bioavailability of PWSDs in the form of either solutions, suspensions, and emulsions. Recently, there has been an increased focus on the utility of self-emulsifying drug delivery systems (SEDDS) and the various strategies for formulation of self-emulsifying systems and their mechanisms of action have been reviewed (Gershanik and Benita, 2000, Gursoy and Benita, 2004).

1.2 Lipid formulation classification system (LFCS)

The lipid formulation classification system (LFCS) was first proposed by Pouton (Pouton, 2000b) and subsequently modified to include Type IV systems (Pouton, 2006b). The LFCS provides a framework for comparing formulations based on the components making up the formulation, which in practice include a wide variety of different excipients. Briefly, Type I formulations are oils (TGs or mixed MG and DGs) which do not disperse and require digestion to facilitate dispersion. Type II formulations generally consist of oils and water-insoluble ester ethoxylates (nonionic surfactants with HLB, hydrophobic-lipophilic balance, values of approximately 11), are self-emulsifying and produce relatively coarse emulsions in the 0.25–2 mm range (Gershanik and Benita, 2000). Type III formulations include water-soluble components, produce micro-emulsions (<100 nm, optically clear), and can be subdivided into Type IIIA and IIIB based on the proportion of water-soluble surfactants and co-solvents used. Type IIIB typically has a greater proportion of water-soluble components than Type IIIA. Type IV formulations do not contain any lipid and consist of a mixture of hydrophilic surfactants and co-solvents.

SEDDS are typically Type II formulations and are isotropic mixtures of natural or synthetic oils with lipophilic or hydrophilic surfactants and co-solvents and which spontaneously emulsify when exposed to the GI tract to form oil-in-water emulsions or micro-emulsions (Pouton, 1997, Pouton, 2006b, Gershanik and Benita, 2000, Gursoy and Benita, 2004, Constantinides, 1995). Apart from their characteristic ability to undergo spontaneous emulsification, or at least very low energy emulsification, SEDDS have an added advantage of being better solvents compared to lipid solutions (with pure TG's) for drugs with intermediate partition coefficient (2<logP<4) due to the fact that SEDDS are typically low in

natural lipids and much greater in amphiphilic surfactants, co-surfactants, and co-solvents (Pouton, 2000b). Improvements in the rate and extent of absorption, and eventually bioavailability, produced by SEDDS are thought to be due to generation of a very high surface area from rapid emulsification under conditions of mild agitation and in the presence of GI fluids. It is acknowledged that the digestion of formulation components may reduce the particle size further. Despite these advantages, relatively few SEDDS formulations have been commercialized [current commercial products include Neoral® (cyclosporine), Norvir® (ritonavir), Fortovase® (saquinavir), and Agenerase® (amprenavir)] (Constantinides, 1995, Pouton, 2000b, Strickley, 2004), perhaps a result of the limited literature describing the performance of SEDDS. In the recent past, *in vitro* dissolution tests and *in vitro* digestion tests that are more reflective of the GI environment have been developed in order to better predict the *in vivo* performance of SEDDS formulations of PWSDs (Porter and Charman, 2001b, Porter and Charman, 2001a, Pouton, 2006b).

Despite having an immense choice in the selection of lipid formulation components, composition and the major advantages mentioned above, the application of SEDDS has been limited due to the lack of an appropriate *in vitro* formulation assessment tool for the prediction of *in vivo* performance. Unfortunately, the current development strategies in the area of lipid-based drug delivery systems LBDDS are mostly empirical, demand a large number of animal experiments, and consume significant amounts of time and money. Hence, a simplified *in vitro* tool to assess the *in vivo* performance of these systems is urgently required. The widespread use of lipid digestion models for *in vitro* evaluation of lipid-based systems has commenced only after the realization that the performance of these systems is affected by digestion and the incorporation of formulation-derived digestion products into endogenous micellar species (Porter and Charman, 2001b, Porter and Charman, 2001a, Porter

et al., 2004b, Porter et al., 2004a, Christensen et al., 2004b, Dahan and Hoffman, 2006b, Reymond and Sucker, 1988, Reymond et al., 1988).

The rate of drug partitioning between the dispersed oil droplets and the aqueous intestinal fluids is dictated by a number of factors including the solubility of the drug in mixed bile salt-lipid micelles, which acts as a reservoir for drug absorption, and the processes determining the formation of these species from the digested dispersed oil droplets. The first requirement of a lipid formulation for increasing the absorption of drugs is to achieve a stable and uniform dispersion, and this can be achieved by self-emulsification or by digestion. Drugs formulated in oils (medium-chain and long-chain) alone e.g., Type I formulations show poor-dispersion characteristics in water but can result in colloidal dispersions in the intestine as a result of good and complete digestibility. In contrast, a poor dispersion which has limited digestibility (e.g., any formulation falling under Type II, Type III, and Type IV) may result in a poor outcome. The Cyclosporine A formulation originally available in the market by the name Sandimmune was reported to have poor dispersion characteristics showing high inter and intra-subject variability in absorption (Ptachcinsky et al., 1986). An alternative to the Sandimmune formulation in the market is the Sandimmune Neoral, a stable microemulsion self-emulsifying formulation. Instant and homogenous dispersion of this formulation in the GI fluids to produce stable micro-emulsion droplets leads to drug partitioning into the aqueous intestinal fluids consistently thereby reducing the inter- and intra-individual variability *in-vivo* (Kovarik et al., 1994). Therefore, a widely used approach is to formulate SEDDS using surfactants such that a homogeneous submicron dispersion is formed, often using materials that are digestible, thereby ensuring that the drug is not trapped in coarse particles. Inclusion of surfactants with oils to produce SEDDS may also increase the solvent capacity of the

formulation when it disperses in the GI fluids (Pouton, 1997) but the fate of drug after digestion also needs to be considered.

The design of self-emulsifying formulations therefore demands adequate solubility of the candidate drug in the formulation and a rigorous *in-vitro* assessment of: a) the dispersion characteristics (ease of dispersion, particle size of the resulting emulsion droplets) of the formulations (Charman et al., 1992, Shah et al., 1994, Hauss et al., 1998, Kim and Ku, 2000, Kim et al., 2000, Kommuru et al., 2001, Kim et al., 2001, Kang et al., 2004, Li et al., 2005, Hong et al., 2006) and b) the impact of lipid digestion on the solubilization capacity of lipid-based formulation to accurately explain the *in-vivo* performance (Reymond and Sucker, 1988, MacGregor et al., 1997, Porter and Charman, 2001a, Zangenberg et al., 2001b, Ljusberg-Wahren et al., 2005, Dahan and Hoffman, 2006b).

1.3 Intra-luminal enzymatic digestion of lipids

Triglyceride (TG) digestion is usually initiated in the stomach by acid-stable lipases (Cohen et al., 1971) i.e. gastric and lingual lipases and was observed previously to be dependent on the species (rat, dog, human, etc.). Lingual lipase secreted by the salivary glands has an optimum pH of 4 (although active up to pH 6-6.5) and preferentially hydrolyzes the TG in the *sn*-3 position (Carey et al., 1983, Phan and Tso, 2001). Gastric lipase operates within an optimal pH range of 4-6 (Hamosh et al., 1981, Abrams et al., 1988) and acts at the oil/water interface of digesting lipid droplets to preferentially hydrolyze the *sn-3* ester bonds of TG (Staggers et al., 1981, Tiruppathi and Balasubramanian, 1982). Gastric lipase is capable of hydrolyzing TG in the absence and presence of phospholipid (PL), however, it is inhibited by the presence of long chain fatty acids (FA), which exists principally as the protonated form at low gastric pH (Bernback et al., 1989). Gastric lipase activity is also significantly reduced in the presence of bile salt (BS) under normal gastric transit conditions. The extent of gastric lipolysis of long chain triglycerides (LCT) is approximately 6.6 – 16.1% with the principal products being diglyceride (DG) and FA (Armand et al., 1996). Whilst the extent of *in vivo* medium chain triglycerides (MCT, esterified FA of 6 to 12 carbons in length) gastric digestion has not been reported, it is likely to be relatively more efficient compared with LCT.

Although the intestinal digestion of intact MCT has been shown to be possible in the absence of lipase *in vitro* (Chow et al., 1990), both LCT and MCT are typically hydrolyzed prior to absorption *in vivo.* In general, hydrolysis of TG by lipase proceeds as a two-step reaction; firstly, hydrolysis of TG yields a single FA and DG, followed by the DG being further hydrolyzed to produce a second FA and the corresponding 2-monoglyceride (2-MG).

Although 2-MG may undergo slow isomerisation to the relatively less lipase-stable, 1-MG, thereby allowing further hydrolysis to yield a third FA and glycerol, this process is usually limited *in vivo* (Mattson and Volpenhein, 1964).

As a whole, efficient emulsification of dietary fats by the mechanical activity of the stomach, the presence of dietary PL, proteins and polysaccharides and the amphipathic products of partial TG lipolysis result in the production of emulsified lipid droplets (1 – 100 µm in size) being delivered into the duodenum (Armand et al., 1996, Shiau, 1987). Pancreatic juice (containing digestive enzymes such as lipase, proteases, glycosidase and nucleases) and bile (containing BS, PL and cholesterol) secreted into the duodenum from the gallbladder subsequently promote further lipid digestion. The enzymes present in pancreatic juice responsible for lipid digestion in the duodenum include carboxyl esterase, phospholipase A_2 and pancreatic lipase and its cofactor, colipase (Shiau, 1987). Carboxyl esterase requires activation by BS and can hydrolyze both water-soluble and water-insoluble esters, such as cholesterol oleate, triolein, lyso-phospholipids and 2-MG. Phospholipase A_2 requires activation by BS and the presence of calcium ions and is responsible for hydrolyzing the ester bond at the *sn*-2 position of PL to produce a lyso-phospholipid and a FA (Shiau, 1987). Carboxyl ester hydrolase (CEH) and pancreatic-lipase related protein 2 (PLRP2) are mainly responsible for digestion of formulation surfactants (Bakala N'Goma et al., 2012b, Borgstrom, 1993b, Carriere et al., 1993). Pancreatic lipase and colipase however, are responsible for the majority of TG hydrolysis in the duodenum (Armand et al., 1996, Carriere et al., 1993). The digestion products formed, together with the endogenous biliary lipids (BS, PL, and cholesterol), self assemble to form various colloidal structures (mixed micelles, uni-lamellar and multi-lamellar vesicles). The hydrophobic core of these colloidal structures provides a

reservoir for lipid solubilization and subsequent absorption. For a graphical representation of this process, see the Figure 1.1 below.

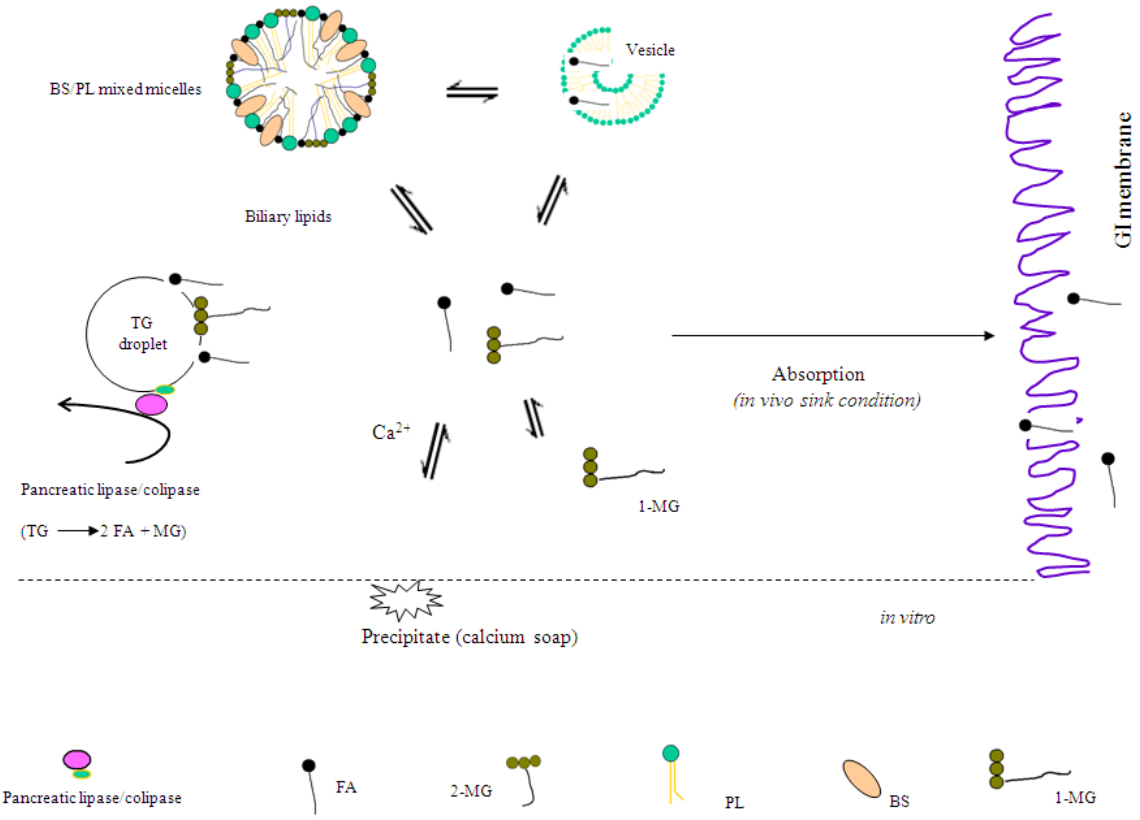


Figure 1.1: A schematic representation of the intraluminal processing of lipids and their absorption.

In a similar fashion, BS and biliary lipids may improve the rate of dissolution and/or aqueous solubility of PWSDs via enhanced wetting and/or solubilization within BS micelles and as a result, increase oral drug bioavailability (Bakatselou et al., 1991, Humberstone et al., 1996). Since dietary and formulation lipids share common physicochemical properties, improved drug solubilization following oral administration of a lipid vehicle (via intercalation into the various colloidal species formed during lipid digestion) has been recognized as a mechanism by which the bioavailability of PWSDs may be enhanced (Bates and Sequeria, 1975, Myers and Stella, 1992).

1.4 *In vitro* assessment of lipid based formulations

A number of studies have been published to date that provide an *in vitro* assessment of the performance of lipid based formulations. Drugs formulated within LBDDS partition from the lipid formulation into the aqueous intestinal environment, then subsequently into the colloidal solubilized intestinal species generated from the lipid digestion products, GI fluids and endogenous biliary lipids. This leads to enhanced solubilization and bioavailability (Carey and Small, 1970, Cistola et al., 1988, Hernell et al., 1990, Staggers et al., 1990, Armand et al., 1996, Kossena et al., 2003a). Hofmann and Borgstrom, 1964, from their work on human intestinal contents collected during fat digestion and absorption, were the first to report that a micellar phase containing BS, PL,FA, and a MG is present during fat digestion and absorption, and that the exogenous lipid is absorbed from the mixed micellar solution (Hofmann and Borgstroem, 1964). Mixed micelles are not absorbed intact (Hoffman, 1970, Simmonds, 1972) and lipid digestion products are thought to be absorbed from a monomolecular inter-micellar phase in equilibrium with the mixed micellar phase (Westergaard and Dietschy, 1976). Formation of multi-lamellar vesicles in the presence of BS

(Rigler et al., 1986) from the liquid crystalline phase formed at the surface of oil/water emulsion droplets was observed by studying the phase behavior of lipid digestion in the duodenum (Patton and Carey, 1979, Patton et al., 1985) and these vesicles were found to be present in equilibrium with BS mixed micelles in model *in vitro* systems and in aspirates of human post-prandial intestinal fluid (Staggers et al., 1990, Hernell et al., 1990). Fatouros et al, 2007a and 2007b, in their recent publications examined the phase changes occurring during lipid digestion of self-emulsifying formulations containing small quantities of lipids. These manuscripts describe the presence of micelles, uni-lamellar vesicles and lamellar and hexagonal liquid crystals during the digestion of the formulation in a model intestinal environment (Fatouros et al., 2007a, Fatouros et al., 2007b).

Influence of digestion of MCT and LCT on the solubilization of a range of PWSDs (griseofulvin, diazepam, danazol, cinnarizine, and halofantrine) using an *in vitro* model of lipid digestion was studied previously by Kaukonen et al. (2004). Drug solubility in the aqueous phase (AP) was greatly enhanced by the intercalation of the MCT and LCT digestion products into the BS/PL mixed micelles present in the simulated intestinal fluid. Solubilization behavior of co-administered drugs on digestion of simple TG solutions was largely dependent on the lipophilicity of the drug. Griseofulvin and diazepam, being less lipophilic drugs, readily partitioned into the AP of the digests when compared to more lipophilic drugs such as cinnarizine and halofantrine, which remained to a greater extent within the undigested oil phase.

Kossena et al. 2003 studied the impact of the apparent solubility of a PWSD as a function of the nature of the colloidal species formed during the digestion of LCT and MCT using griseofulvin, danazol and halofantrine (Kossena et al., 2003a).. The authors found that

mixed micelles, simple micelles and vesicles are formed during MCT digestion, whereas LCT digestion formed only vesicles and mixed micelles. In the LCT digestion, the mixed micellar phase was the predominant solubilizing species for griseofulvin, danazol, and halofantrine, (>70% of the total solubilization capacity) while for the increasingly lipophilic drugs tested, the vesicular phase contributed an increasing proportion of the solubilization capacity. This was attributed to a significant proportion of drug solubilized in the vesicular phase at high BS/PC levels where higher overall lipid load was evident. Kossena et al (2004) reported completely contrasting behavior of the colloidal species during MCT digestion, when both the vesicular phase and the mixed-micellar phase were less able to solubilize the digestion products. A further contrast between MCT and LCT-rich systems was observed at higher BS/PC levels for MCT systems, wherein the capacity of mixed micelles increased over the vesicles reflecting an increase in lipid solubilization by mixed micelles. Kossena et al (2004) concluded from their studies that the drug solubilization was found to be dependent on the lipophilicity of the drugs in LCT systems whereas no such correlation was evident for MCT systems.

The phase behavior of the digestion products of commonly used formulation lipids was characterized previously under model physiological conditions (Kossena et al., 2004). These authors also determined the solubility of a series of PWSDs (hydrocortisone and hydrocortisone esters) within each of these phases. The formulation lipids used were based on caprylic (C8) and lauric (C12) acids (representative of chain lengths commonly seen in medium chain lipids) and oleic acid (C18:1), commonly used in long-chain lipids). In accordance with expectations, when digestion products from formulation derived lipids interacted with intestinal fluids yielding colloidal species, decreasing the FA/MG concentrations resulting from dilution in a model intestinal fluid resulted in phase changes.

These ranged from liquid crystal (which occurs at the oil-water interface of digesting oil droplets) to the colloidal liquid containing mixed micelles and vesicles (located in the bulk of intestinal fluids present in close proximity to the absorptive membrane). At the high lipid concentrations expected to prevail on the surface of a digesting formulation droplet, a cubic liquid crystalline phase was formed by long-chain lipid systems and lamellar structures were obtained for medium-chain lipids. A higher and efficient solubilization capacity for a series of hydrocortisone esters was noted with long-chain lipids (due to the presence of cubic phase) than the capacity of the lamellar phase produced by medium-chain lipids. From this, it was suggested that the drug may readily undergo a precipitation upon dispersion of a TG formulation and the solubilizing capacity is likely to be inferior when medium-chain lipids are used.

Under dilute conditions (purportedly present at the vicinity of the absorptive surface in vivo), where a phase change from liquid crystalline to colloidal liquid phase occurs, the vesicular species persisted for the long-chain lipid containing systems for all the compounds tested, retaining the drug solubilization capacity.. However, this was not evident in the case of the medium-chain lipid systems. These observations may explain the higher systemic exposure of drugs often observed with lipidic formulations containing long-chain lipids when compared with corresponding medium-chain lipid formulations.

(Porter et al., 2004b) studied the effect of lipid load on solubilization of halofantrine using an *in vitro* lipid digestion model**.** Solubilization of halofantrine after *in vitro* lipid digestion of MCT was greatly affected by the mass of the lipid. Decreasing the lipid mass from 25 to 5 mg TG per milliliter of digestion medium resulted in reduced AP solubilization of halofantrine co-administered in MCT. This reduction in solubilization capacity with lower

lipid mass when compared to the *in vitro* data obtained at higher lipid mass (25mg TG/ml digestion medium), was found to correlate with the poor performance of the MCT systems *in vivo.* The authors attributed the lower solubilization capacity of digests of MCT to the rapid digestion of the dose form, relatively poor swelling of the BS/PL mixed micellar species by medium chain FA and MG, and the presence of lipid levels in the AP below that required to form vesicular species. In contrast to the MCT systems, when LCT systems were studied the solubilization capacity of AP for the drug after digestion did not vary as much when the lipid load in the digestion medium was varied between 25mg TG and 5 mg TG per milliliter of digestion medium. Indeed the proportion of the drug in the solubilized phase at the end of digestion period was actually higher at the lower lipid load. The authors suggested that this reflected incomplete digestion at high lipid load, leading to sequestration of halofantrine in undigested oil. This reduced drug partitioning into the AP at higher lipid loads. At lower LCT lipid mass, where digestion was almost complete, the reduced quantity of undigested lipid into which drug could partition led to a slightly higher proportion of drug precipitating than was evident at higher lipid loads. However, a significant proportion of the drug was still maintained in solution (substantially more than the corresponding MCT formulation), most likely resulting from the relatively effective swelling of the BS/PL micelles by the long chain FA and MG digestion products.

1.4.1 *In vitro-in-vivo* correlation studies

(Reymond and Sucker, 1988) studied the effect of lipid vehicles on the intestinal absorption of cyclosporine using an *in vitro* lipid digestion model based on the partition of drug from olive oil or Miglyol 812 (MIG812) into phases of simulated intestinal content. They hypothesized that there was a correlation between the amount of drug soluble in the aqueous phase (AP) with its absorption *in vivo* (Reymond et al., 1988). The *in vivo* absorption

was assessed by the mass of drug excreted in bile and urine. An *in vitro- in vivo* correlation (IVIVC) was not established for this data set. Despite higher solubility of the drug in the *in vitro* AP digest of MIG812, this vehicle did not result in better absorption *in vivo* compared to olive oil. The authors attributed this lack of IVIVC to the rapid lipolysis process, leading to variability in the extent of digestion and the static characteristics of phase quantification in their *in vitro* model. This is the first reported study in the literature wherein an *in-vivo* correlation was attempted from an *in-vitro* digestion model.

Difference in drug bioavailability after oral administration of a MCT, LCT, and a blend of MCT/LCT lipid solution formulation of halofantrine to dogs has been explained using an *in vitro* lipid digestion model (Porter et al., 2004b). Consistent with the *in vivo* data, where the bioavailability of halofantrine was greater after oral administration of the LCT solution than the MCT solution or a blended MCT/LCT solution, higher drug solubilization was observed on *in vitro* digestion of the LCT solution. A similar higher solubilization of LU 28-179 has been observed after *in vitro* digestion of a LCT-based formulation when compared to a MCT formulation (Christensen et al., 2004b). As observed previously, LCTs have been shown to increase solubilization in the GI environment more effectively than MCTs using *in vitro* lipid digestion models (Porter et al., 2004b, Sek et al., 2002a, Kossena et al., 2003a, Porter et al., 2004a). In contrast, higher solubilization of progesterone and vitamin D3 in the AP digests obtained on *in vitro* digestion of a MCT solution was reported when compared to a LCT solution (although digestion of LCT did not reach completion). These data correlated with an increase in drug bioavailability after oral administration of the MCT solution when compared to the LCT solution (Dahan and Hoffman, 2006b).

In-vitro digestion of three lipid-based formulations of danazol was carried out, and subsequently drug distribution was determined across various phases produced by digestion. The data was compared to the corresponding *in-vivo* data obtained following administration of formulations to fasted beagle dogs (Porter et al., 2004a). The formulations used in the study were: a LCT solution based on soybean oil, a medium-chain SMEDDS based on Captex (MCT) and Capmul (a blend of medium-chain mono-, di-, and tri-glycerides), and a long-chain SMEDDS based on soybean oil and Maisine 35-1 (a blend of long-chain mono-, di-, and tri-glycerides). The *in-vitro* results showed very high danazol aqueous phase concentrations (~90%) for a long-chain based SMEDDS formulation whereas in the corresponding medium-chain SMEDDS, the drug precipitated significantly during lipolysis and resulted in only 70% drug in the aqueous phase. The *in-vivo* results were in agreement and supported the *in-vitro* experiments, as the SMEDDS based on a LCT resulted in 5-fold increased AUC in comparison to the MCT based SMEDDS. The authors attributed the higher bioavailability from the LCT based SMEDDS to to the effective solubilization capacity of the long-chain mixed-micelles when compared to their medium-chain counterparts, as also observed previously (Kossena et al., 2003a).

Distribution of progesterone was studied across various phases produced from *in-vitro* lipolysis of LCT, MCT, and a SCT based lipidic systems. These formulations were then evaluated *in-vivo* (Dahan and Hoffman, 2006b). *In-vitro* results showed a performance rank order of MCT>LCT>SCT based on solubilization of drug in the aqueous phase following the lipolysis process. A positive *in-vitro-in-vivo* correlation was evident from their studies as the *in-vivo* results obtained from experiments on rats revealed the same rank order as the *in*-vitro studies. A high correlation (R^2>0.99) between the percent drug solubilized by aqueous phase

and the AUC values was noted by these authors following oral administration of the corresponding lipids (Dahan and Hoffman, 2006b).

Impact of proportion of lipid in a range of self-micro-emulsifying formulations on the solubilization capacity for danazol was studied during *in-vitro* digestion. A reduction in relative lipid content of formulations resulted in significant loss of solubilization capacity of the colloidal species formed post-digestion. In consensus to the *in-vitro* results, the *in-vivo* oral administration of the Danazol SMEDDS to beagle dogs revealed a decrease in oral bioavailability when the lipid quantity present in the delivery system was reduced (Cuine et al., 2007a).

1.4.2 Role of bile

Human bile comprises a mixture of different bile salts (Fausa, 1974). Since, the specific identity of the bile salt (BS) used has been shown to have no appreciable impact on the rate of lipolysis (MacGregor et al., 1997), a single synthetic BS, NaTDC was chosen as the model BS in all the present studies. Digestion media representing low-bile (fasting) or high-bile (fed) intestinal contents were simulated by employing 5 and 20 mM BS respectively in the present study. These concentrations were based on reported concentration found in vivo. BS concentrations of 5.9 ± 1.8 mM in fasting samples and 14.5 ± 9.4 mM (Fausa, 1974) 30 minutes after a test meal, were determined in duodenal contents (Armand et al., 1996). PL for the preparation of BS/PL mixed micelles was included at a molar ratio of 4:1, the ratio reported to be found in bile (Schersten, 1973). PL is an important endogenous amphiphilic compound found in bile and exerts a significant influence on the process of TG digestion (Patton and Carey, 1981, Alvarez and Stella, 1989). (Armand et al., 1996) have reported a PL concentration of approximately 5.75 mM in aspirated human duodenal contents. Since both

BS and PL are secreted in bile, the effect of various concentrations of bile representing low-bile (fasting) and high-bile (fed) conditions on the extent of digestion were examined in the current investigation, keeping the BS:PL molar ratio constant.

It was observed previously that the digestion of a MCT (MIG812) was found to reach completion during the digestion period (30min) under all the conditions, indicating a bile independent effect. In the absence of BS, the initial rate of hydrolysis was found to be slower than under fasting- and fed conditions although the extent of digestion after 30min was not significantly different (Sek et al., 2002a). The higher initial rate of hydrolysis observed in the case of fasting/fed conditions when compared to an absence of bile could be explained by efficient solubilization provided by mixed micelles for the digestion products. Interestingly, for medium chain oils, a very small difference in the initial rate and the extent of digestion was noted between fasting and fed states, implying that a minimal level of micellar solubilization is required for solubilizing the medium chain FAs.

In the case of a LCT (soybean oil), digestion was 'incomplete' even in the presence of bile and resulted in partial digestion under both the conditions representing fasting state and fed state, as 57.9±6% and 39.4±5% of triglyceride respectively was still present 30 min after digestion was initiated (Sek et al., 2002a). These authors also reported that the extent of digestion was 2-fold higher in the fed conditions than that observed under fasting conditions, indicating higher rate and extent of LCT with greater BS concentrations. This observation was in agreement with other studies conducted in the past (Borgstrom and Erlanson, 1973, Alvarez and Stella, 1989, MacGregor et al., 1997).

1.4.3 Role of calcium

The lipolysis of MCT and LCT generates lipolytic products with little aqueous solubility (Patton et al., 1985). These lipolytic products (monoglycerides and FAs) therefore tend to accumulate at the interface of the digesting TG droplet and impede the progression of lipolysis. FA accumulation at the interface of digesting TG droplet (Brockerhoff, 1968, Scow et al., 1979) renders the substrate inaccessible to the pancreatic lipase and thereby preventing further hydrolysis (brockerhoff and Jensen, 1974).

One mechanism by which interfacial FAs are removed is via complexation reaction with a counter ion such as calcium (Alvarez and Stella, 1989) and therefore, the presence of these counter ions in the reaction medium during lipolysis was observed to be essential for the continuation of the lipolysis process. *In vitro,* the formation of calcium soaps was found to take place between FA and Ca^{2+} ions at around 4 mM calcium concentration (Zangenberg et al., 2001a, Lichtenberg et al., 1988). $Ca(FA)_2$ is formed in the presence of Ca^{2+} leading to formation of a precipitate (Patton et al., 1985, Hernell et al., 1990). Moreover, the formation of calcium soaps at the surface of a digesting LCT (olive oil) droplet has been directly visualized via light microscopy (Patton et al., 1985).

Calcium was shown to have a profound influence on the rate of lipolysis (Alvarez and Stella, 1989) in the presence of bile. In a study conducted on lecithin-stabilized TG emulsions, these authors have demonstrated that calcium forms a highly active catalytic form of mixed-micelle-lipase complex in achieving efficient lipolysis of short-chain, medium-chain, and long-chain triglycerides. Activity of the mixed-micelle-lipase complex was shown to be reduced and it was suggested that this was due to inefficient binding to TG emulsion interfaces and that the presence of calcium was crucial to achieve efficient lipolysis.. Calcium

was also reported to be vital in reducing the lag phase and thereby the time to attain the maximal steady-state enzyme activity. Lag phase is caused by a decreased ability of the enzyme to bind and penetrate the interface and a lag of this type has been previously described using emulsions of tributyrin, LCT, and Intralipid (Borgstrom, 1980, Larsson and Erlanson-Albertsson, 1986). It has been suggested that the excess levels of phospholipid dispersed in the aqueous phase of the two phase system partitioned between the emulsion surface and the aqueous medium, causing the observed lag phase. Phospholipids reduce the enzyme levels at the interface by binding the lipase-colipase and preventing its action at the interface (Lairon et al., 1974, Lairon et al., 1978). Moreover, since a lag time was observed in the absence of calcium with many different emulsions (Borgstrom, 1980, Larsson and Erlanson-Albertsson, 1986, Borgstrom, 1977), the effect has been attributed to the general properties of lipase at the emulsion interface, rather than to properties of specific emulsions.

Previously, the particle size of the TG (MCT/LCT) dispersion was reported to influence the lag time, with finer dispersions showing a three-fold higher lag time than coarser dispersions (Armand et al., 1992). Di-valent ions such as calcium reduced the negative charges by overcoming the electrostatic repulsions between the droplets and thereby facilitated the efficient action of the enzyme at the interface (Borgstrom, 1980, Benzonana, 1968, Scow, 1988, Brown et al., 1977).

Calcium also was reported to bring about an enormous improvement in the extent of lipolysis of both medium and long chain triglycerides (MacGregor et al., 1997). One mechanism could be that the fatty acids from the interface of digesting TG droplet are removed by the formation of calcium soaps in a manner proportional to the level of calcium

concentration (MacGregor et al., 1997). A requirement for high calcium levels to activate the lipase completely has also been reported previously (Alvarez and Stella, 1989).

1.4.4 Role of supersaturation

A number of studies have successfully utilized the *in vitro* digestion model for the prediction of *in-vivo* performance of lipid formulations, as described above in section 1.4 (Reymond and Sucker, 1988, Porter et al., 2004a, Porter et al., 2004b, Dahan and Hoffman, 2006b, Cuine et al., 2007a, Dahan and Hoffman, 2007). The majority of studies have reported the performance of lipid-based formulations to be directly related to the colloidal species formed post-digestion, and implicated their potential role of particular species in determining the performance of formulations. Although, a good IVIVC was evident in many cases, correlation was not universal and the in vitro data did not explain the performance of some formulations. It has been acknowledged that the concentration of drug prevailing in the GIT is not always limited to the solubility in the colloidal species, and that the drug may be present in concentrations well above its saturated solubility, i.e. in a supersaturated state (Brouwers et al., 2009).

Drugs in a supersaturated state may either lead to precipitation or have the potential to enhance the bioavailability if present in a meta-stable state. If the concentration of species in solution is above its equilibrium solubility, then the system is supersaturated. The degree of supersaturation is measured by the supersaturation ratio (*S*), which is the ratio of the drug solubility to its equilibrium solubility. A supersaturated solution has the tendency to return to the equilibrium state via precipitation, however this may take a significant length of time if the saturated state is metastable.

The presence of a supersaturated state may contribute to the overall solubilized drug and lead to enhancement of drug flux across the GIT. However, this meta-stable state needs to exist for

sufficient time period for the absorption to take place to have an impact. Higuchi et al (1960) was the first to describe the potential application of the metastable, supersaturated state of drugs and its impact on the drug transport across biological membranes. Therefore, a strategy recognized to enhance the intestinal absorption of drugs which suffer from solubility limited oral bioavailability, has been the generation and maintenance of this meta-stable state of supersaturation. This concept was best described previously using the term 'spring' and 'parachute' approach (Guzman et al., 2007, Guzman et al., 2004). 'Spring' referred to the generation of supersaturation (high energy form of the drug) via formulation approaches such as solutions of drug based on cosolvent or lipid-based formulations, or by the delivery of high-energy solid forms of drugs such as amorphous forms, crystalline salt forms or co-crystals, which provide an accelerated dissolution and/or high apparent solubility in the GIT. Due to the inherent tendency of these high-energy forms to precipitate, maintenance of this form for a time period sufficient for absorption to take place has been deemed to be crucial. This stabilization of the supersaturated state is referred to as 'the parachute'.

Historically, this spring and parachute approach was applied to transdermal formulations (Kondo et al., 1987) and to date has not been explored extensively for oral formulations. Importance of polymers to stabilize high energy forms such as the stabilization of amorphous forms of the drug in formulations (e.g. solid dispersions), and the effect this has on dissolution properties have been described previously (Vasconcelos et al., 2007). A 2003 study of stabilization of the supersaturated state and prevention of precipitation from solid dispersion formulations was the first reported study intended for oral use (Yamashita et al., 2003). Since then several drug delivery systems that induce supersaturation *in-vitro* and which have been investigated for their absorption enhancing capacity have been reported in the literature (Gao et al., 2003, Gao et al., 2004, Gao et al., 2009, Guzman et al., 2007,

Overhoff et al., 2008, Yamashita et al., 2003, Mellaerts et al., 2008b, Mellaerts et al., 2008a, Brouwers et al., 2007, Miller et al., 2008, Kohri et al., 1999, Wire et al., 2006, Vaughn et al., 2006).

1.5 Need for this Investigation/Statement of the problem

Despite, the literature available (described in the 'introduction'), a consensus has not been reached as to the best way to predict the performance of lipid formulations *in-vivo*. Unfortunately, the correlations were not straight forward and predictability of *in vivo* performance based on a limited set of factors have failed to yield a complete insight in understanding the ability of *in vitro* models for the *a priori* prediction of *in vivo* performance. What is required now is to systematically devise studies based upon the valuable information available and arrive at a meaningful model in predicting the *in vivo* performance of drug candidates in various lipid-based formulations. In essence, it appears that the successful development of an *in vitro* model depends primarily on a more detailed understanding of all the existing *in vivo* sink conditions and their simulation *in vitro.*

In the *in vivo* situation, there are three key aspects that contribute to sink conditions. 1. Sink conditions that drive lipolysis to completion, 2. Sink conditions that drive the free drug molecules from surface of the digesting lipid droplet phase to colloidal phase, and 3. Sink conditions that drive drug molecules from colloidal species to physiological compartment (blood).

1.6 Hypotheses

Overall

An appropriate *in vitro* lipid digestion model can provide better *in vitro-in vivo* correlation for lipid-based systems.

Specific

1. Addition of calcium to the in vitro lipolysis model is a valid way to create sink condition *(see Aim 1)*.
2. In vitro assessment of lipid formulations and the selection of proper excipients containing within using dispersion and digestion testing gives detailed insights into the likely fate of formulations in the intestine *(see Aim 2)*.
3. Degree of drug supersaturation prevailing post in vitro digestion has a pronounced influence and a crucial role to play on the performance of lipid-based drug delivery systems *(see Aim 3)*.

Aims and observations

Aim 1

Studies conducted to date and described in the introduction have failed to achieve complete digestion of orally administered lipid-based formulations *in-vitro*, and thus fail to adequately simulate the *in vivo* conditions. One of the aims of this research project was therefore to address this issue of non-completion of lipolysis by adjusting the conditions of the *in vitro* lipolysis model in an attempt to drive lipolysis to completion. Several factors, which are proposed to persist *in vivo* and could play a role in the completion of lipolysis, are

pH, calcium concentration, bile concentration, oil-aqueous phase volume ratio, and pancreatic enzyme activity.

Calcium was used as a primary tool (keeping other variables constant) to drive the reaction to completion by sequestering fatty acids as calcium soaps. Chapter 2: (data published as a paper in International Journal of Pharmaceutics) deals with the effect of increasing calcium and bile salt (BS) concentration on the *in vitro* digestion of a model long-chain triglyceride (soybean oil) in order to understand how these factors affect the solubility of poorly water-soluble drugs in the digested formulation. Results showed that although high calcium concentration may prove beneficial with respect to bringing the lipolysis to completion, the formation of fatty acid soaps interferes with the solubilisation pathway for poorly water soluble drugs. It was therefore concluded that high calcium concentration in the system during in vitro lipolysis results in an outcome that is not likely to correlate with the fate of the drug in vivo.

Aim 2

Historically the primary evaluation tool for orally administered lipid-based systems has been particle size analysis. It has been assumed that the achievement of a homogenous dispersion of fine droplet size following dispersion in the GI fluids would indicate the quality of the formulation. However, in vitro digestion testing has made it very clear that the oil droplet size and the structure and composition of the colloids change during gastrointestinal transit. Owing to the fact that some lipid-based formulations suffer a loss of solubilization capacity following dispersion and/or digestion, a major focus in our laboratory and that of others has been to improve the ability to predict the *in vivo* performance of lipid-based systems. This has been addressed by systematically investigating *in vitro* testing methods,

and establishing which factors play a role in reducing the solubilization capacity of the system during dispersion and digestion, leading to drug supersaturation and eventually the risk of drug precipitation.

In Chapter 3: (data published in the European Journal of Pharmaceutical Sciences) we carried out a thorough assessment of the solubilization capacity of eight different formulations of fenofibrate and investigated how the solubilization capacity changed during dispersion and digestion of the lipid-based formulations. The formulations studied were closely related self-emulsifying lipid-based formulations (Type II and IIIA lipid-based formulations as per LFCS). The formulations were prepared with a minimum number of excipients to facilitate comparison. We have shown that the formulations generate supersaturation during both dispersion and digestion and concluded that the likelihood of precipitation could be predicted by calculating the extent of supersaturation that develops during these processes. Discrimination between formulations was not feasible from our dispersion studies even when high drug loadings (yielding high supersaturation) were employed proving that all the formulations solubilized the drug efficiently with hardly any drug being lost as precipitate and that the colloidal species maintained the high supersaturation conditions very well during *in vitro* dispersion. This, however, was not the case with the formulations post-digestion wherein we observed that the lipid-based formulations underwent considerable stress and that the ability of formulations maintaining drug in solubilized state was highly dependent on *lipid composition* and the *nature of surfactant.*

Aim 3

In an effort to understand the critical significance of the degree of supersaturation during digestion, and the use of this parameter to understand the differences in performance of self-emulsifying lipid-based formulations, we chose a single type of lipid-based system (Type IIIA SEDDS) and explored the influence of surfactant on supersaturation. Maximum supersaturation (SR^M), which was defined as the ratio of total concentration of drug present during digestion in the absence of any precipitation (i.e., drug dose) and the inherent equilibrium drug solubility, was shown to be a remarkable indicator of performance of formulations. The data from this study (Chapter 4) was accepted for publication in the Journal of Pharmaceutical Sciences in December 2013.

Chapter 2: Research paper 1

In vitro digestion testing of lipid-based delivery systems: calcium ions combine with fatty acids liberated from triglyceride rich lipid emulsions to form soaps and reduce the solubilisation capacity of colloidal digestion products.

Abstract

In vitro digestion testing is of practical importance to predict the fate of drugs administered in lipid-based delivery systems. Calcium ions are often added to digestion media to increase the extent of digestion of long-chain triglycerides (LCTs), but the effects they have on phase behavior of the products of digestion, and consequent drug solubilization, are not well understood. The effect of calcium and bile salt concentrations on the rate and extent of *in vitro* digestion of soybean oil was investigated, while the solvent capacity of the digestion products for two poorly water-soluble drugs, fenofibrate and danazol was also determined. In the presence of higher concentrations of calcium ions, the solubilization capacities of the digests were reduced for both drugs. This effect is attributed to the formation of insoluble calcium soaps, visible as precipitates during the digestions. This reduces the availability of liberated fatty acids to form mixed micelles and vesicles, thereby reducing drug solubilization. The use of high calcium concentrations does indeed force *in vitro* digestion of LCTs but may overestimate the extent of drug precipitation that occurs within the intestinal lumen.

2.1 Introduction

While a number formulation and non-formulation strategies have been developed to address the increasing number of new chemical entities that demonstrate oral absorption limited by low aqueous solubility (Williams et al., 2012i), approaches that utilize drug pre-dissolved in a lipid vehicle remain popular. The basis for using lipids stems from a number of studies that noted improved absorption and bioavailability of a poorly water-soluble drug (PWSD) following co-administration with a lipid-rich meal (Charman et al., 1993, Crounse, 1961, Charman et al., 1997, Humberstone et al., 1996, Welling, 1996, Sunesen et al., 2005). In a broad sense, lipid-based drug delivery systems (LBDDS) therefore aim to harness the often positive effect of dietary lipids on oral drug absorption (Porter et al., 2008a, Larsen et al., 2008, Porter et al., 2007a, Hauss, 2007b) by circumventing drug dissolution, which in the case of PWSD is often slow and potentially limits the rate and extent of drug absorption, and by increasing the solubilization reservoir in the GI fluids (Cuine et al., 2007a, Porter et al., 2004a, Porter et al., 2007a, Kleberg et al., 2010).

While there are many different types of LBDDS, discrimination may be made based on their composition and properties following interaction with endogenous GI fluids. In an effort to facilitate this discrimination, Pouton proposed the Lipid Formulation Classification System (LFCS) (Pouton, 2000b, Pouton, 2006b), which classifies LBDDS into five discrete groups (Type I, II, IIIA, IIIB and IV) according to the proportion of oil, lipophilic surfactant, hydrophilic surfactant and co-solvent in the formulation. Type I and II formulations represent the most lipophilic formulations and form coarse and highly turbid emulsions on dispersion in aqueous fluids. Digestion of the dispersed oil phase will therefore benefit their *in vivo* performance since this will force drug to partition from a poorly dispersed oil droplet phase

into more solubilized colloidal phase. In contrast, it is generally well recognized that digestion is not essential to the performance of Type IIIA/B and Type IV systems as they form finer (i.e., nanosized) emulsions and/or micellar phase systems in the GI tract. As LBDDS enter the small intestine, digestion of formulation components is inevitable and may significantly impact the subsequent formulation behavior. Assessment of all types of LBDDS during *in vitro* digestion tests is therefore necessary for a complete understanding of formulation performance.

Lipid digestion occurs primarily in the small intestine through the action of lipases and esterases. Pancreatic lipase is the main enzyme involved in the digestion of triglycerides (Armand et al., 1996, Carriere et al., 1993). Pancreatic lipase is an interfacial enzyme that, in the presence of co-lipase anchor, will effectively bind to the hydrophobic surface of an oil droplet (Erlanson-Albertsson, 1992a). Other enzymes present in the small intestine thought to play a role in lipid digestion include carboxyl ester hydrolaze (CEH), phospholipase A_2 and pancreatic lipase-related protein 2 (PLRP 2). However, these enzymes are believed to contribute more to the hydrolysis of phospholipids, cholesterol esters and formulation surfactants rather than triglyceride (Bakala N'Goma et al., 2012a, Borgstrom, 1993a). Pancreatic lipase hydrolyses molecules of triglyceride (TG) to yield two molecules of fatty acids (FA) and a molecule of 2-monoglyceride (2-MG). Further hydrolysis of 2-MG is limited by the region specificity of pancreatic lipase towards positions 1 and 3 of the TG molecule (Carriere et al., 1997), although CEH and PLRP 2 may also hydrolyze 2-MGto yield a third FA and glycerol(Bakala N'Goma et al., 2012a). However, PLRP 2 has not yet been identified in porcine-derived pancreatin (de Caro et al., 2008) - the source of pancreatic lipase commonly used in *in-vitro* digestion models - while the quantity of CEH in this extract has not be determined. Alternatively, 2-MG may undergo slow isomerization to the relatively less

lipase-stable 1-MG to allow further hydrolysis to yield a third FA and glycerol, however this process is usually limited *in vivo* (Mattson and Volpenhein, 1964). The extent of MG hydrolysis to FA and glycerol *in vitro* is therefore unknown. Natural detergents in the small intestine i.e., bile salts and phospholipids (secreted along with PL from the gall bladder in response to lipids entering the small intestine) form mixed micelles that shuttle the products of lipid digestion from the site of production i.e., the oil: water interface, to the site of absorption, i.e., the enterocyte membrane (Hofmann, 1963).

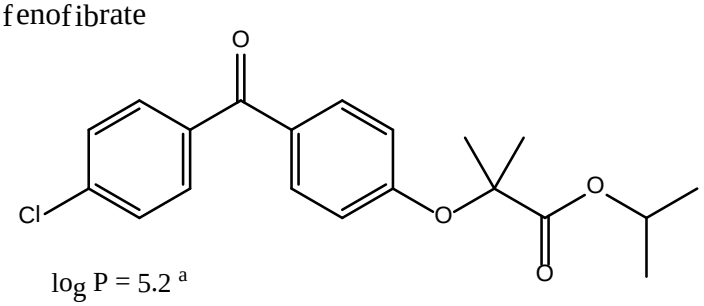
In vitro digestion tests are designed to simulate the above digestion processes so that the fate of drug may be monitored as the physical and chemical nature of LBDDS change. It is also customary to relate this outcome to the extent of formulation digestion. Detailed descriptions of the *in vitro* digestion models have been already provided (Sek et al., 2002a, Williams et al., 2012h). In brief, lipid substrate is dispersed in a digestion medium (maintained at 37°C) consisting of a buffer, bile salt and phospholipid before the addition of pancreatic enzymes that initiate digestion. Digestion is monitored using a pH-stat titrator that detects the progressive decrease in pH as ionized fatty acid is released via the digestion of lipids and target pH is maintained by the stoichiometric titration of base (i.e., sodium hydroxide solution) into the reaction vessel. Samples of the digest may be taken throughout the experiment and centrifuged to separate a poorly dispersed oil phase, a colloidal aqueous phase (containing mixed micelles and vesicles), and a pellet phase. Drug incorporated into the LBDDS, that subsequently precipitates during digestion is contained within the pellet phase, and is thought to represent drug that is poorly available for absorption since re-dissolution is required and the dissolution of PWSD from crystalline solids is usually poor. In contrast, drug that remains solubilized within the colloidal aqueous phase digest is expected to be in rapid

equilibrium with drug in free solution and to provide a reservoir of drug that is highly available for absorption(Porter et al., 2007a, Boyd et al., 2003).

One of the experimental complexities of *in vitro* digestion models is the difficulty in achieving complete digestion of the lipid substrate. This issue is particularly the case for highly lipophilic, long-chain lipid formulations (Williams et al., 2012h, Williams et al., 2012d), and stems from the fact that most *in vitro* digestion models are 'closed' systems. As such, the absence of a sink for removal of digestion products (such as that provided *in vivo* by absorption) causes a progressive increase in the concentration of lipid digestion products which, depending on the solubilization capacity of the digestion medium for the digestion products, ultimately results in accumulation of the lipid digestion products at the oil droplet surface, suppressing further digestion of the remaining oil phase (Brockerhoff, 1968, Scow et al., 1979, Fave et al., 2004, brockerhoff and Jensen, 1974). Since this phenomenon is attenuated *in vivo* by absorption, strategies to 'force' lipid digestion to completion *in vitro* have been sought, and the most common approaches include an increase in the bile salt reservoir (Li et al., 2011) and the addition of a fatty acid complexant, e.g., calcium ions either within the digestion media or via continuous addition (Alvarez and Stella, 1989, Patton and Carey, 1979, Patton et al., 1984, Zangenberg et al., 2001b, Zangenberg et al., 2001a, MacGregor et al., 1997). The effect of increasing calcium concentration on the phase behavior of digested lipids and the resultant impact on drug solubilization, however, is not well understood. The first aim of the current study was therefore to determine whether increasing calcium concentration could be used to push the *in vitro* digestion of a model long-chain triglyceride (LCT) to completion. The second aim was to probe the solubilization capacity of the digests formed by this approach. In the current studies fenofibrate and danazol were employed as model PWSD (Figure 2.1) and soybean oil as a model long-chain lipid

substrate. The data show that increasing calcium concentration in the digestion test is highly effective in pushing the digestion of triglyceride to completion, however the facile formation of insoluble fatty acid – calcium soaps is limiting to the solubilization capacity of the digestion colloids. The use high calcium concentrations may therefore overestimate the extent drug precipitation that may occur *in vivo* in the intestine.

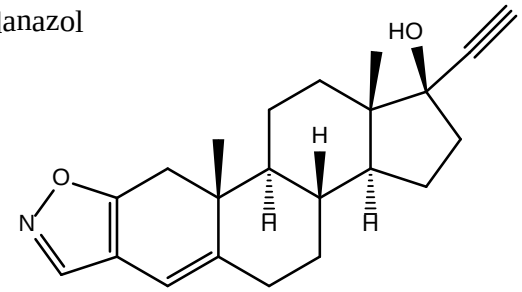
fenofibrate



log P = 5.2 [a]

Solubility in water = 1.3 μg/ml [b]

danazol



log P = 4.5 [c]

Solubility in water = 1.0 μg/ml [c]

Figure 2.1: chemical structure of model drugs danazol and fenofibrate investigated in this study. a – (Munoz et al., 1994) b - Sheu et al. (1994). c –(Bakatselou et al., 1991)

2.2 Materials and methods

2.2.1 Materials

Danazol was obtained from Sterling Pharmaceuticals Pty Ltd (Sydney, Australia). Fenofibrate, soybean oil (the long-chain triglyceride), sodium taurodeoxycholate >95% (NaTDC), pancreatin extract (from porcine pancreas, P7545, 8xUSP specifications activity), calcium chloride dihydrate, Tris-maleate, and the lipid digestion inhibitor 4-bromophenylboronic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). Lecithin (ca. 99.2% egg-phosphatidylcholine (PC), Lipoid E PCS) was purchased from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). 1.0M sodium hydroxide (Univol) was purchased from Ajax Finechem Pty Ltd (Sydney, Australia) and was diluted with water (Milli-Q water purification system, Millipore, Bedford, MA) to achieve a 0.2 M titration solution. Methanol and chloroform used in this work were HPLC grade from Merck (Melbourne, Australia).

2.2.2 In vitro digestion experiments

In vitro digestion experiments were performed as previously described (Sek et al., 2002a). Briefly, 250± 5 mg of soybean oil was directly weighed into a water jacketed-glass reaction vessel (T=37^{o}C) and dispersed in 9ml aqueous digestion buffer (pH 7.5) consisting of 50mMTris-maleate, 150mM sodium chloride and 0mM – 40mMcalcium chloride dihydrate, supplemented with 0mM-100mMbile salt and 0mM-25mMPC. The range in bile salt conditions were chosen to represent the widest range of potential solubilization conditions of the intestine, ranging from ‘extreme fasted’ (i.e., 0mM bile salt and 0 mM PC) to ‘extreme fed’ conditions (i.e., 100mM bile salt and 25 mM PC). In each test, a bile salt: PC ratio of 4:1 was employed. Dispersion of the LCT was provided via a magnetic stirrer, and over 10 min vigorous mixing, the LCT formed a coarse emulsion. During this dispersion phase, the pH

was adjusted to pH 7.5±0.05 with 1.0M NaOH/HCl. Digestion was initiated by the addition of 1ml pancreatin, containing 40000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per ml of digestion medium. The pancreatin extract was prepared fresh on each day of testing from 1g of pancreatin powder thoroughly mixed in 5ml digestion buffer (in the absence of bile salt or PC). To minimize loss of enzyme activity, the prepared pancreatin extract was stored on ice prior to use. The mixture was then centrifuged (1600*g*, 5°C, Eppendorf 5408R, Eppendorf AG) for 15 min, then the supernatant was recovered and the pH adjusted to pH 7.5±0.1 with 5.0M NaOH.

Digestion of the LCT was continuously monitored using a pH-stat automatic titration unit (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.2 M NaOH.

2.2.3 Extent of digestion in vitro

Digestion profiles were firstly corrected for the background fatty acid (FA) released on digestion of the bile salt-PC mixed micelles, the concentration of which were determined in separate experiments undertaken in the absence of LCT. This correction was particularly important in the present study as the concentration of PC used in the digestion medium varied from 0mM to 25mM (and therefore, would have a significant effect on the amount of total FA titrated).

For each mole of liberated FA, one mole of NaOH is titrated to neutralize the acid and maintain the pH at 7.5. Assuming that each mole of TG is digested into two moles of FA and one mole of 2-MG (it is widely recognized that the conversion [by isomerization] of 2-MG into 1-MG and eventually into a third FA and glycerol is restricted only to *in vivo* conditions (Mattson and Volpenhein, 1964) while the extent of 2-MG hydrolysis *in vitro* using porcine pancreatin extract is not known) the fraction FA released was calculated using Equation 1:

$$\text{Extent of digestion (\%)} = \frac{(V_{NaOH} \times M_{NaOH}) \times MW_{TG}}{m_{TG} \times 2} \times 100$$ Equation 1

where M_{NaOH} is the molarity of the NaOH titrant, V_{NaOH} is the total volume of titrant consumed during titration, m_{TG} is the mass of the TG added to the digestion vessel and MW_{TG} is its molar mass.

2.2.4 Drug solubility in the aqueous colloidal phase

The equilibrium solubility of danazol and fenofibrate in the aqueous colloidal phase (AP_{DIGEST}) generated by the digestion of LCT was evaluated after 5, 30 or 60 min digestion (as described above). At these time points, 2x4ml samples were collected from the reaction vessel and immediately treated with digestion inhibitor (9µl per ml of digestion medium of 0.5 M 4-bromophenylboronic acid in methanol) to arrest ongoing digestion. Samples were ultra-centrifuged (400000*g*, 37°C, Optima XL-100K Ultracentrifuge, SW-60 swinging-bucket rotor, Beckman, Palo Alto, CA) in soft-walled polyallomer tubes (Beckman) for 30 min to separate the digestion phases. The sample tubes were pierced near the bottom using a 5ml syringe-23G needle assembly to extract the AP_{DIGEST} to ensure it was not contaminated with the undigested oil phase(Sek et al., 2002a, Williams et al., 2012h). Crystalline drug was then added in excess to 4 ml AP_{DIGEST} before incubation at 37°C in an orbital mixer. After 48 h, the mixtures were centrifuged (1600*g*) for 15 min and 100 µl of the supernatant was diluted with methanol before analysis for drug content by high performance liquid chromatography (HPLC), as described in section 2.2.5 below.

The equilibrium solubility of both drugs were also determined in LCT-free digestion medium, containing (i) 5 mM bile salt/5 mM calcium, (ii) 5 mM bile salt/40 mM calcium, (iii) 100 mM bile salt/5 mM calcium and (iv) 100 mM bile salt/40 mM calcium (with PC at a concentration to provide a 4:1 bile salt: PC ratio). These drug/digestion media mixtures were equilibrated and analyzed for drug content as described above. Solubility was assessed with respect to equilibration time (i.e., samples were removed at 1, 6, 24, 48, and 120 h) and equilibrium solubility defined as being obtained when solubility values at two consecutive time points varied by less than 5%.

2.2.5 HPLC assays

HPLC analysis for danazol and fenofibrate were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, MA). The columns used for both drugs were reverse-phase C18 columns; a Waters Symmetry® column for danazol (150 x 3.9 mm, 5 µm, Waters Alliance Instruments) and a Phenomenex® Luna column for fenofibrate (C18 (2), 150 x 4.6 mm, 3µm, Phenomenox, Torrence, CA).The mobile phase employed for both drugs consisted of methanol and water in a 75:25 v/v ratio pumped through the HPLC columns at 1 ml/min. The sample injection volume was 50µl, with UV detection for danazol and fenofibrate at 288 nm and 286 nm, respectively. All samples and standards were maintained at 10°C and the column temperature maintained at 25°C.

2.3 Results

2.3.1 Effect of bile salt concentration

The effect of increasing bile salt concentration on the quantity of titratable FA during *in vitro* digestion of LCT is shown in Figure 2.2. Titratable FA corresponds to FA released from the LCT in response to digestion by enzymes in the pancreatin extract. Total concentrations of FA titrated over 30 min and the calculated levels of LCT digestion (according to Eq. 1) are summarized in (Table 2.1). In the absence of calcium and bile salt (closed circles; Figure 2.2A),there was a lag period of approximately 10 min before FA was detected. By the end of the digestion period (60 min), 5.6 ± 0.4 mM of FA was titrated. This FA concentration using 0 mM bile salt was similar to the concentration titrated at 5 mM bile salt (7.1 ± 0.9 mM, open circles; Figure 2.2A), however, no lag period was observed in the presence of bile salt. In tests performed using 20 mM and 100 mM bile salt (closed and triangles, respectively; Figure 2.2A), the total concentration of FA titrated increased significantly to 19.2±0.3 and 39.8±2.2mM, respectively. This increase in titrated FA at the higher bile salt concentrations was most pronounced during the first 10 min of the digestion tests. FA titration rate during the remainder of the experiments was, however, generally independent of bile salt concentration and was also much slower.

The dashed horizontal line in Figure 2.2 denotes the theoretical concentration of FA when the extent of LCT digestion is 100% (according to Eq. 1). In the absence of calcium, the extent of digestion was ~10% at 0 and 5 mM bile salt, and 33.4% and 69.3% at 20 and 100 mM bile salt, respectively (Table 2.1).

The results of LCT digestion tests performed in digestion media containing 0-100 mM bile salt, but supplemented with 5mM calcium, are shown in Figure 2.2B. Direct comparison of

titration profiles in Figure 2.2A to those in Figure 2.2B therefore reveal the effect of calcium (5 mM) on LCT digestion. Using the cumulative concentration of titrated FA over 30 min and Eq. 1, the LCT digestion extent at 0 mM bile salt increased from 10% in the absence of calcium, to 64% with 5 mM calcium. At 5 mM bile salt, digestion also increased with the addition of calcium, but to a lesser extent (i.e., from 12% to 24%). With 20 mM and 100 mM bile salt, the addition of 5 mM calcium had no effect on extent digestion.

The addition of 5 mM calcium to the bile salt-free digestion medium led to faster rate of FA titration (closed circles; Figure 2.2 A and B). The total FA concentration in this condition of no bile salt and 5 mM calcium was 37.3 ± 1.2mM. This exceeded the FA concentrations obtained when 5 mM and 20 mM bile salt was added, suggesting that the addition of bile salt (≤ 20 mM) depressed the effects of calcium on LCT digestion.

The highest extent of LCT digestion achieved was 77%, and was at the highest tested bile salt concentration (100 mM). This was slightly higher than the highest extent of digestion obtained in the absence of calcium (69%; Table 2.1). Therefore, despite increasing bile salt to concentrations far-exceeding those in the human small intestine (in both fasted and fed states (Persson et al., 2006, McConnell et al., 2008)), it was not possible to achieve the complete digestion of the LCT *in vitro* when using 0 or 5mM calcium.

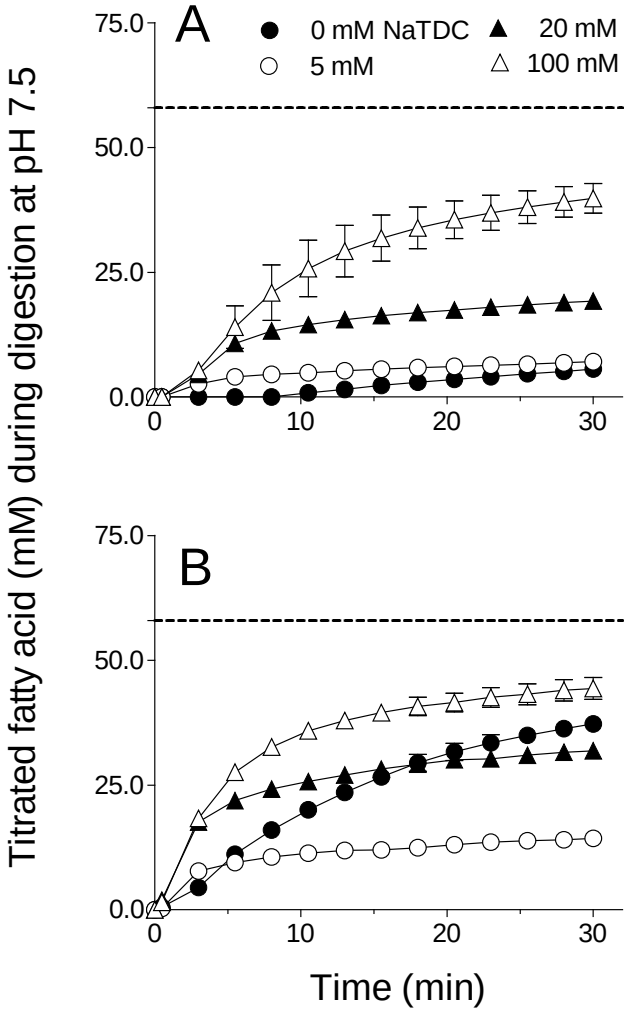


Figure 2.2: Apparent titration of fatty acids (FA) during *in vitro* digestion of soybean oil (a long-chain triglyceride, LCT) in conditions of increasing bile salt (NaTDC) concentration. Digestion tests were performed at A: 0 mM calcium and B: 5 mM calcium. Digestion was initiated at *t*=0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion of the LCT through the addition of 0.2 M NaOH. Values are expressed as means (n = 3) ± SD with background correction for the level of FA released in background digestion tests (no LCT). NaTDC; sodium taurodeoxycholate (the bile salt used in this study). The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. 1).

2.3.2 Effect of calcium concentration

The effect of increasing calcium concentration on titration of FA during *in vitro* digestion of the LCT is shown in . Tests were performed in digestion media containing either no (Figure 2.3A), 5 (Figure 2.3B) or 20 mM bile salt (Figure 2.3C), with the latter two conditions mimicking typical fasted and fed bile salt concentrations, respectively. Data obtained at 0 mM and 5 mM calcium is reproduced from Figure 2.2 and is included to further illustrate the effect of calcium and bile salt concentration on digestion.

In the absence of bile salt (Figure 2.3A), increasing calcium led to a progressive increase in titrated FA. Between 0 and 5 mM calcium, the amount of titrated FA at 30 min increased 6-fold. Further increases in calcium concentration to 20 and 40 mM led to higher titrated FA concentrations, although the effect of calcium on FA titration progressively diminished. Since the total extent of digestion at 30 min at 40 mM calcium reached 106%, it was clearly evident that the use of high calcium concentrations could lead to complete LCT digestion. Therefore, a gradual depletion of available substrate most likely explains the smaller effect of increasing calcium concentration > 20 mM on total LCT digestion.

Increasing calcium also led to a progressive increase in titrated FA in the presence of 5 (Figure 2.3B) and 20 mM bile salt (Figure 2.3C). However, consistent with the previous section, the positive effects of calcium on digestion are attenuated by the bile salt. Therefore, in the presence of bile salt, it was not possible to achieve complete digestion of LCT through increasing calcium concentration. To further illustrate this depressive effect of bile salt on digestion, the total concentration of titrated FA (i.e. values at 30 min from Figure 2.2 and Figure 2.3) is plotted as a function of either bile salt or calcium concentration, as shown in Figure 2.4A and B. Both plots illustrate the depressant effect of bile salt on digestion and it is most marked at 5 mM bile salt. The shape of the digestion profile is also different in the

presence of bile salt, with the initial rate of digestion faster compared to in the absence of bile salt. The rate of digestion in the absence of bile salt at 20 mM and 40 mM calcium was similar, suggesting that the concentration of calcium was not limiting to digestion.

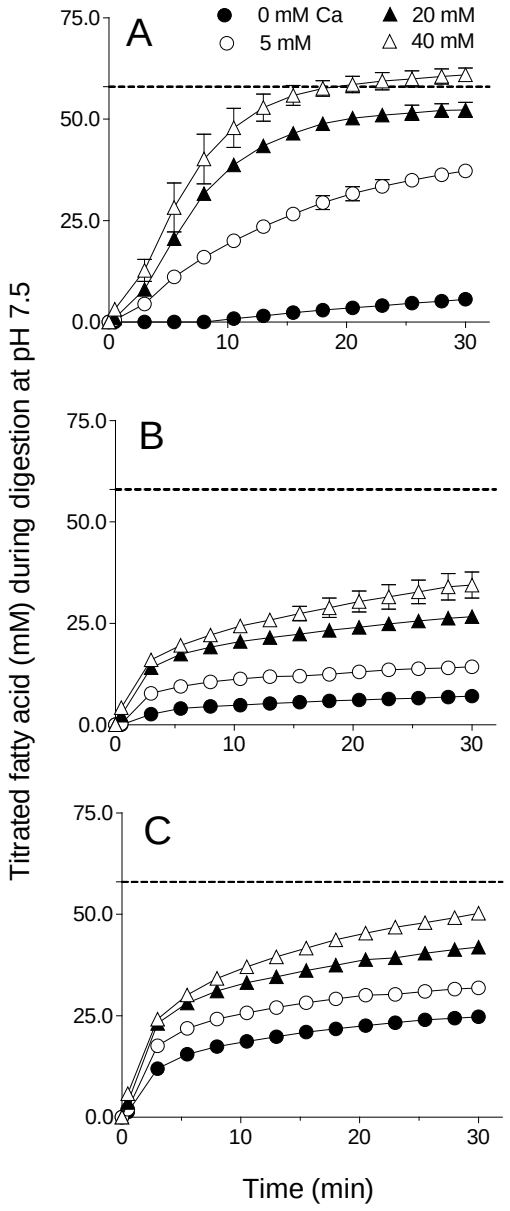


Figure 2.3: Apparent titration of fatty acids during in vitro digestion of soybean oil (a long-chain triglyceride, LCT) in conditions of increasing calcium concentration. Digestion tests were performed at A: 0mM , B: 5 mM and C: 20 mM bile salt (NaTDC). Digestion was initiated at t=0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion of the LCT through the addition of 0.2 M NaOH. Values are expressed as means (n = 3) ± SD with background correction for the level of fatty acid released in background digestion tests (no LCT). NaTDC; sodium taurodeoxycholate (the bile salt used in this study).The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. 1).

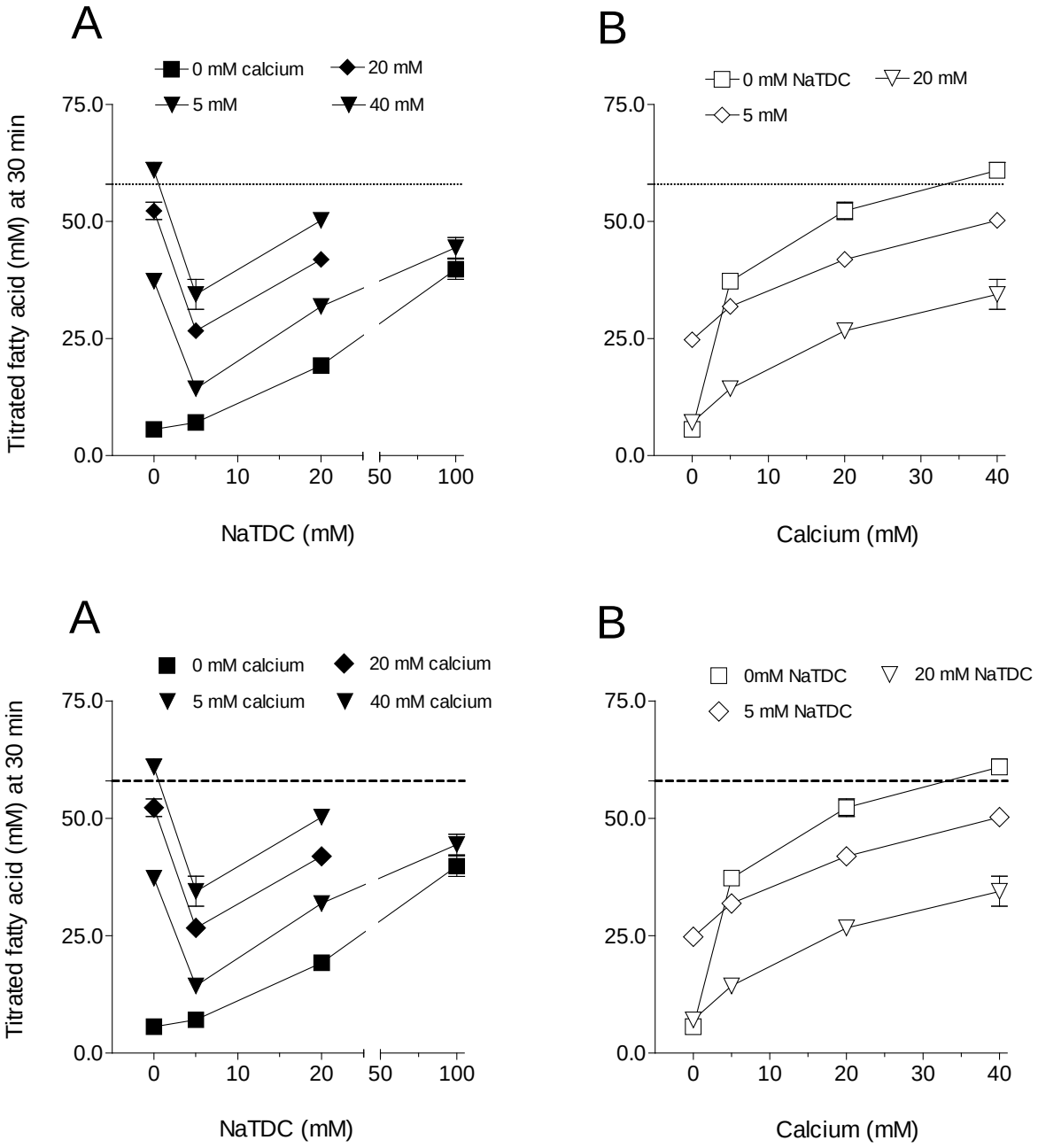


Figure 2.4: Graphical summary of the effect of bile salt (NaTDC) and calcium concentration on the total concentration of fatty acid (FA) titrated over 30 min during the *in vitro* digestion of soybean oil (a long-chain triglyceride, LCT). A: Effect of increasing NaTDC concentration on total titrated FA at four calcium concentrations. B: Effect of increasing calcium concentration on total titrated FA at three NaTDC concentrations. Values are expressed as means (n = 3) ± SD. NaTDC; sodium taurodeoxycholate (the bile salt used in this study). The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. 1).

Table 2.1: Summary of the effect of calcium concentration and bile salt concentration on the digestion of long-chain triglyceride (LCT)

Digestion condition	Fatty acid titrated over 30 min LCT digestion (mM)[a]	Extent of LCT digestion (%)[b]
0 mM calcium, with:		
0 mM bile salt	5.6 ± 1.4	3.3
5 mM	7.1 ± 0.9	12.4
20 mM	19.2 ± 0.3	33.4
100mM	39.8±2.2	69.3
5 mM calcium, with:		
0 mM bile salt	37.3 ± 1.2	64.9
5 mM	14.3 ± 5.2	24.0
20 mM	31.9 ± 1.0	55.5
100 mM	44.4 ± 2.2	77.3
20 mM calcium, with:		
0 mM bile salt	52.3 ± 1.9	91.1
5 mM	26.7 ± 0.1	46.4
20 mM	41.9 ± 0.6	73.0
40 mM calcium, with:		
0 mM bile salt	60.9 ± 1.7	106.1
5 mM	36.1 ± 1.7	60.0
20 mM	51.9 ± 1.6	87.5

[a]Total fatty acid concentration titrated at pH 7.5

[b] Calculated using Eq. 1

2.3.3 Effect of calcium on the drug solubility in AP_{DIGEST}

To assess the effect of calcium on the solubilization capacity of digested lipids, aqueous colloidal phase digests isolated following 5, 30 and 60 min of digestion (AP_{DIGEST_5min}, AP_{DIGEST_30min} and AP_{DIGEST_60min}) were incubated with excess danazol and fenofibrate and solubility assessed over a period of 48 h. Previous work by Kossena et al. has shown that the LCT lipid digestion products swell bile salt-phospholipid mixed micelles which, in turn, increases the solubilization capacity of this bile salt-phospholipid phase (Kossena et al., 2005).

2.3.3.1Low bile salt concentration (5mM)

The upper panel in Figure 2.5 plots digestion as a function of time, and reiterates the increase in LCT digestion in the presence of the higher calcium concentrations (i.e., 40 mM) and the increase in digestion as a function of time. Data points highlighted at 5, 30 and 60 min digestion refer to the time points at which samples of the digest were removed for the solubility studies. The lower panels in Figure 2.5 show the equilibrium solubilities of fenofibrate (bottom left) and danazol (bottom right) in these colloidal digests ($AP_{DIGESTS}$) isolated from either the low or high calcium conditions. To show the effect of calcium on solubility in the absence of LCT and its digestion products, the measured drug solubilities in the equivalent digestion medium (i.e., no LCT) are also shown.

At 5 mM calcium, fenofibrate solubility in the AP_{DIGEST} progressively increased from 47.0 ± 1.4 µg/ml in AP_{DIGEST_5min} to 186.0 ± 20.3 µg/ml in AP_{DIGEST_60min}(lower left panel; Figure 2.5). As fenofibrate solubility in the equivalent digestion medium (i.e., in the absence of digestion products) was only 23 ±2 µg/ml, the increase in solubility in the $AP_{DIGESTS}$ indicates that the digestion products were contributing significantly to drug solubilization. Accordingly,

a degree of ongoing digestion between 5 and 60 min and the resulting higher concentration of digestion products in this phase provides a likely explanation for the increase in solubilization capacity of the AP_{DIGEST} with respect to digestion time.

In contrast, and despite evidence of ongoing digestion between 5 min and 60 min, fenofibrate solubility in $AP_{DIGESTS}$ formed in the presence of 40 mM calcium did not increase. In addition, the solubility values at this higher calcium concentration were generally lower than solubility values measured at the lower calcium concentration (except at 5 min post digestion), and were only marginally above the solubility in digestion medium (i.e., no LCT). The data obtained at the two calcium concentrations therefore suggests that the solubilization capacity of the AP_{DIGEST} was not directly related to the extent of LCT digestion, since increasing calcium increased digestion, but did not always increase drug solubilization. Furthermore, as fenofibrate solubility in the digestion media was not affected by calcium concentration (23 ±2 µg/ml and 23 ±1 µg/ml at 5 and 40mM calcium, respectively), the differences in fenofibrate solubility in the $AP_{DIGESTS}$ (seen in Figure 2.5) could not be directly attributed to the higher concentration of calcium ions.

Consistent with the fenofibrate results, danazol solubility values in the AP_{DIGEST} were lower at 40 mM calcium compared with 5mM calcium (lower right panel; Figure 2.5).These danazol solubility values in the AP_{DIGEST} at 5 and 40 mM calcium were, however, below the solubility in simple digestion media (no LCT), suggesting that the presence of lipid digestion products had a limited impact on danazol solubility in model intestinal fluids at both high and low calcium concentrations. This was in contrast to fenofibrate, which showed a higher solubility in the AP_{DIGEST}. . The difference in danazol solubility between the simple digestion medium and the AP_{DIGEST} was most pronounced at the higher calcium concentration.

However, as seen in Figure 2.5 (lower right panel), danazol solubility in digestion media alone (i.e., no digestion products) was 16 ± 3 µg/ml at 5 mM calcium and 14 ± 1 µg/ml at 40 mM calcium. The solubility of danazol was, therefore, not directly affected by calcium, which is consistent with the fenofibrate results.

Of additional note was the appearance of the pellet phase during the solubility studies. The pellet phase of digestion forms a sediment during ultracentrifugation and consists primarily of insoluble calcium soaps of fatty acid (Sek et al., 2002a, Patton and Carey, 1979).In the present study, the pellet phase at the higher calcium concentration was notably larger compared with the pellet observed at the lower calcium concentration, suggesting the quantity of fatty acid calcium soaps in the presence of 40 mM calcium had increased.

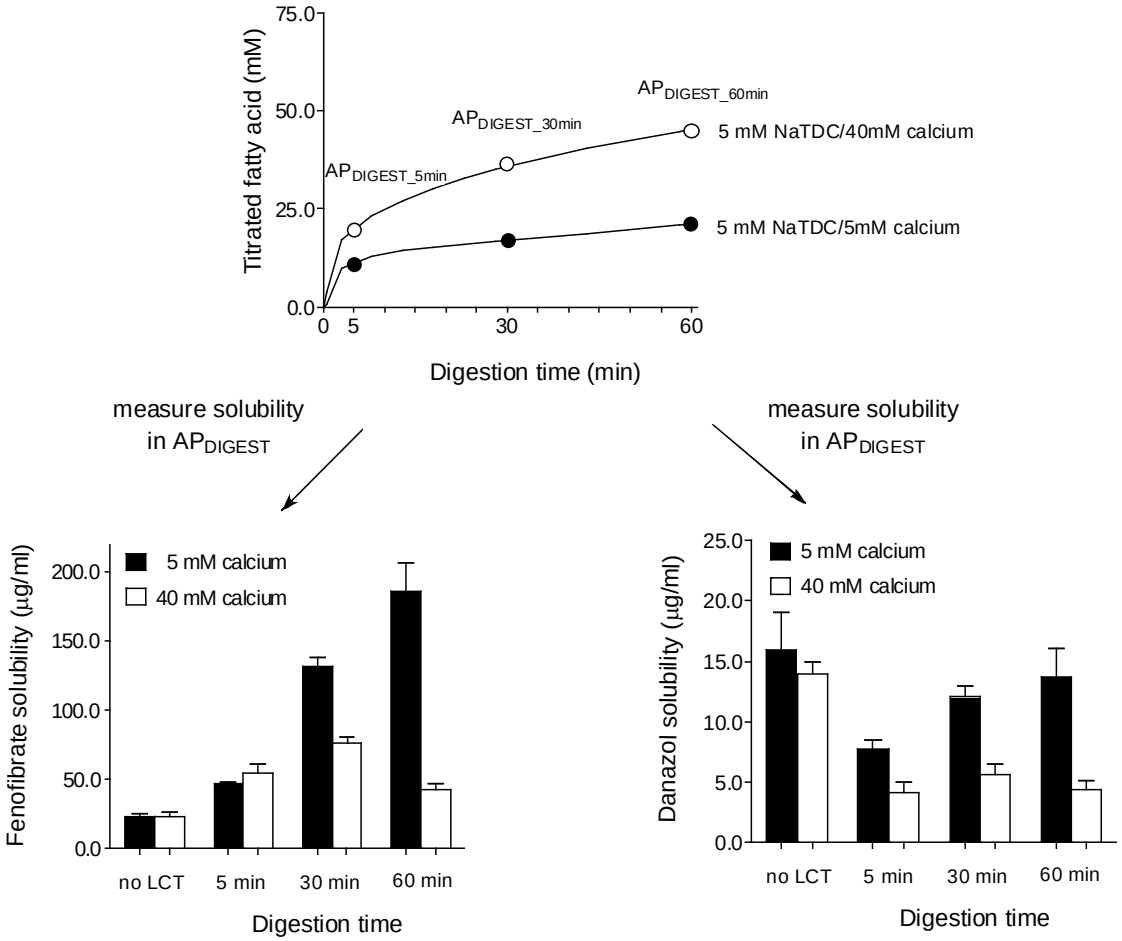


Figure 2.5: Titrated fatty acid profiles describing the digestion of LCT in digestion media containing 5 mM bile salt/5 mM calcium (closed symbols) or 5 mM bile salt/40 mM calcium (open symbols) and the determination of fenofibrate (left) and danazol (right) equilibrium solubility in the AP_{DIGEST} isolated following LCT digestion for 5 min (AP_{DIGEST_5min}), 30 min (AP_{DIGEST_30min}), and 60 min (AP_{DIGEST_60min}). Solubility studies in the $AP_{DIGESTS}$ were performed at 37°C over a 48 h equilibration period. The equilibrium solubility of fenofibrate and danazol in the digestion medium (no LCT) containing 5 mM bile salt/5 mM calcium and 5 mM bile salt/40mM calcium are also shown.

2.3.3.2 High bile salt concentration (20 mM)

The results for aqueous phase solubilization under 20 mM bile salt are shown in Figure 2.6 (c.f. Figure 2.5) with the upper panel indicating that the extent of digestion obtained at 40 mM calcium was higher compared to 5 mM calcium. However, the lower solubilities of fenofibrate (bottom left panel) and danazol (bottom right panel) at the higher calcium concentration indicate that this greater digestion did not translate into higher solubilization capacities for the corresponding $AP_{DIGESTS.}$

The overall effects of calcium on LCT digestion and drug solubility determined at 5 mM bile salt (Figure 2.5; described in section 2.3.3.1) and at the higher 20 mM concentration (in this section) were consistent; while high calcium leads to increased LCT digestion extent, this does correspond to an increase in the solubilization capacity of the isolated $AP_{DIGESTS.}$ This effect was most pronounced for danazol, both at 5 (Figure 2.5) and 20 mM bile salt (Figure 2.6), where solubilities at 40 mM calcium are significantly lower than the values attained at 5 mM calcium. The overall trend, however, of high calcium and a low solubilization capacity of $AP_{DIGESTS}$ was constant for both drugs at both bile salt concentrations. Notably, the effect of calcium on danazol solubility in the AP_{DIGEST} was most pronounced at the higher bile salt concentration, while for fenofibrate, the effect of calcium was most pronounced at lower bile salt concentration.

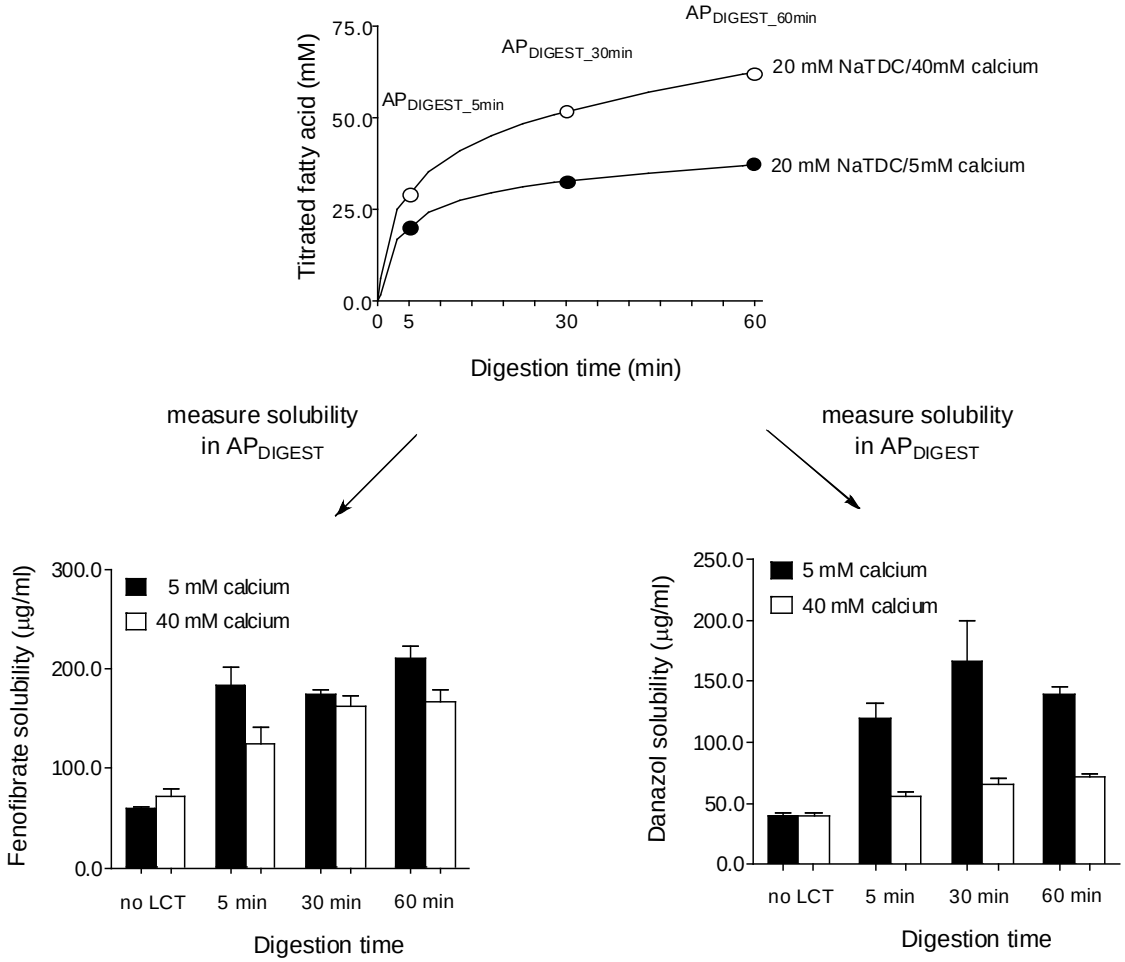


Figure 2.6:Titrated fatty acid profiles describing the digestion of LCT in digestion media containing 20 mM bile salt/5 mM calcium (closed symbols) or 20 mM bile salt/40 mM calcium (open symbols) and the determination of fenofibrate (left) and danazol (right) equilibrium solubility in the AP_{DIGEST} isolated following LCT digestion for 5 min (AP_{DIGEST_5min}), 30 min (AP_{DIGEST_30min}), and 60 min (AP_{DIGEST_60min}). Solubility studies in the $AP_{DIGESTS}$ were performed at 37°C over a 48 h equilibration period. The equilibrium solubility of fenofibrate and danazol in the digestion medium (no LCT) containing 20mM bile salt/5 mM calcium and 20mM bile salt/40mM calcium are also shown.

2.4 Discussion

In vitro digestion models are increasingly being used to assess the performance of lipid-based formulations (LBF) under conditions that mimic lipid digestion in the small intestine (Williams et al., 2012h, Anby et al., 2012d, Larsen et al., 2011b, Williams et al., 2012d, Taillardat et al., 2007b, Thomas et al., 2012a, Ahmed et al., 2012, Tan et al., 2011a). A potential disadvantage of the *in vitro* digestion model is that it is a 'closed' system: as the LBF undergoes enzymatic-mediated hydrolysis, the concentration of lipid digestion products (i.e., FA and MG) will progressively increase, and, in the absence of an appropriate sink (*in vivo*, these digestion products are effectively removed via intrinsic lipid absorption pathways), a build-up of digested lipids at the surface of an oil droplet surface can block further binding of the pancreatic lipase-colipase complex thereby limiting further digestion. This inhibition can complicate the interpretation of in vitro testing of highly oil-rich LBF since a large proportion of drug following 30–60 min digestion of a LBF may remain sequestered within a partially digested oil phase (Williams et al., 2012d).

By binding to FA and forming insoluble complexes, calcium ions can effectively strip digestion products from the oil droplet surface (Alvarez and Stella, 1989, Armand et al., 1992). While this property explains why high concentrations of calcium are often added to digestion tests to drive digestion of long-chain triglycerides (LCTs) and other lipid substrates (Zangenberg et al., 2001b, Zangenberg et al., 2001a, Christiansen et al., 2010b, Hwang et al., 2009), the effects calcium ions have on phase behavior and solubilization properties of colloidal lipids are not well understood.

Soybean oil, a LCT widely used in lipid drug delivery, was used to represent a slowly digesting lipid substrate (at least, *in vitro*), and was digested under conditions of varying bile

salt and calcium concentration (see Figure 2.2 and Figure 2.3). In the absence of calcium, increasing bile salt concentration led to a progressive increase in the rate and extent of LCT digestion (Table 2.1), which is consistent with the previous reports of increased digestion of LC lipids with increasing bile salt (Carey et al., 1983, Bernback et al., 1990, Williams et al., 2012d, MacGregor et al., 1997). Very high bile salt concentrations (i.e., > 20 mM), in excess of likely concentrations in the small intestine in both fasted and fed conditions (Persson et al., 2006, McConnell et al., 2008), were utilized in order to further examine the potential for increases in solubilization capacity of the aqueous phase, to increase the extent of digestion. However, digestion of the LCT by the end of the experiment was (at best) only 75% complete. An initial period of fast digestion was observed in the presence of bile salt, but this was not sustained beyond 10 min. Digestion profiles are therefore 'biphasic' (Figure 2.2). Similar profiles have been observed previously during *in vitro* digestion testing of LCT under the same (Sek et al., 2002a, Han et al., 2009a) and similar (Li et al., 2011) experimental conditions, and this slow and incomplete digestion of LCT is known to manifest through a mechanism that involves the accumulation of digestion products (i.e., FA and MG) at the oil droplet surface limiting access of the lipase-colipase complex to the substrate (Patton and Carey, 1979, Scow et al., 1979, brockerhoff and Jensen, 1974).

The fact that increasing bile salt concentration allows the LCT to reach a higher extent of digestion suggests that the presence of a larger solubilization reservoir for digestion products allowed greater binding between the pancreatic lipase-colipase complex and the lipid substrate. A recent study by Williams et al. noted, however, that correlations between concentrations of titrated (i.e., ionized) FA and total lipid digestion were not always apparent when bile salt concentration was increased (Williams et al., 2012d). Underpinning this effect was the partial ionization of long-chain FA at pH 6.5 (the experimental pH used by Williams

et al),and consequently, FA ionization that was highly sensitive to changing solubilization conditions attained on increasing bile salt concentration. In the present study, however, we have used a higher experimental pH (pH 7.5) to ensure more efficient titration of FA (Patton and Carey, 1981, Sek et al., 2001); a lower sensitivity of FA to changes in the degree of ionization in the presence of bile salt is therefore expected. Furthermore, the effects of bile salt on FA ionization described by Williams et al. were most pronounced at low bile salt concentrations (i.e., < 5mM), and became less evident at higher bile salt concentrations (i.e., up 10 mM). The effects of bile salt on concentrations of titrated FA in the present study are therefore on balance more likely to result from the continued digestion of the residual oil phase.

An alternative approach to bile salt concentration, namely the use of higher calcium concentrations, was taken to increase the extent of digestion of LCT. Calcium has been used previously to push the digestion of long-chain lipid substrates towards completion (Hwang et al., 2009, Christensen et al., 2004b, Zangenberg et al., 2001a) and increasing calcium concentration in the present study led to increased digestion of the LCT (Figure 2.3) is consistent with these previous studies; with digestion complete at the highest calcium concentration (40 mM). Underpinning the pronounced effect of calcium on digestion is its capacity to allow pancreatic lipase to continually access the surface of the oil droplet by binding to and subsequently removing FA from this surface in the form of calcium soaps (Alvarez and Stella, 1989, Patton and Carey, 1979). Alternative roles of calcium include the capacity to reduce the surface charge of oil droplets (which may reduce the electrostatic repulsion between the enzyme and its substrate therefore promoting binding (Armand et al., 1992, Wickham et al., 1998)) and being required as a co-factor to activate pancreatic lipase (Alvarez and Stella, 1989, Kimura et al., 1982). However, studies by Macgregor (MacGregor

et al., 1997) have shown that calcium effects on lipid digestion are most prominent for LC lipid substrates, and consequently, a general consensus that the role of calcium in promoting *in vitro* digestion primarily involves the efficient removal of digestion products from the oil droplet surface has emerged. In the present study, LCT digestion on the addition of 5 mM calcium increased by >6-fold (Table 2.1). Since this increase in digestion induced by a low calcium concentration is too great to be explained solely through FA complexation, the calcium effect (at this low concentration) probably reflected complexation and its capacity to increase pancreatic lipase activity (Kimura et al., 1982, Whayne and Felts, 1971, Alvarez and Stella, 1989).

While the use of high concentrations of calcium ions in the present study led to the complete LCT digestion (~106% at 40 mM calcium, (Table 2.1), digestion extent decreased dramatically to 61% on the addition of 5 mM bile salt and to 88% at 20 mM bile salt. Thus, the beneficial effects of calcium to LCT digestion were attenuated by bile salt at a concentration most likely reflecting true conditions of the fasted small intestine (Hofmann and Mysels, 1992, Lindahl et al., 1997, Persson et al., 2006).

The apparent interplay between bile salt and calcium on LCT digestion rate and extent was not the focus of the present study, and while many explanations may conceivably describe these findings, such possibilities are only briefly discussed. These include the activity of pancreatic lipase is reversely decreased in the presence of micellar quantities of bile salts and phospholipids (Patton and Carey, 1981) due to bile salt-phospholipid mixed-micelles providing an alternative binding site for the pancreatic lipase (Patton and Carey, 1981, Patton and Carey, 1979), the ability of phospholipid to coat the oil droplet surface and therefore prevent lipase binding(Patton and Carey, 1981), and solubilization and subsequent displacement bound lipase at the surface of the oil droplet by bile salt-phospholipid micelles

(Borgstroem et al., 1963, Bauer et al., 2005). Therefore, while increasing bile salt and PC concentration led to increased LCT digestion in the present study, it is also plausible that lipid digestion was simultaneously inhibited through the effects described above. The inhibitory effects of bile salt and phospholipid mediated at the surface of the oil droplet are however thought to be mitigated by colipase, since the pancreatic lipase-colipase complex shows an enhanced binding affinity towards hydrophobic surfaces (Patton et al., 1978, Bezzine et al., 1999), and since colipase is present within the crude porcine pancreatin extract used in the present study (Patton et al., 1978), and these inhibitory affects may have been reduced.

Aside from effects on lipase, conjugated bile acids have been shown to interact and form salts with calcium ions (Hofmann and Mysels, 1992, Gu et al., 1992, Jones et al., 1986). There are also reports of decreasing bile salt solution concentration with the continuous titration of calcium during *in vitro* lipid digestion studies (Christensen et al., 2004b, Zangenberg et al., 2001b). The combined use of calcium with bile salts, and the subsequent formation of poorly soluble calcium salts of bile acid, may therefore decrease the available concentration of both species. However, the aforementioned studies that associated decreasing bile salt concentration with increasing calcium have utilized a crude bile salt extract (i.e., porcine derived) containing a mixture of glycine- and taurine-conjugated bile salts. In contrast, taurodeoxycholate was the only bile salt used in the present study. Since calcium salts of taurine-conjugated bile acids are much more soluble than those glycine-conjugated bile acids (Hofmann and Mysels, 1992, Gu et al., 1992, Jones et al., 1986), bile salt precipitation in the present study was less likely.

In some industries, it is common to use calcium ions to achieve higher concentrations of MG during enzymatic hydrolysis of oils, where build-up of FA in the absence of calcium is usually limiting to the MG yield (Hwang et al., 2009). From a drug delivery perspective, the

fate of both FA and MG are important since they are known to contribute to the solubilization capacity of the intestinal milieu (Kossena et al., 2005, Porter et al., 2007a, Charman et al., 1993). Indeed, several recent studies have discussed the link between solubilization properties of a digested lipid formulation and the risk of drug precipitation (Anby et al., 2012d, Williams et al., 2012d, Porter et al., 2011). We have therefore used the measured drug solubility in the digests to probe for the solubilization capacity of the phase. Digests were formed under varying bile salt and calcium conditions to determine if drug solubilization could be related to the extent of lipid digestion.

The solubility results (Figure 2.5 and Figure 2.6) compare the solubilization capacity of $AP_{DIGESTS}$, formed under conditions of varying bile salt and calcium concentration, towards the model PWSDs and show that the solubilization capacity of the digests is markedly decreased at high calcium concentrations. This lower solubilization capacity of the digests formed under higher calcium concentration was in spite of a higher extent of digestion. While conventional understanding of lipid digestion reasons that increasing the digestion of LC lipids will lead to an enrichment of the colloidal aqueous phase with lipid digestion products (and an increased solubilization capacity of this phase), the results of the present study show that such enrichment does not occur at high calcium. Instead of enhancing the solubilization capacity of the bile salt-phospholipid phase, the presence of calcium ions leads to the greater part of liberated FA from LCT forming insoluble complexes, and as described schematically in

Figure 2.7: , an AP_{DIGEST} depleted of digestion products.

Since the solubility of danazol and fenofibrate in simple bile salt – phosphatidylcholine media was not influenced by the concentration of calcium ions, the potential for calcium induced bile acid precipitation (Gu et al., 1992, Hofmann and Mysels, 1992) to affect solubility results

in the present study is small. Furthermore, the reduction in solubilization capacity of the AP_{DIGEST} at the high calcium concentration was evident both at 5 mM and 20 mM bile salt concentrations, and as described earlier, bile salt precipitation is likely to be more relevant to those glycine-conjugated bile salts (rather than taurine-conjugated which were used here).

The implications of these findings are that the use of high calcium concentrations during *in vitro* testing of LBDDS does promote digestion, however the sink phase produced by calcium may be biased towards the digestion products only. As a result, calcium creates an imbalance between the solubilization capacity of a digesting lipid formulation (by depleting the colloidal phases of digestion products) and the solubilized drug concentration (which is not affected directly by calcium), with the eventual outcome of an increase likelihood of drug precipitation. The use high calcium concentrations may therefore overestimate the extent drug precipitation that may occur *in vivo* in the intestine, potentially to a point where correlations between *in vitro* and *in vivo* data sets cannot be made. Sinks that provide sink conditions for digestion products and drug (i.e., those mimicking the absorptive membrane in the GI tract) may therefore avoid the imbalance provided by a preferential sink such as calcium.

Relationship between the lipid digestion product fate and drug solubilization of the AP_{DIGEST} *in vitro:*

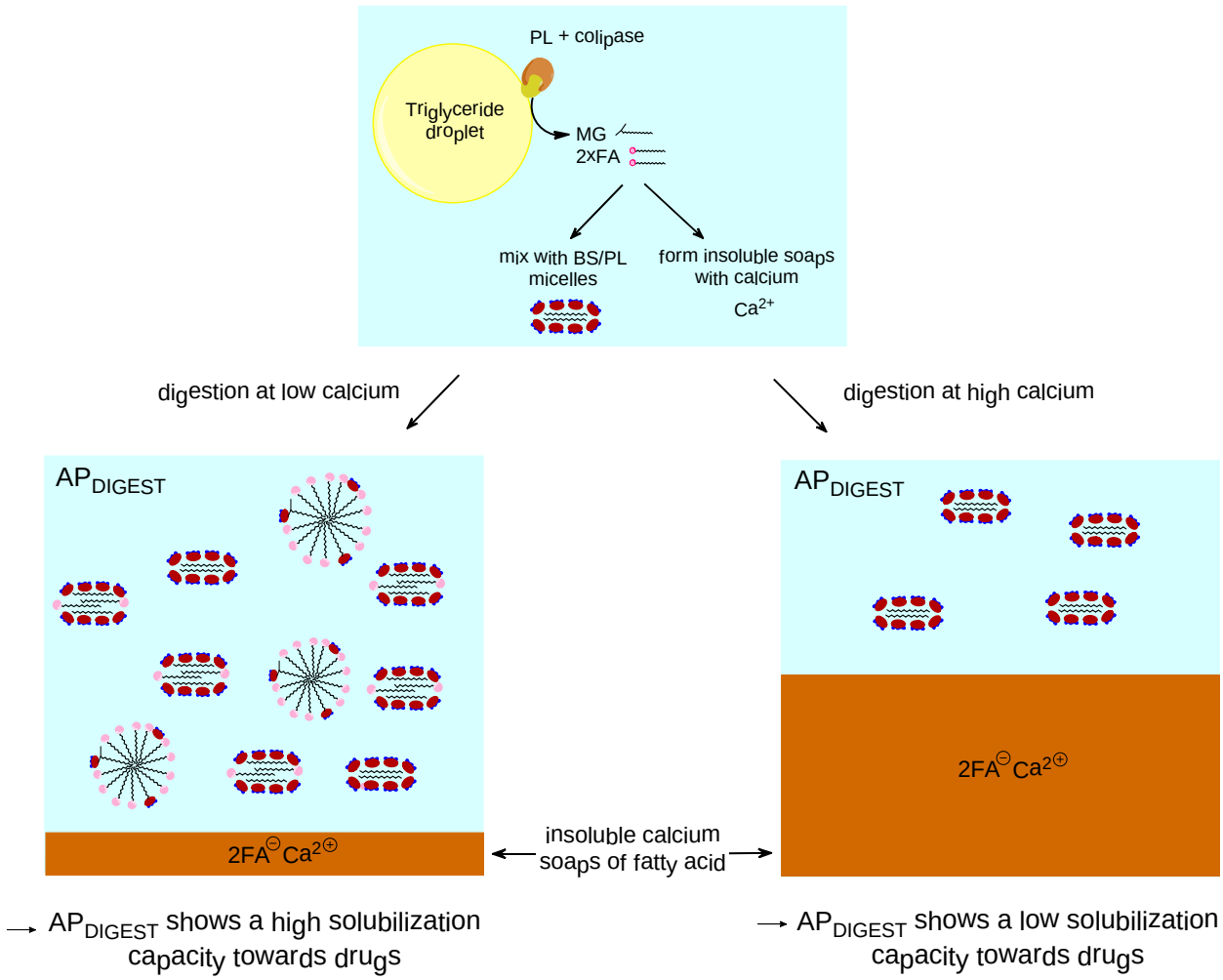


Figure 2.7: Schematic to illustrate the general process of triglyceride digestion under the action of pancreatic lipase (PL)-colipase complex (upper panel) and the subsequent fate of digestion products (fatty acid (FA) and monoglyceride (MG)). Digestion of triglyceride in the intestine is mediated primarily via PL and its cofactor, colipase. Digestion of one LCT molecule liberates two molecules of FA and one molecule of monoglyceride. Molecules of FA may become incorporated into bile salt-phospholipid mixed micelles in the bulk aqueous phase. These colloids swell in size following incorporation of digestion products and contribute significantly to the solubilization capacity of the AP_{DIGEST}. However, liberated FA shows a tendency of forming insoluble soaps in the presence of calcium ions. FA are therefore not contributing to the solubilizing properties of the AP_{DIGEST}

2.5 Conclusions

In vitro digestion models are increasingly being utilized to predict the *in vivo* performance of lipid-based drug delivery systems (LBDDS). While some models have in the past utilized calcium ions to push the lipid digestion process to completion, the consequence of such an approach on drug solubilization has not been investigated. In this study, we showed that increasing calcium is a much more effective approach in promoting LCT digestion compared with increasing bile salt concentration. However, the much lower solubilities of danazol and fenofibrate in the digests formed under conditions of high calcium indicate that this use of calcium is to the detriment of drug solubilization. We have attributed this finding to calcium selectively removing fatty acids from solution (by precipitating fatty acid calcium soaps), leading to an effective decrease in the concentration of fatty acids that are involved in micellar and vesicular drug solubilization. Therefore, the implication to *in vitro* testing of LBBDS is that the use of calcium to promote digestion may exaggerate the decrease the solubilization capacity of the lipid formulation as it digests and, in turn, overestimate the extent of drug precipitation that occurs in the intestine.

Chapter 3: Research paper 2

In vitro assessment of of drug-free and fenofibrate-containing lipid formulations using dispersion and digestion testing gives detailed insights into the likely fate of formulations in the intestine.

Abstract

The solubilizing properties of lipid-based formulations (LBFs) can change dramatically following dispersion and digestion of the formulation components. This study investigated the performance of self-emulsifying LBFs consisting of four different long-chain (LC)/medium-chain (MC) lipid blends formulated with the lipophilic drug fenofibrate and either a water-insoluble surfactant polysorbate 85 (Tween® 85) or its more hydrophilic relative, polysorbate 80 (Tween® 80). These components allowed closely related Type II and IIIA LBFs of fenofibrate to be evaluated during *in vitro* dispersion and *in vitro* digestion testing. Initial assessment of the solvent capacity of drug-free LBFs during dispersion and digestion revealed that the solubility of fenofibrate was more dependent on the surfactant type rather than lipid composition. Type II LBFs in the dispersed state were generally better at solubilizing fenofibrate than equivalent Type IIIA LBFs, regardless of lipid composition. However, even when high drug loadings were used, supersaturation/drug precipitation after dispersion of Type II or Type IIIA LBFs was only moderate. In contrast, digestion of both Type II and IIIA LBFs led to much higher levels of drug supersaturation, and this resulted in drug precipitation. After digestion the ability of each LBF to maintain drug in a solubilized state was highly dependent on lipid composition as well as the choice of surfactant. Notably, MC lipids exhibited very good solubilizing properties in the dispersed state, but resulted in a higher degree of supersaturation on digestion, leading to higher susceptibility to drug precipitation. This study showed that replacing LC lipids with MC lipids in Type II and IIIA LBF, in the proportions used in this study, has little effect on fenofibrate solubilization during dispersion, but is likely to promote supersaturation on digestion. Without careful consideration of drug loading and choice of surfactant in Type II/IIIA MC lipid formulations, there is a high risk of precipitation of drug in the intestine.

3.1 Introduction

It is well recognized that low solubility and/or a slow dissolution rate can be severely limiting to drug absorption from the gastro-intestinal (GI) tract. Unfortunately, an increasing number of molecules in drug discovery and development exhibit these hydrophobic characteristics (Williams et al., In press). However, lipid-based formulations (LBFs), particularly those administered as liquid-filled capsules, present a formulation strategy through which the oral bioavailability of poorly water-soluble drugs (PWSD) may be improved (Porter et al., 2007b, Porter et al., 2004d, Hauss, 2007a, Charman et al., 1992).The primary mechanism through which LBFs enhance drug absorption is that the drug is pre-dissolved in the lipid formulation, eliminating the dissolution required by solid phase delivery systems. LBFs can therefore provide a high concentration of solubilized drug in the GI lumen, which enhances the rate and extent of drug absorption (Porter et al., 2008b, Porter et al., 2007b, Charman et al., 1992). Depending on the LBF composition and its location within the GI tract, the co-administered drug is likely to be solubilized within many different colloidal species, ranging from emulsified oil droplets in the stomach to smaller and less lipophilic micellar phases, which result from the digestion of lipids from the formulation in the small intestine. The digestive processing of a LBF is a consequence of the natural way in which the GI tract responds to the presence of lipids. Therefore an understanding of the fate of the drug during the dispersion/emulsification process and subsequent digestion of its lipid-based vehicle is necessary in order to fully predict the *in vivo* performance of a LBF (Porter et al., 2007b).

During dispersion of a LBF, hydrophilic formulation components are likely to partition into the aqueous GI fluids. This will decrease the solubilizing capacity of the formulation for hydrophobic drug, in turn creating the risk of drug precipitation in instances where drug concentrations exceed solubilizing capacity (i.e., supersaturation). Precipitation is

undesirable as this regeneration of the solid-state re-introduces the need for the drug to dissolve prior to absorption. The second critical process is digestion of the LBF, which occurs primarily in the small intestine (Bakala N'Goma et al., 2012a). Both lipid components and surfactants containing ester groups from the LBF are highly susceptible to enzymatic hydrolysis. Since hydrolysis will significantly alter their physicochemical properties, *in vitro* digestion models are widely used to better understand the *in vivo* performance of LBFs (Dahan and Hoffman, 2008, Thomas et al., 2012a, Anby et al., 2012e, Tan et al., 2012a, Cuine et al., 2008b, Sassene et al., 2010). Efforts to establish standardized digestion conditions are also underway in an initiative supported by the LFCS Consortium (Williams et al., 2012g, Williams et al., 2012c).

To model digestion *in vitro*, the LBF is dispersed in a medium that is representative of the contents of the upper small intestine, and digestion is then initiated by the addition of a porcine-derived pancreatic extract containing pancreatic lipase and other pancreatic enzymes. An immediate descriptor of LBF performance is the rate and extent of LBF digestion, determined from the rate of addition of sodium hydroxide required to maintain a designated pH, to correct for the effect of liberated fatty acid which decreases the pH of the medium. Samples may be removed at intervals during the digestion test, and centrifuged to allow separation of three distinct phases, namely; a pellet phase consisting of insoluble calcium soaps of fatty acid; an aqueous colloidal phase consisting of amphiphilic digestion products, bile salt and phospholipids; and an oily phase containing a mixture of incompletely digested lipid and more lipophilic digestion products. To determine the solubilization capacity of the digested formulation, excess crystalline drug may be mixed with the aqueous colloidal phase formed by digestion of a drug-free LBF. Alternatively, if a drug is included in the LBF prior

to digestion, the distribution of drug within the three phases produced during digestion can be quantified to evaluate the effect of digestion on the fate of an incorporated drug.

Several studies have correlated evidence of drug precipitation during *in vitro* dispersion/digestion with decreased *in vivo* bioavailability (Anby et al., 2012e, Porter et al., 2004d, Porter et al., 2004c, Han et al., 2009b, Dahan and Hoffman, 2006a, Cuine et al., 2008b, Cuine et al., 2007b), and while there are some examples to the contrary (i.e., examples when formulations have demonstrated *in vivo* performance that surpassed expectations based on *in vitro* studies), these could be explained by the formation of a fast-dissolving non-crystalline drug precipitate (Thomas et al., 2012a), or dose-dependency in first-pass drug metabolism (Anby et al., 2012b). Most of the published studies indicate that LBFs that result in drug precipitation *in vitro* are likely to have poorer *in vivo* performance when compared to more robust LBFs.

The aim of the present work was to explore a series of closely related formulations of fenofibrate to determine the factors that affect the performance of LBFs in *in-vitro* dispersion and digestion tests, with a particular emphasis on drug solubilization/precipitation. This approach is an ideal way to identify formulations that might be expected to perform well or poorly *in vivo*, and will facilitate selection of a limited number of formulations to take forward into *in vivo* studies, to explore whether *in vitro-in vivo* correlation can be achieved using *in vitro* digestion testing. The LBFs investigated were all Type II or Type IIIA, as defined by the Lipid Formulation Classification System (LFCS) (Pouton, 2000a, Pouton, 2006a). A minimum number of excipients were used so that LBFs were closely related in term of chemical composition. Type II formulations are highly lipophilic formulations that form turbid emulsions on dispersion in aqueous fluids, typically with a mean droplet diameter in the range 250-3000nm. Type IIIA formulations may contain the same lipid composition as

those in Type II, but in addition contain hydrophilic components such as a water-miscible surfactant or co-solvent. The incorporation of these additional hydrophilic components is associated with improved dispersion and the potential of forming ultrafine (i.e., nano-sized) emulsions with aqueous media. The solubilizing properties of a series of Type II and IIIA LBFs toward the model drug fenofibrate (Figure 3.1) was investigated. With a clinical dose in excess of 100 mg (Keating and Croom, 2007) and low aqueous solubility (Vogt et al., 2008), fenofibrate is a BCS class II compound. Fenofibrate, however, exhibits a high solubility in lipids (>75 mg/g), and together, this low aqueous solubility/high lipid solubility combination is characteristic of the most ideal candidates for oral lipid drug delivery. The study was divided into two parts; the solubilization studies in Part 1 providing the necessary data to allow the subsequent digestion studies of fenofibrate LBFs (Part 2) to be interpreted in terms of drug supersaturation.

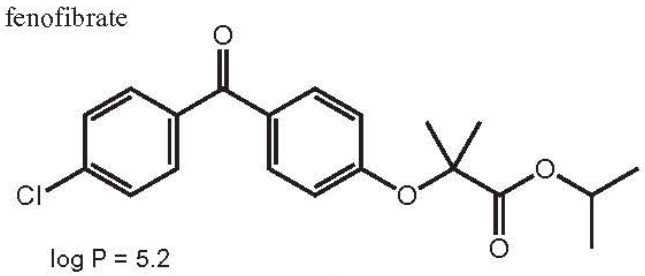


Figure 3.1: The chemical structure and properties of the model drug, fenofibrate

a – Vogt et al. (2008); b - this work.

3.2 Materials and methods

3.2.1 Materials

Fenofibrate, soybean oil (a long-chain triglyceride), polyoxyethylene (20)-sorbitanmonooleate (polysorbate 80, Tween® 80, HLB 15), polyoxyethylene (20)-sorbitantrioleate (polysorbate 85, Tween® 85, HLB11), sodium taurodeoxycholate > 95% (NaTDC), pancreatin extract (from porcine pancreas, P7545, 8 x USP specifications activity), calcium chloride dihydrate, Tris-maleate, and the lipid digestion inhibitor 4-bromophenylboronic acid (4-BPB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Miglyol® 812 (a medium-chain triglyceride) and Imwitor® 988 (a blend of medium-chain mono- and diglycerides) were supplied by Sasol Germany GmbH (Werk Witten, Witten-Germany). Maisine^{TM}35-1 (a blend of long-chain mono-, di and some triglyceride) was supplied by Gattefosse (Saint-Priest, France). Lecithin (ca. 99.2% egg-phosphatidylcholine (PC), Lipoid E PCS) was purchased from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). 1.0M sodium hydroxide (Univol) was purchased from Ajax Finechem Pty Ltd (Sydney, Australia) and was diluted with water (Milli-Q water purification system, Millipore, Bedford, MA) to achieve a 0.6M titration solution. Methanol and chloroform used in this work were HPLC grade from Merck (Melbourne, Australia).

3.2.2 Lipid formulations

The composition of the eight lipid-based formulations (LBFs) investigated in this study are shown in Table 3.1. Each LBF contained 50% lipid and 50% surfactant. The lipid component in the LBFs consisted of 35% long-chain triglyceride (LCT; soybean oil) or medium-chain triglyceride (MCT; Miglyol® 812), and 15% long-chain glyceride mixture (LCMix; Maisine^{TM} 35-1) or medium-chain glyceride mixture (MCMix; Imwitor® 988). As seen in

Table 3.1, LBFs contained either all LC lipids, all MC lipids, or a blend of LC and MC lipids. For Type II LBFs, the water-insoluble surfactant Tween®85 was used. Type IIIA LBFs used the more hydrophilic Tween® 80 surfactant.

Table 3.1: composition of the Type II and IIIA LBFs investigated in this study

Lipid Blend	**Formulation component (% w/w)**					
	Soybean oil (LCT)	Miglyol® 812 (MCT)	Maisine™ 35-1 (LCM)	Imwitor® 988 (MCM)	Tween® 85	Tween® 80
Type II:						
LCT/LCM	35%	-	15%	-	50%	-
LCT/MCM	35%	-	-	15%	50%	-
MCT/LCM	-	35%	15%	-	50%	-
MCT/MCM#	-	35%	-	15%	50%	-
Type IIIA:						
LCT/LCM	35%	-	15%	-	-	50%
LCT/MCM	35%	-	-	15%	-	50%
MCT/LCM	-	35%	15%	-	-	50%
MCT/MCM #	-	35%	-	15%	-	50%

– the properties of these Type II and IIIA LBF on dispersion have also been investigated by Mohsin et al. (2009a).

3.2.3 Part 1: Fenofibrate solubility assessment

3.2.3.1 Anhydrous excipients and investigated LBFs

Crystalline fenofibrate was added in excess to glass sample tubes containing 3 g of each of the anhydrous excipients and LBFs shown in Table 3.1. Drug-excipient/LBF slurries were vortex-mixed and then incubated at 37^{o}C in an orbital mixer (Ratek Instruments, Melbourne, Australia) to provide continuous mixing during the equilibration period. At 24 h intervals over 6 days, a 0.5 g sample was removed and centrifuged (Eppendorf 5408R, Eppendorf AG, Hamburg, Germany) at 1600*g* for 15 min at 37^{o}C. Centrifugation separated the samples into a solid pellet phase and a particle free supernatant. Accurately weighed samples were removed from the supernatant, transferred to 5 ml volumetric flasks and made up to volume with chloroform: methanol (2:1 v/v). Aliquots (50-100 µl) were subsequently diluted >100 fold with methanol. Analysis of fenofibrate content in all cases was conducted using a UV spectrophotometer (Cecil CE 3021; Cecil Instruments, Ltd., UK) measuring absorbance at 286nm, with the exception of fenofibrate/Tween® samples, which were analyzed for fenofibrate content by HPLC (section 3.2.7). Equilibrium solubility in the anhydrous excipients and LBFs was defined as the value attained when consecutive solubility values differed by <5%.

3.2.3.2 Dispersed and digested surfactants Tween® 85 and Tween® 80

The solubilization capacity of the surfactants Tween® 80 and Tween® 85 post-dispersion and post-digestion was determined by equilibrium solubility measurements. A series of Tween® 85/Tween® 80 surfactant solutions (0.5%, 1.25% and 2.5% w/v) were prepared in a digestion medium (pH 7.5, 50 mM Tris-maleate, 150 mM sodium chloride, 5 mM calcium chloride dihydrate, 5 mM NaTDC and 1.25 mM PC). Fenofibrate was added in excess to 10 ml

samples of the dispersed surfactant preparations and subsequently incubated at 37°C in an orbital mixer (Ratek Instruments). After a 48 h period of equilibration, 1 ml samples were removed and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min to separate suspended drug from the solutions or colloidal dispersions of surfactants. A 50-100 µl aliquot of the homogenous supernatant was diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see section 3.2.7).

To assess the solubilization capacity of the surfactants (at 1.25%) post-digestion, *in vitro* digestion tests were carried out using a method described previously (Devraj et al., 2012a, Sek et al., 2002b). In brief, 0.125 g of surfactant was dispersed in 9 ml digestion medium (pH 7.5, see above) for 10 min followed by the addition of 1 ml pancreatin containing 10 000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per ml of digestion medium. Digestion of the surfactant was continuously monitored using a pH-stat automatic titration unit (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.6M NaOH. We chose to use 5 mM NaTDC and 1.25 mM PC deliberately to represent the fasted state. This is because drugs such as fenofibrate are absorbed best in the fed state and suffer from low bioavailability when administered to a fasted stomach. Our intention is to use the *in vitro* tests to examine the likely fate of drugs in the fasted intestine.

After 30 min digestion, 2 x 4 ml samples were collected from the reaction vessel and quenched using the lipid digestion inhibitor 4-BPB (0.5M in methanol, 9µl/ml of sample). Samples were then ultra-centrifuged (400 000*g*, 37°C, Optima XL-100K Ultracentrifuge, SW-60 swinging-bucket rotor, Beckman, Palo Alto, CA) in soft-walled polyallomer tubes (Beckman) for 30 min to separate each digestion sample into a colloidal aqueous phase (AP_{DIGEST}) and a pellet phase. Fenofibrate was added in excess to 2 ml samples of each

AP_{DIGEST} and its solubility determined using the equilibration/sampling method described above for the dispersed surfactant solutions.

3.2.3.3 Dispersed and digested LBFs

The solubilization capacity of the LBFs shown in Table 3.1 post-dispersion and post-digestion was determined by equilibrium solubility measurements. The principles described in section 3.2.3.2 were used, however the more complex phase behavior of the LBFs post-dispersion and post-digestion required a variations in the methods, which are described below.

In the dispersion tests, 1 g of LBF was weighed into 100 ml volumetric flasks and made up to volume with water. Flasks were subsequently incubated at 37°C in an orbital mixer (Ratek Instruments). After mixing for 30 min to allow complete dispersion of the LBF, 3 x 10 ml aliquots were removed, mixed with excess fenofibrate and incubated at 37°C in an orbital mixer (Ratek Instruments). At intervals (i.e., 24 and 48 h), 1 ml samples were removed and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min. In instances where the centrifugation process resulted in the phase-separation of an oily cream phase from the aqueous phase (which was typical for the Type II LBFs containing LC lipids), this cream was gently re-dispersed with the bulk of the aqueous phase using an adjustable pipette (so as to not disturb any pellet phase). A 50-100 µl aliquot of the homogenous supernatant was removed and subsequently diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see section 3.2.7).

In the digestion experiments, 0.25 g of LBF was digested in 9 ml digestion medium and 1 ml pancreatin, using the method described in section 3.2.3.2. Digestion samples (2 x 4 ml) were removed after 30 min and separated by ultracentrifugation (also as described in

section3.2.3.2) to separate the digestion phases, namely a poorly dispersed oil phase (in the case of the more lipophilic LBF), a colloidal aqueous phase (AP_{DIGEST}) and a pellet phase. Sample tubes were pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the AP_{DIGEST}. This approach was essential to ensure that an oil phase (which collected at the top of the sample) was not carried over into the AP_{DIGEST}. Fenofibrate was added in excess to 3ml AP_{DIGEST}, and equilibrated for 48 h, during which 1 ml samples were removed at intervals (i.e., 4, 8, 24 and 48 h), centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min. A50-100 µl aliquot of each homogenous supernatant was diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see section3.2.7). Equilibrium solubility in the dispersed/digested LBFs was defined as the value attained when consecutive solubility values differed by <5%.

3.2.4 Part 2: In vitro assessment of fenofibrate-containing LBF

3.2.4.1 Drug incorporation

All LBFs in Part 2 of this study were loaded with fenofibrate at 80% of its equilibrium solubility in the respective anhydrous formulation (determined method described in section 3.2.3.1 and results presented in section3.3.1.1). The required mass of fenofibrate was weighed directly into clean screw-top glass vials and drug-free LBF was added up to the target mass loading. Vials were sealed, vortex-mixed and incubated at 37°C for at least 24 h prior to testing.

The fenofibrate content in the formulation was verified (in triplicate) on the day of testing using the sampling procedure (without centrifugation) described in section 3.2.3.1 before the fenofibrate content was determined by HPLC (section3.2.7).

3.2.4.2 In vitro dispersion testing

Dispersion testing of fenofibrate containing LBF was conducted in accordance with the method described in section 3.2.3.3: The use of the same methods allowed direct comparison of the solubility results (Part 1) to the evaluation during dynamic studies (Part 2), where 1g of LBF containing fenofibrate at 80% solubility was dispersed in 100ml water, and incubated at 37°C in an orbital mixer (Ratek Instruments). To measure the concentration of fenofibrate that remained solubilized in these tests, 1 ml samples were withdrawn at intervals (0.5, 1, 2, 4, 8, 16, 24, 32, 48, 72, and 96h) and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min to sediment any drug precipitate. Consistent with section 3.2.3.2, in instances where the centrifugation process resulted in the phase-separation of an oily cream phase from the aqueous phase, this cream was gently re-dispersed with the bulk of the aqueous using an adjustable pipette so as to not disturb any pellet phase. A 100 µl aliquot of the homogenous dispersion was diluted >10 fold before analysis of fenofibrate content by HPLC (see section 3.2.7).

3.2.4.3 In vitro digestion testing

In vitro digestion experiments were performed in accordance with the method described in section 3.2.3.3, where 0.25g LBF containing fenofibrate at 80% solubility was digested for 30 min in 9ml digestion medium (plus 1 ml pancreatin). In these experiments, the fenofibrate concentration across each of the digestion phases in the centrifuged samples was determined using the following method. Firstly, the oil phase in the digestion samples was carefully aspirated using an adjustable pipette and transferred to a 10 ml volumetric flask, followed by 50 µl of 1M HCl and chloroform-methanol mixture (2:1 v/v) up to volume. The sample tubes were then pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the

AP_{DIGEST}. Finally, to remove the pellet, the polyallomer tube was cut just above the mass of the pellet phase and suspended in 100 µl chloroform-methanol mixture (2:1 v/v). The pellet was then transferred to a 5 ml volumetric flask followed by 50 µl of 1M HCl and chloroform-methanol mixture (2:1 v/v) up to volume. Each of the recovered phases was further diluted >10-fold in methanol prior to HPLC analysis (see section 3.2.7) to determine the fenofibrate content in individual phases.

In certain digestion experiments, it was necessary to remove more than two 4 ml samples. In these tests, experiments were scaled up to 0.5 g of LBF and 18 ml digestion medium, prior to the addition of 2 ml pancreatin, as described above.

3.2.5 Extent of surfactant and LBF digestion

Digestion profiles were corrected for the background fatty acid released upon digestion of the bile salt/phospholipid mixed-micelles; this concentration was determined in separate experiments undertaken in the absence of surfactant/LBFs. The total concentration of fatty acid titrated over 30 min (corrected for the background fatty acid) was compared to the theoretical quantity of fatty acid that could be liberated if the surfactants/LBFs were completely hydrolyzed to provide an estimation of the extent of digestion using Equation 1. In-line with previous work (Cuine et al., 2008b), it was assumed that on digestion of lipids, one triglyceride molecule released two fatty acid molecules, and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a single fatty acid molecule. In the case of the surfactants, it is assumed that all fatty acids are available for hydrolysis, which is consistent with previous work.

$$\text{Extent (\%) of digestion} = \frac{\text{titrated fatty acid (mmoles)} \times 100}{\text{Theoretical maximum titratable fatty acid (mmoles)}} \quad \text{Equation 1}$$

3.2.6 Supersaturation

Solubility values in the dispersed and digested LBFs determined in Part 1 of this study were used to calculate the supersaturation ratio (SR) during dispersion/digestion testing of fenofibrate-containing LBFs in Part 2 via Equations 2a or 2b.

During dispersion:

$$SR = \frac{\text{Fenofibrate dissolved (mg)}}{\text{Fenofibrate solubility in dispersed LBF (mg)}} \quad \text{Equation 2a}$$

During digestion:

$$SR = \frac{\text{Fenofibrate dissolved in } AP_{DIGEST} \text{ (mg)}}{\text{Fenofibrate solubility in } AP_{DIGEST} \text{ (mg)}} \quad \text{Equation 2b}$$

Equations 3a or 3b were used to calculate the maximum supersaturation ratio (SR^M), which is the ratio between the fenofibrate dose in the LBF (maximum theoretical concentration of solubilized drug in the absence of any drug precipitation) and drug solubility in dispersed or digested (i.e., AP_{DIGEST}) LBF:

During dispersion:

$$SR^M = \frac{\text{Fenofibrate dose (mg)}}{\text{Fenofibrate solubility in dispersed LBF (mg)}} \quad \text{Equation 3a}$$

During digestion:

$$SR^M = \frac{\text{Fenofibrate dose (mg)}}{\text{Fenofibrate solubility in } AP_{DIGEST} \text{ (mg)}} \quad \text{Equation 3b}$$

Each of the values used to calculate SR^M values during dispersion and digestion are shown in Table 3.2

Table 3.2: Maximum supersaturation ratios (SR^M) for fenofibrate produced by dispersion and digestion of Type II and IIIA LBFs.

Formulation	Fenofibrate dose (mg) in 1g LBF [a]	Fenofibrate solubility (mg) in 1g LBF following:		SR^M	
		Dispersion [b]	Digestion (AP_{DIGEST})[c]	Dispersion [d]	Digestion [e]
Type II:					
LCT/LCM	80.3	80.7	7.6	1.0	10.6*
LCT/MCM	87.8	72.0	6.0	1.2	14.6*
MCT/LCM	101.3	97.9	5.2	1.0	19.5*
MCT/MCM	82.0	96.6	2.4	0.8	34.2
Type IIIA:					
LCT/LCM	85.1	48.5	9.2	1.8	9.3*
LCT/MCM	92.4	29.6	4.0	3.1	23.1
MCT/LCM	102.4	68.5	3.6	1.5	28.4
MCT/MCM	88.7	72.1	2.4	1.2	37.0

[a] 80% of the solubility in anhydrous LBFs; values in Figure 3.5A.

[b] 1 in 100 dilution; values in Figure 3.5B

[c] 1 in 40 dilution; values in Figure 3.5C

[d] fenofibrate dose in the LBF divided by solubility in the dispersed LBF (i.e., Equation 3)

[e] fenofibrate dose in the LBF divided by solubility in the LBF AP_{DIGEST} (i.e., Equation 3)

* SR^M values are overestimated since an oil-phase evident on digestion meant that the solubility in the AP_{DIGEST} does not reflect the entire solubilization capacity of the digested LBF.

3.2.7 HPLC assay

HPLC analysis for fenofibrate were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, MA), with a Phenomenex® Luna column (C18 (2), 150 x 4.6 mm, 3 µm, Phenomenex, Torrence, CA). The mobile phase consisted of methanol and water in a 75:25 v/v ratio pumped in isocratic mode through the HPLC column at 1 ml/min. The sample injection volume was 50 µl, with UV detection at 286 nm. All samples and standards were maintained at 10°C and the column temperature maintained at 25°C.

3.2.8 Polarized light microscopy

A Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters was used to analyse selected dispersion/digestion pellets containing fenofibrate. Following centrifugation, the pellet was carefully removed from the sample tube and placed on a microscope slide. Samples were analysed under normal light and cross-polarized light at 20x magnification, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan). Pellets were isolated and analysed in the manner described above on the same day.

3.3 Results

3.3.1 Part 1: Fenofibrate solubility studies

3.3.1.1 Anhydrous excipients and surfactants

Figure 3.2 shows the equilibrium solubility of fenofibrate in the excipients used in this study. The lowest solubility of 94.9 ± 6.3 mg/g was evident in the LCT, soybean oil. In contrast, the

highest solubility of 137.0 ± 6.2 mg/g was evident in the MCT, Miglyol® 812. Consistent with this solubility dependence on lipid chain length, fenofibrate solubility in the mixed LC glyceride, Maisine™ 35-1 (110.9 ± 4.7 mg/g) was lower than the solubility in the equivalent mixed MC glyceride, Imwitor® 988 (133.4 ± 4.0 mg/g). The investigated surfactants were Tween® 80 (polyoxyethylenesorbitanmonooleate) and the more lipophilic Tween® 85 (polyoxyethylenesorbitantrioleate) with fenofibrate solubility in the surfactants was highly comparable at 99.0 ± 2.4 mg/g and 102.1 ± 3.3 mg/g, respectively.

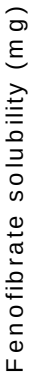


Figure 3.2: Fenofibrate equilibrium solubilities in the anhydrous lipid excipients at 37°C. SBO - soybean oil; M812 -Miglyol® 812; MSE - Maisine™ 35-1; I988 - Imwitor® 988; T80 - Tween® 80; T85 - Tween® 85. Mean± SD (n = 3).

3.3.1.2 Dispersed and digested surfactant solutions

Figure 3.3 shows the solubilization capacity of dispersed and digested Tween® 80 and Tween® 85 surfactants for fenofibrate. To allow a direct comparison with the solubility of fenofibrate in the anhydrous surfactants (taken from Figure 3.2), solubilities are expressed as

the mass of fenofibrate dissolved by 1 g of dispersed/digested surfactant. Surfactant concentrations of 0.5% w/v and 1.25% w/v represent the concentration present during the LBF dispersion studies (i.e., 1 g of the LBFs shown in Table 3.1 dispersed in 100 ml) and digestion studies (i.e., 1 g of the LBFs shown in Table 3.1 digested in 40 ml), respectively. To enable direct comparison of the solubilization capacity of dispersed and digested surfactants, solubility studies were performed in the digestion medium (containing bile salt and phospholipid). However additional studies confirmed that solubility trends were not dependent on the presence of these endogenous solubilizers (see Supplementary Information).

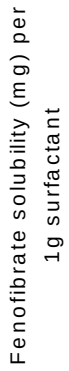


Figure 3.3: The effect of dispersion and digestion on the solubilization capacity of 1g of Tween® 85 (black bars) and 1g Tween® 80 (white bars) for fenofibrate. Solubility is expressed as mass of fenofibrate dissolved by 1g of surfactant, undiluted and at three different dilutions and after digestion (as described in methods). Mean± 1 SD (n = 3).

As expected, the fenofibrate solubility in Tween® 80 and Tween® 85 decreased with dilution in the aqueous medium. In the dispersed state, Tween® 85 exhibited a higher solubilization capacity than Tween® 80, This higher affinity of a lipophilic drug for a more hydrophobic surfactant is consistent with previous work (Alvarez-Nunez and Yalkowsky, 2000, Yalkowsky, 1999).

A direct comparison of the solubility in the dispersed and digested 1.25% surfactant solutions firstly reveals that digestion lowered the solubilization capacity of both surfactants. Secondly, as there was a more marked effect of digestion on the solubility in Tween® 85 (a 3.2-fold decrease), the difference in solvent capacity between Tween® 85 and Tween® 80 was less pronounced after digestion. These observations correlate well with the digestion profiles for the two surfactants, shown in Figure 3.4.

Titratable fatty acid in this figure corresponds to fatty acid released from the surfactant in response to digestion of surfactant esters by pancreatic enzymes (Fernandez et al., 2007, Bakala N'Goma et al., 2012a, Cuine et al., 2008b). The concentration of fatty acid released from Tween® 85 after 30 min digestion was 1.4-fold higher than Tween® 80. This was expected since Tween® 85 is a polyethoxylated sorbitan tri-ester, and therefore, contains more fatty acid esters (per unit mass) than Tween® 80. An approximation of the extent of digestion of these surfactants (assuming that each Tween® 80 molecule releases one fatty acid molecule, and Tween® 85 potentially releases three fatty acid molecules) was 35.6% for Tween® 80 and 24.1% for Tween® 85.

Titrated fatty acid (mM)

Figure 3.4: Apparent titration of fatty acids during *in vitro* digestion of Tween® 85 and Tween® 80. Digestion was initiated at t=0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion. Data were corrected for the level of fatty acid released in digestion tests in the absence of surfactant. Mean ± 1 SD (n = 3).

The results therefore show that the lower solubilization capacity of a surfactant following dispersion may be further reduced by digestion (in-line with previous work (Cuine et al., 2008b)). It was during dispersion that the greatest differences in solubilization by Tween® 85 and Tween® 85 were evident.

3.3.1.3 Anhydrous, dispersed and digested LBFs

Figure 3.5 shows the equilibrium solubility of fenofibrate in 1g of each LBF in the anhydrous form (Figure 3.5A), following dispersion in water (Figure 3.5B), and following 30 min digestion (Figure 3.5C).

Fenofibrate solubility in the anhydrous LBFs ranged from 72 mg/g to 126 mg/g. For both Type II and IIIA LBF, the highest solubilities were attained using the MCT/LCM lipid blend. This most likely reflects the higher fenofibrate solubility in MCT over the other lipids (Figure 3.2). However, the lowest solubility was also evident in LBFs containing MCT (i.e., the MCT/MCM formulations), highlighting the complexity of attempting to relate solubility in LBFs based on solubilities of the individual excipients (Williams et al., 2012g). The solubility differences within each of the four lipid Type II/IIIA LBFs pairs was insignificant (Figure 3.5A), which is consistent with the observation that fenofibrate solubilities in anhydrous Tween® 80 and Tween® 85 were similar (i.e., Figure 3.2).

Figure 3.5: Fenofibrate solubilities at 37^{o}C in the eight LBFs in the anhydrous, diluted, or digested forms. A: Solubilities in anhydrous LBFs. B: Solubilities in 1 g LBFs after dispersion in 100 ml. C: Solubilities in 1 g digested LBFs (in 40 ml). Mean ± SD (n = 3). Type II LBFs contained Tween®85, TypeIIIA contained Tween®80 (see Table **3.1**).

The solubility of fenofibrate in 1 g LBF that was dispersed in 100 ml water is shown in Figure 3.5B. A comparison of solubility values in Figure 3.5A to those in Figure 3.5B firstly reveals that dispersion of the LBFs lowered their solubilization capacity for fenofibrate. As dispersed Type II LBFs could solubilize between 75 mg and 96 mg of fenofibrate, and Type IIIA LBFs between 40 mg and 70 mg fenofibrate, it is also apparent that the decrease in LBF solubilization on dispersion was most marked for the Type IIIA LBFs. As the only difference between Type II and IIIA LBFs was the surfactant, this lower solubilization capacity of dispersed Type IIIA can be attributed to the lower solubilization capacity of dispersed Tween® 80. This is consistent with solubility results using individual surfactants, shown in Figure 3.3, and with previous studies by Mohsin et al. (2009a) and Williams et al. (2012c), both of which reported a lower solubilization capacity of hydrophilic LBFs/excipients on dilution.

For both Type II and IIIA LBFs, those containing the highest proportion of MC lipid, namely MCT/LCM and MCT/MCM, exhibited the highest solubilization capacity in the dispersed state. Variation in solubility across the four lipid blends was more marked in the case of the Type IIIA LBFs suggesting that the solubilizing properties of the lipids were more important when using the hydrophilic surfactant Tween® 80. The fact that surfactants demonstrate a higher solubility in water compared to lipids meant overall that it was the nature of the surfactant that most strongly affected solubilization of a dispersed LBF (rather than the composition of the lipids).

Figure 3.5C shows the solubility of fenofibrate in the aqueous colloidal phase (AP_{DIGEST}) obtained following 30 min digestion (values are normalized to 1 g LBF in 40 ml to allow comparison). To aid in the interpretation of the results, the digestion profiles for each LBF are

shown in Figure 3.6. Profiles in the upper panels plot concentrations of titrated fatty acid, while those in the lower panels plot the % digestion of the LBFs (calculated using Eq. 1).

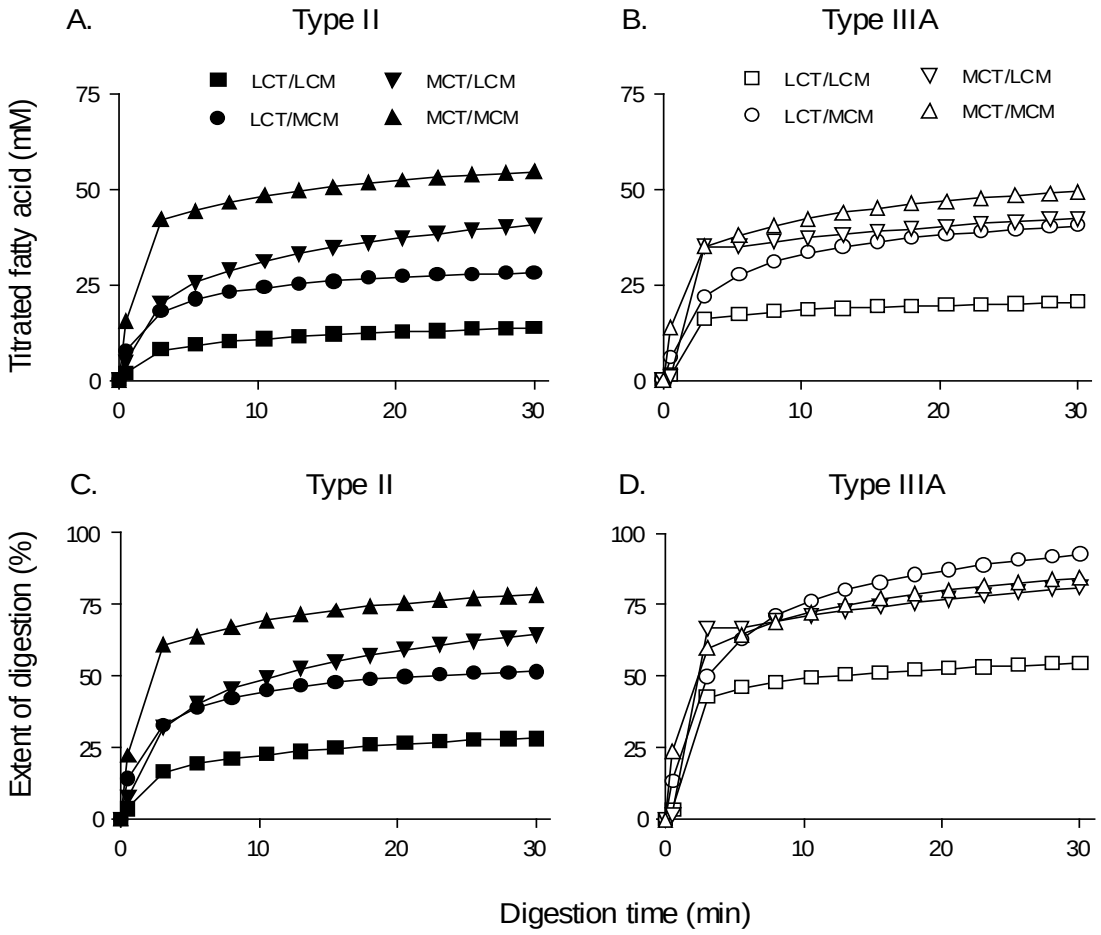


Figure 3.6: *In vitro* digestion of the eight LBFs. A and B:Apparent titration of fatty acids released during *in vitro* digestion of Type II and IIIA LBFs. Data were corrected for the level of fatty acid released in digestion tests in the absence of LBF. C and D: Data are plotted as estimated % LBF digestion, calculated using Eq. 1.

The fenofibrate solubilities in digested LBFs were lower than those in the dispersed state (i.e., compare white bars in Figure 3.5B to those in Figure 3.5C). This decrease in solubilization

was observed despite the use of a higher LBF concentration in the digestion tests (i.e., 2.5% w/v, compared with only 1% during dispersion) and, consistent with previous work (Williams et al., 2012c), digestion of LBF-containing MC lipids led to a more marked decrease in solubilization. For example, the Type IIIA MCT/MCM was capable of solubilizing 72 mg fenofibrate in a dispersed state, but after 30 min digestion, solubilized only 2.4 mg fenofibrate, representing a >30-fold decrease in solubility. Therefore, though it had the highest solubilization capacity on dispersion, the Type IIIA MCT/MCM formulation exhibited the lowest solubilizing capacity following digestion.

Digestion of the Type II MCT/MCM formulation led to a 40-fold decrease in solubilization capacity, further illustrating the lower solubilizing properties of digested MC lipids. Despite differences in surfactant, there was no difference in the solubilization capacity of digested Type II and IIIA LBF containing MCT/MCM lipids.

The use of the fenofibrate solubility in the AP_{DIGEST} to assess the solubilization capacity of other digested Type II LBFs was complicated by the formation of an oil phase during centrifugation of the respective digestion samples. As this oil phase is expected to include a mixture of undigested triglyceride and highly lipophilic digestion products such as diglyceride, monoglyceride and any protonated fatty acids (Sek et al., 2002b, Williams et al., 2012g, Williams et al., 2012c), the greater prevalence of an oil phase in the case of the Type II LBFs over Type IIIA LBFs (only the Type IIIA LCT/LCM formulation formed a very small oil phase) can be attributed to the lower extent of digestion of Type II LBFs (see lower panels in Figure 3.6). This results in a greater amount of undigested triglyceride, and differences in the capacity of each surfactant phase to solubilize lipid. As these phase-separated lipids will exhibit a high capacity to solubilize fenofibrate, measured solubilities in

Type II $AP_{DIGESTS}$ are not representative of the solubilization capacity of the completely digested LBF, which, in turn, prevents a direct comparison of solubilization capacities of digested Type II LBFs (which form an oil phase) with their Type IIIA equivalents.

The solubilities in the Type II $AP_{DIGESTS}$ shown in Figure 3.5C nevertheless reveal that increasing MC lipid content in the formulation progressively decreased the solubilization capacity of this phase and that the solubilities in the Type II and III $AP_{DIGESTS}$ were similar, despite the evidence of an oil phase in the case of the Type II LBF. The decreasing solubility of fenofibrate in digested LBFs with increasing MC lipid was consistent for both Type II and IIIA LBF, and also coincided with increasing digestion of the LBF (particularly the Type II LBF).

3.3.2 Part 2: Evaluation of drug-containing LBFs

3.3.2.1 In vitro dispersion testing

The results of *in vitro* dispersion testing of Type II and IIIA LBFs containing fenofibrate at a concentration equivalent to 80% of the equilibrium solubility in the formulation (i.e., 80% of the values in Figure 3.5A), are shown in Figure 3.7. For Type II LBFs (Figure 3.7A and Figure 3.7B), with the exception of the Type II LCT/MCM formulation, practically all of the drug remained in a solubilized state for the entire 96 h. In the case of the Type II LCT/MCM, ~82% drug remained solubilized, indicating that almost 20% of the dose had precipitated on dispersion. Precipitation did not occur to a significant extent during the first 4 h, and the large standard deviation bars indicate that precipitation was variable. There was a greater propensity for precipitation in the case of equivalent (but more hydrophilic) Type IIIA LBFs (Figure 3.7C and Figure 3.7D). For all formulations apart from the Type IIIA MCT/MCM, this precipitation commenced within the first 4 h, and was more prevalent after 4 h.

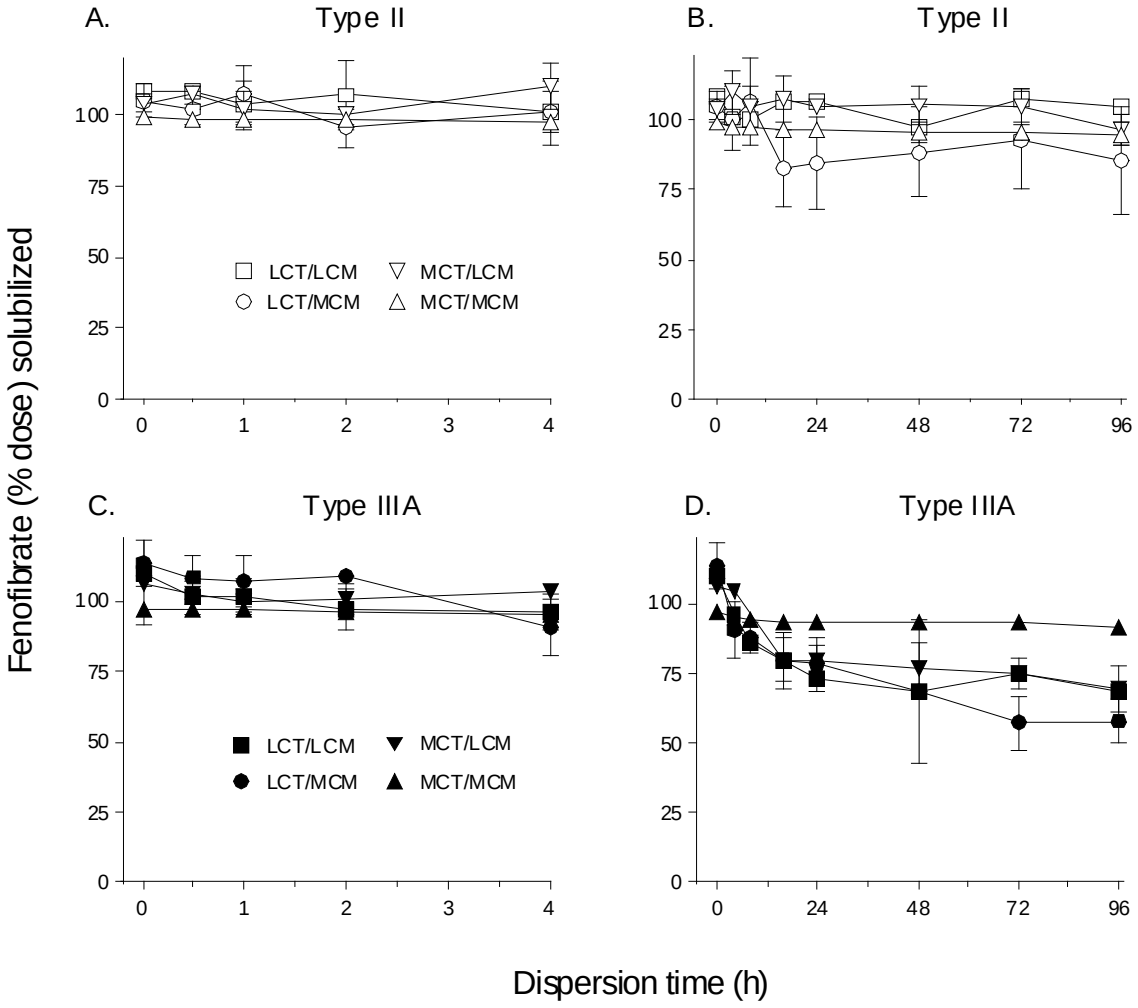


Figure 3.7: Percentage of the fenofibrate dose solubilized in the aqueous phase during *in vitro* dispersion of LBFs. A and B - Type II LBFs; C and D - Type IIIA LBFs. A and C – 4h period; B and D – 96h period. Means± SD (n = 3). Each LBF contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

Figure 3.8 shows the absolute mass of fenofibrate that remained in the solubilized form at selected time points during dispersion of Type II (Figure 3.8A) and Type IIIA (Figure 3.8B) LBFs. Having measured the solubilization capacity of the dispersed LBFs in Part 1 of this study (i.e., data included in Figure 3.5B and shown in Figure 3.8 by the dashed horizontal line), it is possible to evaluate the performance of respective LBFs in terms of the degree of supersaturation generated during their dispersion and digestion.

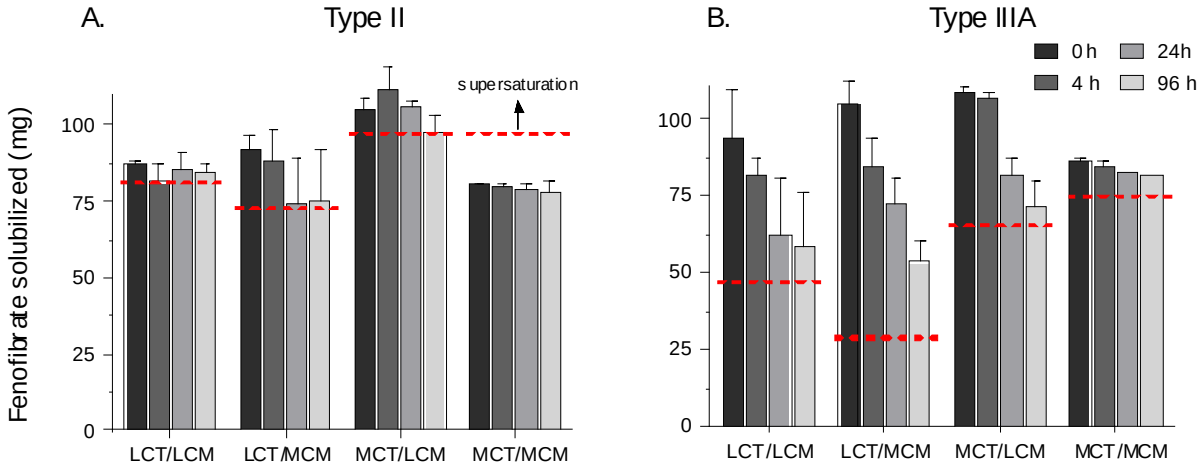


Figure 3.8: Mass of fenofibrate solubilized as a function of time during *in vitro* dispersion. A - Type II LBFs, B - Type IIIA LBFs. Mean ± SD (n = 3). Each LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation. The dashed horizontal lines denote the solubilization capacities of the dispersed LBFs. Values above this line indicate supersaturation.

The supersaturation ratio (SR) can be calculated at any of the sample time points using Eq. 2. The parameter SR^M describes the maximum level of supersaturation in the absence of drug precipitation(Eq. 3), and can be utilized to describe the driving force of drug precipitation (Williams et al., 2012c, Anby et al., 2012e).Calculated SR^M values for dispersion of the LBFs

are shown in Table 3.2.. The results show that for all Type II LBFs, except the Type II LCT/MCM formulation, dispersion did not lead to supersaturation ($SR^M \leq 1$, Table 3.2). In other words, the dose utilized was not high enough to exceed the solubilization capacity of the dispersed formulation, and the lack of supersaturation in turn explains the lack of drug precipitation (Figure 3.7A/B and Figure 3.8A).The lower solubilization capacity of the dispersed Type II LCT/MCM formulation was responsible for the generation of supersaturation (SR^M 1.2). During the extended dispersion study, this modest degree of supersaturation was sufficient to cause precipitation.

The SR^M values for Type IIIA LBFs were 1.8 (LCT/LCM), 3.1 (LCT/MCM), 1.5 (MCT/LCM) and 1.2 (MCT/MCM) (Table 3.2). Type IIIA LBFs therefore produced higher SR^M values compared to equivalent Type II LBFs because the more hydrophilic Type IIIA LBFs have lower solubilization capacities on dispersion (illustrated clearly in Figure 3.5B and tabulated in Table 3.2). Type IIIA LBFs that generated the highest SR^M values (LCT/LCM and MCT/LCM) were the first to show evidence of precipitation (by 4 h). In accordance with the higher SR^M values, the extent of precipitation was also greatest for these two formulations. Of the Type IIIA formulations, the MCT/MCM variant generated the lowest SR^M value, and as a consequence resulted in the least amount of precipitation.

In summary the results of the *in vitro* dispersion tests revealed that Type II LBFs out-performed Type IIIA equivalents, and that this observation was explained by the degree of supersaturation obtained following dilution of the formulation. Since performance differences within the Type II/IIIA LBFs classes were small, the performance of these particular LBFs on dispersion were therefore most dependent on the nature of the surfactant and not the composition of the lipid component.

3.3.2.2 In vitro digestion testing

The effect of 30 min *in vitro* digestion on fenofibrate solubilization by Type II and IIIA LBFs is summarized in Figure 3.9. The results are presented as the % of the drug dose contained in each of the isolated digestion phases, namely; an oil phase, the colloidal AP_{DIGEST} and the pellet phase (containing any precipitated drug).

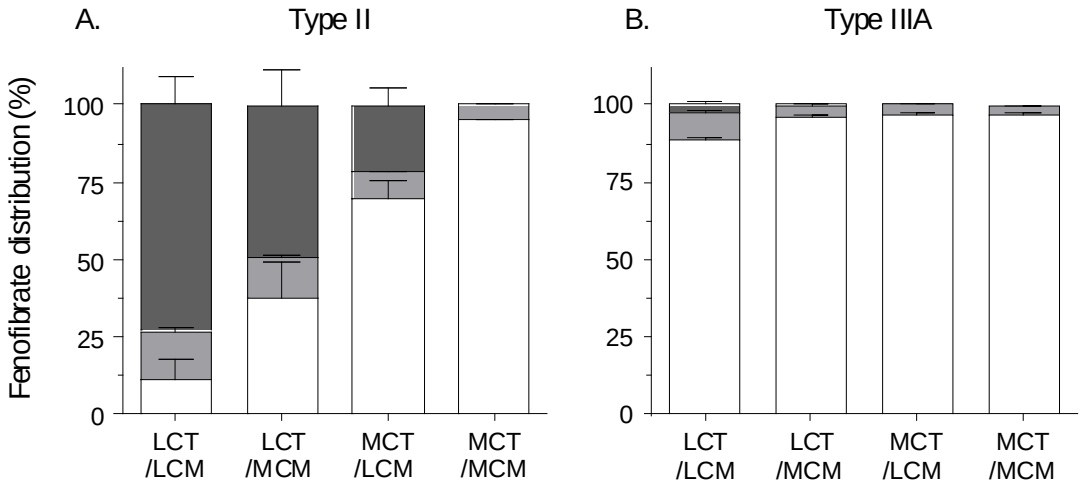


Figure 3.9: The effect of *in vitro* digestion on the fate of fenofibrate in A: Type II and B: Type IIIA lipid formulations. Box-plots show the distribution of fenofibrate across the three phases assayed: a poorly dispersing oil phase (dark shaded bars); colloidal aqueous phase, $AP_{DIGEST,}$ (light shaded bars); and pellet phase (white bars). Mean ± SD (n = 3). LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

Decreasing the quantity of LC lipid in the Type II LBFs resulted in decreases in the % of fenofibrate in the oil phase (Figure 3.9A). This directly correlated (r^2 = 0.9798) with the extent of digestion for respective Type II LBFs (calculated via Eq. 1, and shown in Figure

3.6C). Coincidently, the decreased quantity of fenofibrate in the oil phase, caused by reducing LC lipid/increasing MC lipid, also correlated with an increase in drug precipitation, which increased from 11.3 ± 6.2 % in the case of the LCT/LCM formulation to 95.0±0.3 % for the MCT/MCM formulation. The increased likelihood of drug precipitation from MC over LC lipid formulations on digestion is consistent with previous work (Porter et al., 2004c, Williams et al., 2012c, Dahan and Hoffman, 2006a). However, the present study also revealed that by substituting as little as 15% LC lipid for MC lipid (i.e., comparing LCT/LCM to LCT/MCM formulations in Figure 3.9A) a formulator could inadvertently produce a poorer formulation that results in a marked increase in precipitation (~3-fold in this case). In contrast the substitution of 15% MC lipid for LC lipid (i.e., comparing MCT/MCM to MCT/LCM formulations in Figure 3.9A) can have the opposite effect of decreasing drug precipitation. Therefore, despite increasing the proportion of MC lipids in the LBF to increase digestibility and to increase the lipid concentration in the AP_{DIGEST}, the fenofibrate concentrations in the respective $AP_{DIGESTS}$ were highest when LBFs contained the most LC lipid (250.4 ± 18.9 µg/ml (LCT/LCM) and 248.5 ± 33.5 µg/ml (LCT/MCM) compared with 182.3 ± 16.1 µg/ml (MCT/LCM) and 83.6 ± 3.2µg/ml (MCT/MCM)).

For all Type IIIA LBFs, 30 min digestion caused >80% of the incorporated fenofibrate to precipitate (Figure 3.9B). Differences in performance after 30 min between the different Type IIIA LBF were negligible, however the removal of samples at earlier time points during digestion, 5 and 15 min, revealed that drug precipitation during digestion of the LBF containing only LC lipid was slower compared with the MC lipid counterpart (Figure 3.10). Indeed, in the case of the LCT/LCM Type IIIA LBF, ~50% of the incorporated dose had precipitated after 5 min of digestion (Figure 3.10A), whereas for the MCT/MCM formulation

(Figure 3.10B), >90% of the dose had precipitated in this time. The use of LC lipids therefore offered some resistance to precipitation, though after 15 min, the small differences in the performance of Type IIIA LBFs werenegligible.

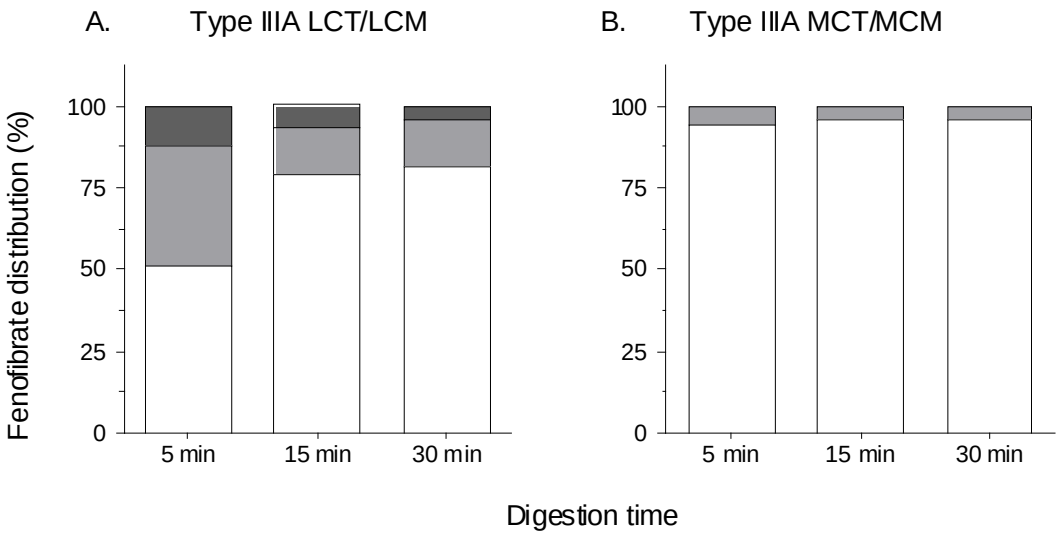


Figure 3.10: The effect of *in vitro* digestion time on the fate of fenofibrate in A: Type IIIA LCT/LCM and B: Type IIIA MCT/MCM LBFs. Box-plots show the distribution of fenofibrate across the three phases assayed: a poorly dispersing oil phase (dark shaded bars); colloidal aqueous phase, $AP_{DIGEST,}$(light shaded bars); and pellet phase (white bars). LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

Analogous to the dispersion study, the theoretical maximum supersaturation ratio (SR^{M}) achieved on digestion was calculated using Eq. 3, producing the values shown in

Table 3.2. SR^M values were typically higher for Type IIIA LBFs compared with Type II LBFs, and typically increase with decreasing LC lipid/increasing MC lipid in the formulation. It is also apparent that SR^M values produced on digestion were much higher than those produced by dispersion. As respective LBFs tested in dispersion and digestion experiments (in part 2) contained the same fenofibrate dose, this difference in SR^M can be ascribed to the lower solubilization capacity of the digested formulations (previously described in section 3.3.1.3). For example, dispersion of the Type II MCT/MCM formulation did not lead to supersaturation (SR^M 0.8), however the marked decrease in solubilization of this LBF on digestion led to a greater than 40-fold increase in SR^M (Table 3.2), and in turn, extensive drug precipitation. Similar, albeit less pronounced, increases in SR^M were apparent for all other LBFs. Direct comparisons of SR^M attained on dispersion and digestion were complicated in situations where LBFs formed an oil phase on digestion (denoted by * values in Tabl3 3.2). As discussed in section 3.3.1.3, the solubilization capacity of the digested LBF in these cases is underestimated (and therefore, SR^M values are overestimated) because a proportion of the formulation is sequestered in the oil phase. As shown in Figure 3.9A, this oil phase contributes significantly to drug solubilization. Nonetheless, SR^M values for Type II LBFs are lower in all cases than those produced on digestion of Type IIIIA counterparts.

3.3.2.3 Assessment of fenofibrate precipitate under cross polarized light

Figure 3.11 shows micrographs of the pellet phase from samples removed following digestion of the Type IIIA LCM/MCM formulation containing fenofibrate (Figure 3.11A) and the drug-free equivalent Type IIIA LCM/MCM formulation (Figure 3.11B). Pellets were viewed under cross-polarized light, with evidence of birefringence used to identify areas of crystallinity.

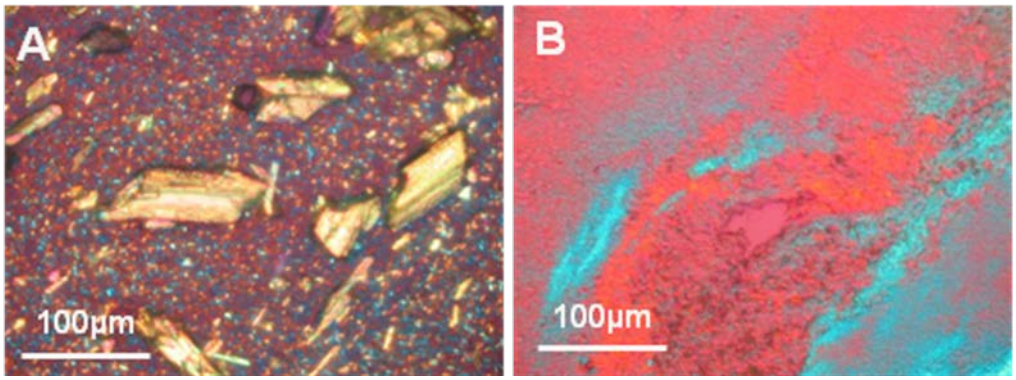


Figure 3.11: Micrographs of the pellet phase following digestion of the Type IIIA LCT/MCM formulation. A and B: pellet phase after 30 min digestion. In A the formulation contained fenofibrate at 80% saturation. In B, the formulation did not contain fenofibrate.

3.4 Discussion

In vitro testing of lipid-based formulations (LBFs) is increasingly focused on the need to monitor the fate of incorporated drug during critical events that occur in the gastro-intestinal (GI) tract, namely dispersion of the formulation in aqueous fluids and subsequent digestion of the formulation components. This focus stems from the fact that dispersion and digestion can dramatically alter physicochemical properties of a LBF and, in turn, trigger drug precipitation. Incidences of precipitation will decrease the concentration of solubilized drug and, where the precipitate consists of a slow dissolving crystalline solid, formulations with a higher tendency to precipitate are expected to result in lower bioavailability *in vivo*.

In the present study LBFs that were either Type II or IIIA according to the Lipid Formulation Classification System (LFCS) were evaluated in *in-vitro* dispersion and *in-vitro* digestion tests. Both Type II/IIIA LBFs may be described as self-emulsifying drug delivery systems (SEDDS), yet due to differences in the amount of hydrophilic excipients, Type II/IIIA LBFs can show marked differences in emulsification and drug solubilization properties following dispersion and digestion. The tendency of Type IIIA LBFs to produce ultrafine dispersions, has resulted in these formulations being the more favored option within industry. It has been assumed that fine particles would promote rapid absorption, though the interplay between this and their fate during digestion has not been studies in detail.

The aim of the present work was to discern the effects of lipid composition and surfactant type on the solubilization properties of Type II and IIIA LBFs during dispersion and digestion. This study follows on from work described by Mohsin et al.(2009a), and more recently by Anby et al. (2012e) and Williams et al. (2012c), with each showing that the degree of supersaturation attained on dispersion and/or digestion of a LBF can be used to

explain the fate of drug during *in vitro* testing. The present study was therefore divided in two parts. In Part 1, a thorough assessment of the solubilization capacity of eight LBF, and their respective solubilization capacities following dispersion and digestion was performed. Subsequently, in Part 2, the performance of drug-containing LBFs during dynamic dispersion and digestion tests was evaluated. The results of the solubility studies in Part 1 therefore allowed the performance of drug-containing LBF in Part 2 to be discussed in terms of supersaturation. Fenofibrate (Figure 3.1) was used as a model lipophilic drug. Four different lipid mixtures consisting of long-chain (LC) and medium-chain (MC) lipids were investigated, and were mixed with either Tween® 85 surfactant to generate Type II LBFs or the more hydrophilic Tween® 80 surfactant to form Type IIIA LBFs, providing for a total of eight LBFs (Table 3.1). It is stressed here that the experiments described in Part 1 were performed with drug-free formulations whereas in Part 2, LBFs were incorporated with fenofibrate at a level equivalent to 80% of the equilibrium solubility in the anhydrous formulation. As the fenofibrate solubility in Type II and IIIA LBFs of the same lipid component were highly comparable, the absolute fenofibrate loading in Type II/IIIA formulation pairs was similar in all cases. Due to some differences in solubility in MC and LC lipids, the target dose across the four Type II and four IIIA LBFs ranged from 80.3 to 101.3 mg and 85.1 to 102.4 mg, respectively. The results show that the lower solubilization capacity of dispersed Type IIIA LBFs compared with Type II equivalents introduced supersaturation and therefore the risk of drug precipitation. Digestion of the all Type IIIA LBFs and Type II LBFs containing predominantly MC lipid led to extensive drug precipitation, which could be rationalized once again by supersaturation.

3.4.1 LBF performance on dispersion

A Type II LBF by strict definition should contain no water-miscible components (Pouton, 2006a), however it remains possible that a small proportion of the excipients, such as the nonionic surfactant Tween® 85 investigated here, will partition into an aqueous medium to in turn lower the solubilization capacity of the LBF towards a hydrophobic drug (Mohsin et al., 2009a). In the present study, the solubilization capacity of Type II LBFs decreased on dispersion (Figure 3.5B), however the extent of this decrease was small considering that LBFs were diluted 100-fold. As a result, dispersion of fenofibrate-containing Type II LBFs generally did not generate supersaturation (Table 3.2, Figure 3.8A), and with the exception of one formulation (that did generate supersaturation and show evidence of precipitation), dispersed Type II LBFs were shown to maintain practically all of the drug dose in a solubilized form over four days (Figure 3.7). This lack of precipitation occurred despite the use of a high drug loading, equivalent to 80% of the equilibrium solubility in the anhydrous LBF. In contrast to Type II LBFs, dispersion of Type IIIA LBFs led to a more marked decrease in solubilization capacity (Figure 3.5B) and higher degrees of supersaturation on dispersion of the fenofibrate-containing formulations (Table 3.2, Figure 3.8B) that led to some evidence of precipitation. This can be rationalized by the fact that Type IIIA LBFs contained the more hydrophilic surfactant Tween® 80. This surfactant has a greater affinity for aqueous media, readily forming a micellar solution, and therefore might be expected to lose a greater proportion of its bulk solubilization properties on dispersion.

More hydrophilic IIIB and IV types of LBF have been reported to undergo a decrease in solubilization on aqueous dispersion that covers two or more orders of magnitude (Mohsin et al., 2009a, Williams et al., 2012c, Pouton, 2006a). The decrease in solubilization capacity of Type IIIA LBFs in the present study were more modest, as evidenced by the maximum

supersaturation ratios (SR^M; Eq. 3) obtained on dispersion, that were all less than 3.1 (Table 3.2). The limited drug precipitation during the initial 4 h of dispersion of Type IIIA LBFs therefore suggests that higher supersaturation ratios are required for more rapid precipitation.

Such higher degrees of supersaturation may be produced on dispersion if drug loading in the LBF is increased. However, the Type IIIA LBFs investigated here contained high fenofibrate loadings, equivalent to 80% saturation. The fact that the Type IIIA LBFs generated relatively small degrees of supersaturation on dispersion suggests that the high proportion of lipid in these formulations (50% in this study) may attenuate the loss of solubilization that might be expected when using a water-miscible surfactant, even at high drug loadings. Therefore, unless rendered more hydrophilic through the addition of co-solvent or polar oils (such as MC monoglycerides), limited precipitation following the dispersion of a Type IIIA fenofibrate formulations is expected. Indeed there may be no precipitation in the stomach before the formulation is emptied from the stomach into the intestine. Thus it can be concluded that for fenofibrate formulations, the design of both Type II and Type IIIA LBFs should be focused on the solubilization capacity of the excipients in the digested state (rather than dispersion).

3.4.2 LBF performance on digestion

As the quantity of lipid in LBFS is sufficient to stimulate gall bladder secretion, and therefore, highly efficient digestion processes (Kossena et al., 2007), digestion of lipid components of a LBF within the small intestine is inevitable. Although the high lipid content in Type II and IIIA formulations may prevent drug precipitation on dispersion, digestion of these lipids will decrease the LBF solubilization capacity, increase supersaturation and, depending on the drug loading, may promote rapid and extensive precipitation of the incorporated drug (Figure 3.9). The results presented here also showed that supersaturation

and precipitation is intensified by the use of high amounts of MC lipids. The finding that the use of MC lipids is associated with increased risk of precipitation is consistent with several other studies (Williams et al., 2012c, Anby et al., 2012e, Porter et al., 2004c, Dahan and Hoffman, 2006a, Han et al., 2009b). The present work also suggests that substitution of as little as 15% LC lipid for MC lipid is sufficient to significantly increase the rate and extent of drug precipitation.

Triglycerides are readily digested in the small intestine by pancreatic lipase into monoglycerides and fatty acids. Digestion will therefore render a LBF travelling through this region of the GI tract increasingly more hydrophilic and, since the solubilization capacity of this digested LBF towards a hydrophobic drug is inevitably lower than the solubilization capacity of the bulk and/or dispersed LBF, digestion can lead to supersaturation. Supersaturation has two opposing potential roles in drug absorption; either increasing drug absorption through increasing the thermodynamic activity of the absorbable fraction of drug or decreasing drug absorption by driving precipitation (Williams et al (in press)). Critical to the optimal *in vivo* performance of a LBF is the need to avoid factors that promote precipitation, principally a high degree of supersaturation, since supersaturation lowers the thermodynamic barriers to precipitation. By lowering the solubilization capacity of the LBF, digestion was shown to generate high supersaturation ratios for both Type II and IIIA LBFs (Table 3.2). Supersaturation ratios were higher when LBFs contained MC lipids. This was because the solubilization difference between the anhydrous/dispersed LBF and the digested LBF is generally more pronounced on using MC lipids, which reflects the relative hydrophilicity of monocaprylin/monocaprin and caprylic/capric acid, and the lower tendency of these digestion products to interact with and supplement the bile salt/phospholipid solubilizing phase(Kossena et al., 2003b). In the *in vitro* conditions employed here, the high

supersaturation ratios generated by digestion of MC lipids could not be maintained for 30 min, and sometimes, not for even 5 min (Figure 3.10B).

The use of MC lipid in Type II and IIIA formulations is effective for the generation of LBFs that result in minimal loss of solubilization on dispersion, and that subsequently generate supersaturation rapidly on entering the intestine. In some instances, this supersaturation may be highly effective in driving drug absorption, though if too high, supersaturation may lead to extensive precipitation before drug absorption occurs. The use of LC lipids reduces the risk of precipitation by slowing the rate and extent of LBF digestion and by increasing solubilization. However, as evidenced in the present study, this approach alone may not be sufficient. Alternative strategies that may also attenuate drug precipitation from Type IIIA LBFs include lowering drug loading (Williams et al., 2012c) (though this may not be practically possible), use of polymer precipitation inhibitors (Anby et al., 2012e) or judicious selection of the surfactant (Cuine et al., 2008b).

The latter strategy is important since formulation surfactants such as the Tween® surfactants investigated here are substrates to pancreatic enzymes including carboxyl ester hydrolase (CEH) and phospholipase (Cuine et al., 2008b, Christiansen et al., 2010a, Fernandez et al., 2008b, Bakala N'Goma et al., 2012a). Surfactants are digested to more polar and less amphiphilic molecules, which can lower the solubilization capacity of the LBF (Cuine et al., 2008b). This appeared to be the case in the present study where it was shown that the solubilization capacity of Tween® 85 and Tween® 80 surfactant solutions decreased following digestion (Figure 3.3). The overall drop in solubilization was more marked in the case of Tween® 85 by virtue of its higher digestibility/number of ester groups (Figure 3.4). Thus, the digestion of the formulation surfactant can add to the lower solubilization capacity

of LBFs following digestion of triglycerides. . The varied solubilization capacity of surfactants, and therefore, capacity to affect supersaturation and precipitation dependent on their digestibility calls for a better understanding of the surfactant effects in LBFs, which is the subject of a separate study (Devraj et al., 2012b).

3.5 Conclusions

The results of the current study show that dispersion and digestion of lipid-based formulations (LBFs) can generate supersaturation. The extent of supersaturation on dispersion was dependent on the hydrophilicity of the formulation components, whereas the extent of supersaturation reflected the digestibility of the LBF and the solubilization capacity of the digestion products. High supersaturation ratios are strongly promoting of precipitation and, in the present study, high supersaturation ratios attained on digestion led to widespread crystallization of the model drug fenofibrate from lipid-rich formulations that did not contain any co-solvent. Therefore, while the use of high lipid concentrations in the LBFs (50% in this case) will minimize risk of precipitation on dispersion, judicious selection of the type of lipid and surfactant is necessary to ensure that the positive effect of supersaturation to drug absorption can be exploited before the drug crystals form in the GI tract. The study emphasizes the value of conducting *in vitro* digestion tests on LBFs and the predictive power that can be gained by calculating the maximum supersaturation ratio that occurs during digestion.

Chapter 4: Research paper 3

Choice of nonionic surfactant used to formulate Type IIIA self-emulsifying drug delivery systems and the physicochemical properties of the drug have a pronounced influence on the degree of drug supersaturation that develops during *in vitro* digestion

Abstract

The performance of self-emulsifying drug delivery systems (SEDDS) is influenced by their tendency to generate supersaturated systems during dispersion and digestion in the gastro-intestinal tract. This study investigated the effect of drug loading on supersaturation during digestion of fenofibrate or danazol SEDDS, each formulated using long-chain lipids and a range of nonionic surfactants. Supersaturation was described by the maximum supersaturation ratio (SR^M) produced by *in vitro* digestion. This parameter was calculated as the ratio of the total concentration of drug present in the digestion vessel versus the drug solubility in the colloidal phases formed by digestion of the SEDDS. SR^M proved to be a remarkable indicator of performance across a range of lipid-based formulations. SEDDS containing danazol showed little evidence of precipitation on digestion, even at drug loads approaching saturation in the formulation. In contrast, fenofibrate crystallized extensively on digestion of the corresponding series of SEDDS, depending on the drug loading. The difference was explained by the generation of higher SR^M values by fenofibrate formulations. A threshold SR^M of 2.5-2.6 was identified in 6 of the 7 SEDDS. This is not a definitive threshold for precipitation but in general when $SR^M > 3$ fenofibrate supersaturation could not be maintained.

4.1 Introduction

Examples of lipid-based formulations commonly used in oral drug delivery include simple oil solutions, self-emulsifying drug delivery systems (SEDDS) and co-solvent/surfactant mixtures, each of which have been used to improve the oral absorption of poorly water-soluble drugs (PWSD).(Williams et al., In press, Porter et al., 2007b, Pouton and Porter, 2008b, Hauss, 2007a) SEDDS, consisting of a mixture of drug, oil (s), surfactant (s) and sometimes co-solvent, are perhaps the most widely used type of lipid formulation; Neoral® (the Novartis SEDDS formulation of cyclosporine) is a well-known commercial example. SEDDS are designed to emulsify spontaneously on addition to an aqueous phase, generating colloidal oil-in-water dispersions. The size of the colloidal oil droplets is dependent on the composition of the formulation, particularly the lipid: surfactant ratio and the type of surfactant used.(Thomas et al., 2012c, Cuine et al., 2008b, Khoo et al., 1998, Williams et al., 2012g) While the average particle size of these systems immediately following dispersion is often determined by formulators (Gao et al., 1998b, Pouton and Porter, 2008b), the reality is that oil droplet size and the overall structure and composition of the colloids is continually changing during gastrointestinal transit, as the formulation encounters the digestive system, and as individual components are absorbed. In recent years, other, more robust measures of SEDDS performance have been sought. Methods for assessment of the fate of the drug during either *in vitro* dispersion or *in vitro* digestion are increasingly being used to predict the *in vivo* performance of lipid-based systems. (Mohsin et al., 2009a, Thomas et al., 2012a, Tan et al., 2012a, Anby et al., 2012e, Cuine et al., 2008b) The rationale for such *in vitro* tests stems from the knowledge that SEDDS and other types of lipid formulations may suffer a loss of solubilization capacity following dispersion in the aqueous fluids in the GI tract (Chiang et al., 2011b, Pouton and Porter, 2008b, Pouton, 2006a) or following digestion of lipids and/or

surfactants in the intestine. (Williams et al., 2012c, Devraj et al., 2012c, Anby et al., 2012e, Cuine et al., 2008b) Dependent on drug loading, loss of solubilization capacity can lead to drug supersaturation, and the risk of drug precipitation.

The Lipid Formulation Classification System (LFCS), proposed by Pouton (Pouton, 2006a, Pouton, 2000a) provides some initial guidance on SEDDS performance during dispersion and digestion. The LFCS describes four different classes of lipid formulations. Depending on the excipients used, SEDDS fall into either Type II or Type III according to the LFCS. Type II formulations consist of oils and water-insoluble surfactant(s), and form turbid dispersions of oil droplets that typically range from 0.25 – 2 μm in diameter. Due to the lack of water-soluble components, Type II formulations typically result in minimal loss of solubilization capacity on dispersion (Devraj et al., 2012c, Williams et al., 2012c, Pouton, 2006a). Type III formulations consist of oils mixed with water-soluble (high HLB) surfactant(s) and sometimes also a water-miscible co-solvent. Type III formulations are therefore more hydrophilic. They may form ultrafine dispersions (< 100 nm) but typically lose solvent capacity on dispersion and digestion. Type III A/B sub-classes have also been introduced to better differentiate between Type III formulations showing high (IIIA) or low (IIIB) lipid contents. The high lipid content (>40%) in Type IIIA formulations is often able to prevent rapid and extensive precipitation on dispersion (Williams et al., 2012c, Devraj et al., 2012c, Mohsin et al., 2009a) unless the formulation contains high drug loadings and/or co-solvent, (Anby et al., 2012e, Cuine et al., 2008b). However, oils present in SEDDS (both Type II and Type IIIA/B) are likely to be readily digested by pancreatic lipases in the small intestine, (Bakala N'Goma et al., 2012a, Hur et al., 2011) causing the physicochemical nature of the SEDDS to change dramatically. More specifically, at the molecular level, digestion involves the enzymatic hydrolysis of esters in triglyceride and diglyceride molecules and the formation

of less lipophilic monoglyceride and fatty acid molecules. This process at a formulation level causes a progressive depletion of an oil droplet phase and the enrichment of bile salt/phospholipid mixed micellar phase(s) which include the digestion products. Digestion has the effect of 'forcing' drug to partition from the oil reservoir, which is rapidly decreasing in volume, into the micellar phase. Since lipophilic drugs typically have lower affinity towards the more hydrated micellar phases, the transfer of drug from an oil-rich phase by digestion is associated with a decrease in drug solubility. This is analogous to other events that are known to create supersaturation by shifting the position of equilibrium, such as solvent-shift phenomena (Warren et al., 2010, Brouwers et al., 2009).

We and others have shown that digestion of Type IIIA SEDDS can dramatically lower their solubilization capacity for hydrophobic drugs to a point where drug precipitation occurs (Williams et al., 2012c, Anby et al., 2012e, Devraj et al., 2012c, Thomas et al., 2012a, Thomas et al., 2012c). The effect of precipitation on drug absorption is dependent on the physical form of the drug in the precipitate. The emergence of a crystalline solid with a slow rate of re-dissolution (often the case for PWSD) is likely to be associated with decreased bioavailability (Williams et al., 2013, Cuine et al., 2008a, Anby et al., 2012c). Rational lipid formulation design therefore requires an awareness of the factors that may contribute to drug precipitation, the critical factor being the extent of supersaturation generated by a loss of solubilization upon dispersion and digestion.

In our previous study (Devraj et al., 2012c), the performance of a Type IIIA SEDDS consisting of long-chain lipids (soybean oil and Maisine^{TM} 35-1), the surfactant Tween® 80, and a high loading (~85 mg/g) of the poorly water-soluble drug fenofibrate, was examined *in vitro*. Precipitation of fenofibrate during dispersion was moderate (<25% over 24 h). However, during 30 min of digestion, due to exposure to pancreatin and bile, more than 85%

of the drug crystallized from solution. The substantial increase in precipitation observed during digestion tests was attributed to a marked increase in the degree of supersaturation caused by digestion of the SEDDS, which decreased the solubilization capacity (Devraj et al., 2012c). The present study was designed to extend our understanding of the performance of Type IIIA SEDDS during *in vitro* digestion testing, by further exploring whether the degree of supersaturation attained during digestion could explain differences in drug precipitation. Model drugs were chosen with high (fenofibrate) or lower (danazol) solubility in anhydrous SEDDS, which allowed a wide range of drug loadings to be evaluated. In this study we explored the influence of the choice of surfactant. Each SEDDS consisted of long-chain lipids combined with one of seven different nonionic surfactants. The surfactants included various digestible materials (Cuine et al., 2008a) (Cremophor® EL, Cremophor® RH40, Tween® 80 and Solutol® HS-15, D-α-tocopherol polyethylene glycol [TPGS] 1000 succinate) and non-digestible materials (Brij® 97 and Brij® 98). The focus on the choice of surfactant is timely, given that recent studies have suggested that the digestibility of the surfactant in SEDDS can dramatically influence performance *in vitro* and *in vivo* (Porter et al., 2011, Cuine et al., 2008b, Fernandez et al., 2009a). Other recent studies have compared various nonionic surfactants and reported their differential capacity to affect the activity of intestinal digestion enzymes (Christensen et al., 2004b, Cuine et al., 2008b), the interfacial properties at the oil: water interface (Mun and McClements, 2006, Sandra et al., 2008, Lesmes et al., 2010) and cytochrome mediated drug metabolism in the GI tract (Christensen et al., 2011, Bakken et al., 2009, Jurgens et al., 2002). These studies all reiterate the need for judicious selection of formulation surfactant in SEDDS. The studies presented herein aimed to investigate the extent of precipitation of two drugs, fenofibrate and danazol, from a range of formulations that differed only in the identity of the surfactant used to form Type IIIA

lipid-based delivery systems. The emphasis of the study was to evaluate precipitation as an unbiased measure of performance, and to ask whether there was any relationship between the extent of precipitation and the degree of supersaturation generated during digestion of the formulations.

4.2 Materials and methods

4.2.1 Materials

Details of the nonionic surfactants used in the study are presented in **Table 4.1**. Fenofibrate, soybean oil (a long-chain triglyceride), sodium taurodeoxycholate >95 % (NaTDC), porcine pancreatin extract (P7545, 8 x USP specifications activity), calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), Tris-maleate and the lipid digestion inhibitor 4-bromophenylboronic acid (4-BPB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Danazol was kindly supplied by Sterling Pharmaceuticals (Sydney, New South Wales, Australia). Maisine^{TM} 35-1 (a blend of long-chain mono-, di and some triglyceride) was supplied by Gattefosse (Saint-Priest, France). Lecithin (approximately 99.2 % egg-phosphatidylcholine (Zidar et al.), Lipoid E PCS), was purchased from Lipoid GmBH (Ludwigshafen, Germany). 1.0 M sodium hydroxide (Univol) was purchased from Ajax Finechem Pty Ltd (New South Wales, Australia) was diluted with water (Milli-Q water purification system, Millipore, Bedford, MA) to produce a 0.6M titration solution. Methanol and chloroform used in this work were HPLC grade and were obtained from Merck (Victoria, Australia).

4.2.2 Lipid formulations

The lipid-based formulations investigated in this study were Type IIIA SEDDS as defined by the Lipid Formulation Classification System (Pouton, 2006b, Pouton, 2000b). SEDDS used in this study contained 50% w/w long-chain lipids (soybean oil and Maisine^{TM} 35-1 in a 7:3 w/w

ratio) and 50% w/w of one of the nonionic surfactants listed in **Table 4.1**. All of the formulations emulsified rapidly to produce fine sub-micron dispersions under conditions of gentle agitation. The particle size distributions of dispersions were not evaluated because they change extensively as soon as digestion is initiated.

Table 4.1: Details of the nonionic surfactants used in the Type IIIA SEDDS

Surfactant	Chemical name	Quoted HLB value/range
Brij® 97[a]	Polyoxyethylene (10) oleyl ether	~12
Brij® 98[a]	Polyoxyethylene (20) oleyl ether	15
Cremophor®EL[b]	Polyethylene glycol (35)-glycerol ricinooleate	12-14
Cremophor® RH40[b]	Polyethylene glycol (40)-glycerol hydroxystearate	14-16
Solutol® HS-15 [b]	Polyethylene glycol (15)-hydroxy stearate	14-16
Tween® 80[a]	Polyoxyethylene (20) sorbitanmonooleate	15
TPGS[a]	D-α-tocopherol polyethylene glycol (23) succinate	~13

[a] Obtained from Sigma-Aldrich, St. Louis, MO.

[b] Obtained from BASF, Washington, NJ.

Note: The oxyethylene content of each material is quoted using a common nomenclature, not necessarily used by the manufacturers, where the number in brackets represents the approximate number of $–CH_2CH_2O-$ groups per molecule. However the materials are not synthesized by common methods. The oxyethylene chains are a varied chain length due to

their polymeric nature and the materials, particularly the esters, may contain complex mixtures of molecules.

To measure the danazol and fenofibrate solubility in anhydrous surfactants and SEDDS, crystalline drug was added in excess to 3g anhydrous surfactant or SEDDS. Mixtures were incubated with continuous mixing at 37°C in an orbital mixer (Ratek Instruments, Melbourne, Victoria, Australia). At 24 h intervals over 6 days, a ~0.5 g sample was removed and centrifuged (Eppendorf 5408R, Eppendorf AG, Hamburg, Germany) at 1600*g* for 15 min. Accurately weighed samples of the supernatant were dissolved in 5 ml chloroform: methanol (2:1 v/v), and aliquots (100 µL) were then diluted >10-fold in methanol. For fenofibrate in the SEDDS, samples were analyzed for drug content using a UV spectrophotometer (Cecil CE 3021; Cecil Instruments, Ltd., UK) measuring absorbance at 286 nm. All other samples were analyzed for drug content by content by HPLC (see section 4.2.7). Equilibrium solubility in the anhydrous excipients and SEDDS was defined as the value attained when consecutive solubility values differed by <5%.

To prepare SEDDS containing one of the two drugs, the required mass of drug was weighed directly into clean screw-top glass vials and drug-free SEDDS was added up to target mass. Vials were sealed, vortex-mixed and incubated at 37°C for at least 12 - 24 h prior to testing.

4.2.3 In vitro digestion testing

In vitro digestion experiments were performed as previously described (Devraj et al., 2012a, Sek et al., 2002b). In brief, 0.25 g of SEDDS or 0.125 g of surfactant was dispersed in 9 ml digestion medium (50 mM Tris-maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM NaTDC, 1.25 mM PC, pH 7.5) for 10 min followed by the addition of 1 ml pancreatin containing 10 000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per

ml of digestion medium. Digestion of the surfactant was continuously monitored using a pH-stat titrator (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.6M NaOH.

After 30 min, 2 x 4 ml samples were collected from the reaction vessel and digestion was inhibited in these samples using a lipid digestion inhibitor (0.5M 4-BPB in methanol, 9µl/ml of digestion sample). Samples were then ultracentrifuged (400,000*g*, 37°C, Optima XL-100K Ultracentrifuge, SW-60 swinging-bucket rotor, Beckman, Palo Alto, CA) in soft-walled polyallomer tubes (Beckman) for 30 min to separate the digestion samples into a poorly dispersed oil phase, a colloidal aqueous phase (AP_{DIGEST}) and a pellet phase. We chose to stop digestion at 30 min because the differences between 30 min and 60 min experiments were limited. Approximately 90% of the digestion occurs within 15 min.

In instances where a drug-containing SEDDS was digested, all three digestion phases were isolated and analyzed for drug content. Firstly, the oil phase (where present) in the digestion samples was carefully aspirated using an adjustable pipette and transferred to a 10 ml volumetric flask, followed by 50 µl of 1M HCl and chloroform-methanol mixture (2:1 v/v) up to volume. The sample tubes were then pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the AP_{DIGEST}. Finally, to remove the pellet, the polyallomer tube was cut just above the mass of the pellet phase and suspended in 100 µl chloroform: methanol (2:1 v/v). The pellet was then transferred to a 5 ml volumetric flask followed by 50 µl of 1M HCl and chloroform: methanol (2:1 v/v) up to volume. Each of the recovered phases was further diluted >10-fold in methanol prior to HPLC analysis (see section 4.2.7) to determine the fenofibrate content in individual phases. In instances where a drug-free SEDDS was digested, only the AP_{DIGEST} was isolated. Drug solubility in the AP_{DIGEST} was determined according to the method described in section 4.2.4.

4.2.4 Drug solubility in AP_{DIGEST}

Drug-free SEDDS (0.25 g) was digested for 30 min according to the method described in section 4.2.3. Fenofibrate/danazol was subsequently added in excess to 3 ml AP_{DIGEST}, and equilibrated for 48 h at 37°C in an orbital mixer (Ratek Instruments). At intervals (i.e., 4, 8, 24 and 48 h), 1 ml samples were removed, centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min. 50-100 µl aliquots of each homogenous supernatant were diluted >10-fold with methanol before analysis of drug content by HPLC (see section 4.2.7). Equilibrium solubility in the digested SEDDS was defined as the value attained when consecutive solubility values differed by <5%.

4.2.5 Estimated extent of SEDDS digestion

Digestion profiles were corrected by subtracting for the fatty acid released upon digestion of the bile salt/phospholipid mixed-micelles (i.e. the fatty acid titrated in blank experiments in the absence of formulations); this concentration was determined in separate experiments undertaken in the absence of surfactant/SEDDS. The total concentration of fatty acid titrated over 30 min (corrected for the background fatty acid) was compared to the theoretical quantity of fatty acid that could be liberated if the surfactants/SEDDS were completely hydrolyzed. This provided an estimation of the extent of digestion using Equation 1. In-line with previous work (Devraj et al., 2012c, Williams et al., 2012g, Christensen et al., 2004a, Li and McClements, 2010), it was assumed that on digestion of lipids *in vitro*, one triglyceride molecule released two fatty acid molecules (plus a non-digestible 2-monoglyceride), and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a single fatty acid molecule. In the case of the surfactants, it is assumed that all fatty acids are available for hydrolysis, which is consistent with previous work (Christiansen et al., 2010a, Cuine et al., 2008b). The estimated fatty acid content in Cremophor® EL, Cremophor®

RH40 and Tween® 80 were taken from Cuine et al (Cuine et al., 2008b). For TPGS monoesters (molecular weight of 1513), it was assumed that each molecule can liberate one fatty acid molecule. Solutol® HS-15 consists of polyethoxylated 12-hydroxystearic acid and some free polyethylene glycol (30%) (BASF, 2012). As ethoxylation may occur at both the carboxyl moiety and the hydroxyl moiety of 12-hydroxystearic acid, Solutol® is a mixture of monoesters (molecular weight of 961) or diesters (molecular weight of 1244). Complete digestion of 0.125 g of Solutol® HS-15 (the amount of surfactant present in the digestion studies) would therefore liberate 9.1 mM and 14.1 mM of fatty acid if consisting entirely of mono- or diesters, respectively. In the interest of simplicity, it is assumed that Solutol® consists of 70% monoesters of 12-hydroxystearic acid and 30% polyethylene glycol.

$$\text{Extent (\%) of digestion} = \frac{\text{titrated fatty acid (mmoles)} \times 100}{\text{Theoretical maximum titratable fatty acid (mmoles)}} \quad \text{Equation 1}$$

4.2.6 Supersaturation

To calculate the supersaturation ratio (SR) after 30 min of digestion of LBFs containing drug, the concentration of drug in $AP_{DIGESTS}$ after 30 min digestion (AP_{30MIN}) was divided by the drug solubility in the $AP_{DIGESTS}$ (determined in section 4.2.4):

$$SR = \frac{\text{Drug concentration in } AP_{30\ MIN}\ (\mu g/ml)}{\text{Drug solubility in } AP_{DIGEST}\ (\mu g/ml)} \quad \text{Equation 2}$$

Equation 3 was used to calculate the maximum supersaturation ratio (SR^M), which is the ratio between the maximum drug concentration in the $AP_{DIGESTS}$ (AP_{MAX}) in the absence of any drug precipitation (i.e., drug dose divided by test volume) and drug solubility in the AP_{DIGEST}:

$$SR^M = \frac{AP_{MAX}\ (\mu g/ml)}{\text{Drug solubility in } AP_{DIGEST}\ (\mu g/ml)} \qquad \text{Equation 3}$$

4.2.7 HPLC detection of model drugs

All HPLC analyses were performed using a Waters Alliance system comprising a 2695 Separation Module and model 486 tunable absorbance detector (Waters Alliance Instruments, Milford, MA). The column used for fenofibrate assays was a Phenomenex® Luna C_{18} column (150 x 4.6 mm, 3µm Phenomenex, Torrence, CA). The column used for danazol assays was a Waters Symmetry® C_{18} column (150 x 15 mm, 5 µm, Waters Symmetry®) with a C_{18} security guard cartridge (4 x 2.0 mm, Phenomenex). For both drugs the injection volume was 50 µL. UV detection was at 288 nm for fenofibrate and 286 nm for danazol. The mobile phase consisted of methanol and Milli-Q water in a 75:25 $^{v}/_{v}$ ratio and was pumped through the column at a flow rate of 1 ml/min.

4.2.8 Polarized light microscopy

A Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters was used to analyze selected digestion pellets containing danazol or fenofibrate. Each pellet was carefully removed from the sample tube and placed on a microscope slide. Samples were analyzed under cross-polarized light at 20x magnification, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan). Pellets were isolated and analyzed in the manner described above (section 4.2.3) on the same day.

4.3 Results

4.3.1 In vitro digestion of SEDDS in the absence of drug

Profiles of fatty acid titrated during *in vitro* digestion of Type IIIA SEDDS formulated with different nonionic surfactants are shown in Figure 4.1. Titratable fatty acid corresponds to fatty acid liberated in response to digestion of the formulation components by pancreatic enzymes. $SEDDS_{TPGS}$ and $SEDDS_{CRH40}$ formulations show a near-linear rate of digestion over 25 min, whereas digestion of the other SEDDS was most rapid during the first 5 min of the test. The total concentration of fatty acid titrated varied from 18.7 mM for $SEDDS_{BR97}$ up to 30.1 mM for the $SEDDS_{SOLUTOL}$. Complete digestion of the lipid components in the SEDDS would lead to 28.0 mM of liberated fatty acid (marked by the dashed horizontal line in Figure 4.1). Using this value as a measure of complete SEDDS digestion for formulations containing non-digestible Brij surfactants, the calculated extent of digestion of $SEDDS_{BR97}$ and $SEDDS_{BR98}$ (via Eq. 1) was 66% and 102%, respectively. Similar calculations for the remaining SEDDS are complicated by potential surfactant-derived fatty acid, since Cremophor®, Tween® and Solutol® surfactants all contain ester groups that may be

hydrolyzed in *in vitro* digestion tests (Cuine et al., 2008b, Williams et al., 2012g, Devraj et al., 2012c, Christiansen et al., 2010a). The fatty acid titrated in Figure 4.1 for the equivalent SEDDS is therefore likely to be derived from both lipid and surfactant. By calculating the maximum and minimum mass of available fatty acid (i.e. in the case of either complete digestion or no digestion of the surfactant), the calculated extents of digestion of the remaining SEDDS are (in increasing order of digestion); 58%-87% for $SEDDS_{CRH40}$, 62%-97% for $SEDDS_{CREL}$, 76%-102% for $SEDDS_{T80}$ and 81-108% for $SEDDS_{SOLUTOL}$. From these values, and from Figure 4.1, it is evident that the $SEDDS_{CRH40}$ was digested to a lesser extent and more slowly when compared to the equivalent $SEDDS_{CREL}$, which is consistent with previous work (Cuine et al., 2008b). However, by the end of the test, differences in the extent of digestion amongst all SEDDS, with the exception of $SEDDS_{BR97}$, were modest. PEG-succinic acid esters in TPGS have been shown to be resistant to hydrolysis by pancreatic enzymes (Christiansen et al., 2010a). Similarly to the SEDDS containing the Brij® surfactants, which contain only non-digestible ethers, titrated fatty acids from $SEDDS_{TPGS}$ are likely to be derived only from the mixed glycerides.

Figure 4.1: Apparent titration of fatty acids released during *in vitro* digestion of Type IIIA SEDDS, each containing one of seven nonionic surfactants. Formulations were; ▼$SEDDS_{BR97}$, ▽$SEDDS_{BR98}$, ■$SEDDS_{CREL}$, □$SEDDS_{CRH40}$, ●$SEDDS_{T80}$, ○ $SEDDS_{SOLUTOL}$◆ $SEDDS_{TPGS}$. Each SEDDS contained 50 % w/w lipid (soybean oil:Maisine^{TM} 35-1, 7:3) and 50% surfactant. Digestion was initiated at 0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during the test. Titrated fatty acid has been corrected for background fatty acid (liberated mainly by digestion of phospholipids) determined in background digestion tests (i.e., digestion medium in the absence of SEDDS). Data for the $SEDDS_{T80}$ has been reproduced from (Devraj et al., 2012c)

4.3.2 Drug solubility in the anhydrous surfactants and SEDDS

Danazol and fenofibrate equilibrium solubilities in the seven surfactants investigated and the corresponding SEDDS are shown in Table 4.2. Danazol solubility in the pure surfactants was higher (1.4- 2.9 fold) than in the respective SEDDS, reflecting its higher solubility in hydrophilic surfactants over long-chain lipids (Williams et al., 2012f). The highest danazol solubility was determined in the SEDDS containing the Brij® surfactants (22.1 mg/g and 23.5 mg/g for $SEDDS_{BR97}$ and $SEDDS_{BR98}$, respectively) and the lowest solubility value was 12.2 mg/g in $SEDDS_{SOLUTOL}$.

Fenofibrate solubility was highest in the $SEDDS_{T80}$ and lowest in the $SEDDS_{CRH40}$. In contrast to the corresponding data for danazol the narrow range of fenofibrate solubility in the SEDDS indicate that its solubility was much less sensitive to the chemistry of the surfactant. The log P values for danazol (log P 4.5 (Bakatselou et al., 1991)) and fenofibrate (log P 5.2 (Munoz et al., 1994)) are both high, yet fenofibrate exhibited much higher (5-10 fold) solubility in each lipid formulation.

Table 4.2: Equilibrium Solubilities of Danazol and Fenofibrate in the Pure Surfactants and the Corresponding SEDDS

Surfactant	Danazol solubility [a] (mg/g)		Fenofibrate solubility [a] (mg/g)	
	pure surfactant	SEDDS[b]	pure surfactant	SEDDS[b]
Brij® 97	34.6 ± 1.1	22.1 ± 0.3	141.7 ± 0.3	100.0 ± 2.5
Brij® 98	34.5 ± 1.4	23.5 ± 0.4	134.3 ± 3.3	105.6 ± 3.0
Cremophor® EL	31.7 ± 0.4	20.7 ± 0.9	113.6 ± 5.8	97.6 ± 4.0
Cremophor® RH40	33.0 ± 1.3	14.2 ± 2.4	117.1 ± 3.3	96.2 ± 3.8
Solutol® HS-15	35.1 ± 2.1	12.2 ± 1.7	124.7 ± 1.5	100.7 ± 4.5
Tween® 80	31.5 ± 2.6[c]	14.2 ± 2.4[c]	102.1 ± 3.3[c]	106.4 ± 8.0[c]
TPGS	30.5 ± 1.4	21.2 ± 0.4	114.8 ± 3.3	101.8 ± 1.2

[a]Solubility was determined at 37◦C and values are expressed as means (*n* = 3) ± 1 SD.

[b]SEDDS contained 50% (w/w) lipid (soybean oil–MaisineTM 35-1, 7:3, w/w) and 50% (w/w) of the listed surfactant.

[c]Data for the SEDDST80 have been reproduced from (Devraj et al., 2013a)

4.3.3 In vitro digestion of SEDDS containing drugs at 80% saturation

Danazol or fenofibrate was incorporated into each of the seven SEDDS at a load equivalent to 80% of the equilibrium solubility values shown in Table 4.2. These formulations were used to investigate the impact of 30 min digestion on the fate of the drug in each case.

4.3.4 Drug distribution following digestion

Figure 4.2 shows the effect of *in vitro* digestion of SEDDS on the fate of incorporated danazol (Figure 4.2A) or fenofibrate (Figure 4.2B). The results are presented as the percentage of the

dose recovered from each phase produced by digestion, namely; an oil phase consisting of any undigested triglyceride and lipophilic digestion products; the colloidal AP_{DIGEST} consisting of the majority of the amphiphilic digestion products (but not undigested oil droplets), bile salt and phospholipid; and, lastly, the pellet phase consisting of insoluble calcium soaps of fatty acid and any precipitated drug.

For both danazol and fenofibrate, digestion samples from $SEDDS_{BR97}$ contained the largest volume of oil phase, which is consistent with the lower extent of digestion of this particular formulation (Figure 4.1). Due to better digestibility, the digestion samples from all other SEDDS contained little or no oil phase. Following digestion of the SEDDS, the majority (>90%) of danazol was solubilized in the AP_{DIGEST} (Figure 4.2A). This outcome, which represents good, or desirable performance *in vitro* (i.e., a low precipitation tendency), is in agreement with recent work by Williams *et al* on formulation of danazol in Type IIIA SEDDS containing long-chain lipids (Williams et al., 2012b, Williams et al., 2012f).

The results of identical experiments carried out using fenofibrate (Figure 4.2B) however reveal that 55 – 88% of the fenofibrate dose was recovered from the pellet phase, indicating that the drug had precipitated extensively during the digestion experiment. Precipitation was lowest in the cases of $SEDDS_{BR97}$ and $SEDDS_{TPGS}$ (though it still amounted to >50%); this was concurrent with the presence of an oil phase post-digestion, and was consistent with previous work (Devraj et al., 2012c).

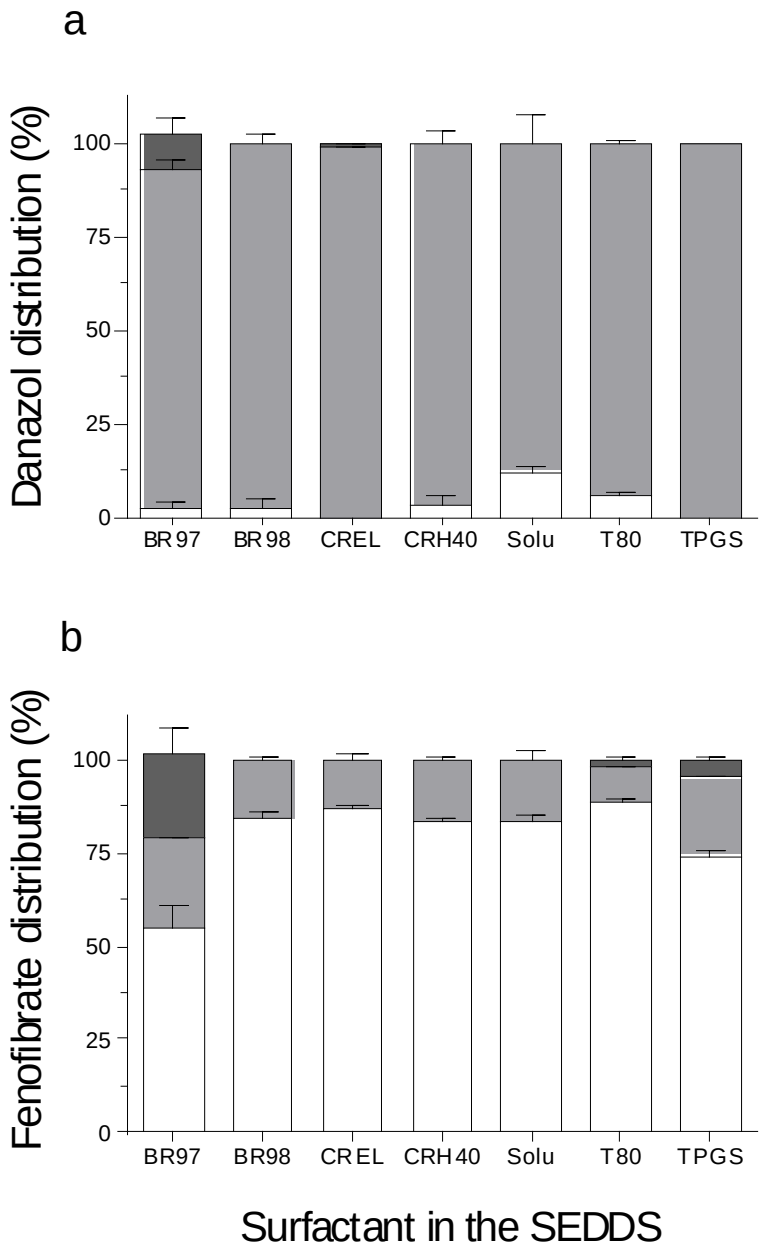


Figure 4.2: The effect of *in vitro* digestion on the fate of danazol (A) and fenofibrate (B) in SEDDS containing various nonionic surfactants. Surfactants were; Brij® 97 (BR97), Brij® 98 (BR98), Cremophor® EL (CREL), Cremophor® RH40 (CRH40), Solutol® HS-15 (Solu), Tween® 80 (T80) and TPGS. The stacked box-plots show the % of total drug distribution within a poorly dispersing oil phase (dark shaded bars), colloidal aqueous phase, AP_{DIGEST} (light shaded bars) and pellet phase (white bars). Values are expressed as means (n =3) ± SD. In all cases each SEDDS contained a drug load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation. The compositions of the formulations can be found in Table 4.1. Data for the $SEDDS_{T80}$ containing fenofibrate has been reproduced previously (Devraj et al., 2012c).

4.3.5 Supersaturation

To determine whether the differences in performance between danazol and fenofibrate-containing SEDDS shown in Figure 4.2 could be explained by the degree of supersaturation produced during digestion, the drug solubility in drug-free $AP_{DIGESTS}$ (obtained by digestion of drug-free SEDDS) was determined, and SR and SR^M values were calculated according to Eq. 2 and 3, respectively. Results are presented in Table 4.3 for danazol and in Table 4.4 for fenofibrate.

Danazol solubility values in respective $AP_{DIGESTS}$ (Table 4.3) are within a 125- 202 µg/ml range, lowest in the case of $SEDDS_{T80}$ and highest in the case of the $SEDDS_{TPGS}$. As danazol concentrations in the $AP_{DIGESTS}$ following digestion of equivalent drug-containing SEDDS (AP_{30MIN}) are in excess of these solubility values, it was apparent that digestion led to supersaturation. SR values, however, are modest, ranging from 1.3 ($SEDDS_{SOLUTOL}$) to 2.3 ($SEDDS_{CRRH40}$). The higher SR values in this range are a reflection of a lower solubility in the AP_{DIGEST} (e.g., $SEDDS_{CRH40}$) and/or a slightly higher absolute drug loading because of a higher solubility in the anhydrous formulation (e.g., $SEDDS_{CREL}$). SR^M, the ratio between the maximum danazol concentration in the AP_{DIGEST} in the absence of drug precipitation (AP_{MAX}) and drug solubility in the AP_{DIGEST} captures the maximal driving force of drug precipitation during *in vitro* digestion (Williams et al., 2012c, Anby et al., 2012e, Devraj et al., 2012c). As danazol showed a low propensity to precipitate, values for SR^M are similar to respective SR values (Table 4.3), slight differences being due to either a small amount of precipitation (e.g., $SEDDS_{SOLUTOL}$), the collection of drug in some cases in a phase-separated oil phase (e.g., $SEDDS_{BR97}$), or some incomplete recovery of drug from the AP_{DIGEST} (although total drug recoveries in the case of danazol was typically > 90%).

In contrast to danazol, SR^M values for fenofibrate were considerably higher (Table 4.4), ranging from 5.0 ($SEDDS_{SOLUTOL}$) up to 9.3 ($SEDDS_{T80}$). This marked difference in SR^M values between the two drugs can be explained by the higher (between 5-10 fold) solubility of fenofibrate in each anhydrous SEDDS, giving an equivalent increase in AP_{MAX} (since all of the SEDDS contained drug at 80% of its respective solubility in each SEDDS), relative to the much smaller (~2-fold) difference in fenofibrate solubility values in the $AP_{DIGESTS}$.

Since the majority of the incorporated fenofibrate precipitated during the digestion experiments (Figure 4.2B), SR values after 30 min digestion were already well below SR^M. In many cases, SR was below or close to unity, indicating that the extent of this precipitation was such that any supersaturation was effectively removed by precipitation within 30 min. The fact that many SR values are <1 may reflect the non-equilibrium conditions generated during dispersion. SR values would be expected to return to unity at equilibrium.

In summary the supersaturation values in Table 4.3 and Table 4.4 together with the results in Figure 4.2 taken together indicate that digestion of the SEDDS led to supersaturation of both danazol and fenofibrate, but the higher SR^M values for fenofibrate formulations (SR^M>5.0) were sufficiently high to promote drug precipitation.

Table 4.3: Supersaturation ratios(SR) and maximum supersaturation ratios (SR^M) for danazol, resulting from *in vitro* digestion of SEDDS containing various nonionic surfactants. Danazol solubility in the AP_{DIGEST}, relative to measured (AP_{30MIN}) and maximum danazol concentrations (AP_{MAX}) in AP_{DIGEST} allow calculation of SR and SR^M respectively.

Formulation	Solubility (ug/ml)			SR^d	SR^{Me}
	AP_{DIGEST}[a]	AP_{30MIN}[b]	AP_{MAX}[c]		
$SEDDS_{BR97}$	184 ± 0.3	307.0 ± 33.7	442	1.7	2.4
$SEDDS_{BR98}$	187 ± 0.3	311.4 ± 21.2	470	1.6	2.5
$SEDDS_{CREL}$	131 ± 2.7	281.6 ± 46.5	414	2.1	3.1
$SEDDS_{CRH40}$	130 ± 1.5	298.3 ± 35.5	270	2.3	2.1
$SEDDS_{SOLUTOL}$	135 ± 1.3	182.7 ± 6.5	244	1.3	1.8
$SEDDS_{T80}$	125 ± 7.5[f]	233.8 ± 5.5[f]	284[f]	1.8[f]	2.3[f]
$SEDDS_{TPGS}$	202 ± 2.3	286.8 ± 15.5	424	1.4	2.1

[a]Drug solubility in the AP_{DIGEST} obtained following 30 min *in vitro* digestion of drug-free SEDDS.

[b]Measured drug concentration in the AP_{DIGEST} following 30 min digestion of the drug-containing SEDDS.

[c]Maximum theoretical concentration (i.e., in the absence of drug precipitation) attained in the AP_{DIGEST} during digestion, and is calculated using drug load in the formulation divided by the volume of the test.

[d]Ratio of drug in AP_{30MIN} to the drug solubility in AP_{DIGEST} (see Eq.2). Values shown in the table correspond to those obtained using a 80% saturation level in the formulation.

[e]Ratio of AP_{MAX} to drug solubility in AP_{DIGEST} (see Eq.3).

SEDDS contained 50% (w/w lipid (soybean oil: Maisine^{TM}35-1, 7:3) and 50% w/w of the listed surfactant.

[f]This data point is taken from a published study –(Devraj et al., 2012c)

Table 4.4: Supersaturation ratios(SR) and maximum supersaturation ratios (SR^M) for fenofibrate, resulting from *in vitro* digestion of SEDDS containing various nonionic surfactants. Fenofibrate solubility in the AP_{DIGEST}, relative to measured (AP_{30MIN}) and maximum fenofibrate concentrations (AP_{MAX}) in AP_{DIGEST} allow calculation of SR and SR^M respectively.

Formulation	Solubility (ug/ml)			SR^d	SR^{Me}
	AP_{DIGEST} [a]	AP_{30MIN} [b]	AP_{MAX} [c]		
$SEDDS_{BR97}$	330 ± 3.0	346.6 ± 10.5	2000	1.1	6.1
$SEDDS_{BR98}$	300 ± 8.2	221.7 ± 17.1	2011	0.7	6.7
$SEDDS_{CREL}$	371.1 ± 5.7	212.4 ± 21.7	1952	0.6	5.3
$SEDDS_{CRH40}$	342.8 ± 9.8	224.1 ± 21.4	1924	0.6	5.6
$SEDDS_{SOLUTOL}$	400 ± 9.0	245.2 ± 28.8	2014	0.6	5.0
$SEDDS_{T80}$	230.0 ± 3.4	180.8 ± 11.0	2128	0.8	9.3
$SEDDS_{TPGS}$	370 ± 1.2	321.3 ± 19.4	2036	0.9	5.5

Details of [a-e] are provided below Table 4.3.

4.3.6 In vitro digestion of SEDDS containing various fenofibrate loadings

To explore the link between SR^M and the fate of drug during *in vitro* digestion of SEDDS, fenofibrate loading in selected SEDDS was reduced by half from 80% to 40% saturation. In accordance with Eq. 3, this also decreases SR^M by half, and therefore, reduces the driving force of precipitation. The results are presented in Figure 4.3, which shows the data obtained at 80% and 40% saturation to allow comparison of the effect of fenofibrate load (and SR^M) on SEDDS performance. The SEDDS were selected to provide the widest possible range of SR^M values, ranging from 2.5 for $SEDDS_{SOLUTOL}$ up to 4.7 for $SEDDS_{T80}$when the drug load was equivalent to 40% saturation.

The results show that lowering fenofibrate loading led to a reduction in drug precipitation during digestion (calculated as % of dose). For $SEDDS_{CREL}$ and $SEDDS_{SOLUTIOL}$, the reduction in precipitation effectively represented a reversal in the quality of performance of the formulation, such that the majority (>90%) of fenofibrate at 40% saturation was solubilized within the AP_{DIGEST}. Fenofibrate AP_{30MIN} values were 969 ± 56µg/ml and 606 ± 40µg/ml for $SEDDS_{CREL}$ and $SEDDS_{SOLUTIOL}$, respectively, >2-fold higher than the respective AP_{30MIN} values determined at the higher drug loading of 80% saturation (shown in Table 4.4). By decreasing SR^M, and therefore, the propensity for precipitation, higher solubilized drug concentrations and more sustained supersaturation was attained (at 30 min; SR was 2.6 for $SEDDS_{CREL}$ and 1.5 for $SEDDS_{SOLUTOL}$).

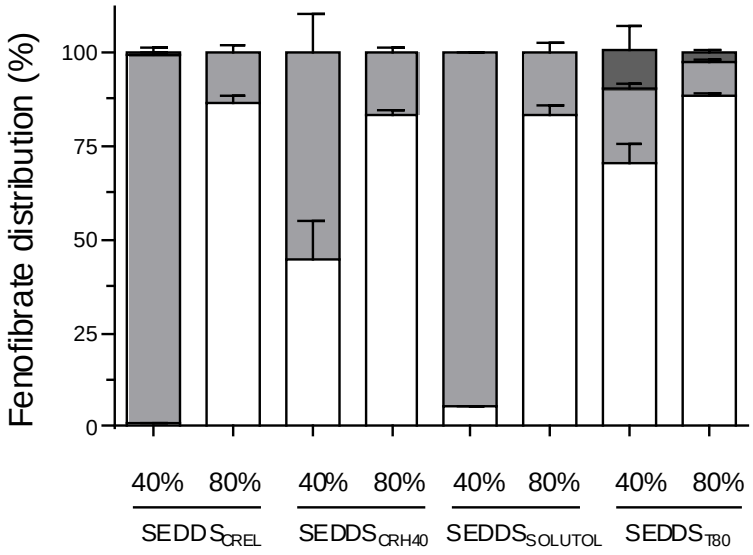


Figure 4.3: The effect of SR^M produced by each SEDDS on the fate of fenofibrate following 30 min *in vitro* digestion. Fenofibrate loading is expressed as the SR^M values generated using 40% or 80% of the equilibrium solubility in the anhydrous SEDDS, each containing one of four surfactants. The results for 80% loading, now expressed in terms of SR^M are duplicated from Figure 4.2. SR^M values at 40% and 80% saturation were; 2.6 and 5.3 ($SEDDS_{CREL}$), 2.8 and 5.6 ($SEDDS_{CRH40}$), 2.4 and 4.8 ($SEDDS_{SOLUTOL}$), and 4.7 and 9.3 ($SEDDS_{T80}$), respectively.

The legend for Figure 4.2 contains details of the layout of this figure. Values are expressed as means (n =3) ± SD.

Reducing the fenofibrate loading in$SEDDS_{CRH40}$ and $SEDDS_{T80}$also reduced the % drug precipitation, though the change in performance in these cases was more modest, with >40% drug precipitation evident in both cases (Figure 4.3). Values for SR at 30 min were SR 1.3 for $SEDDS_{CRH40}$ and 0.8 for $SEDDS_{T80}$, indicating that this precipitation practically removed all supersaturation. This observation that the change in performance with $SEDDS_{CRH40}$ and $SEDDS_{T80}$ was less pronounced can be attributed to the fact that a smaller amount of drug precipitation is required to remove supersaturation.

For the SEDDS that showed little evidence of drug precipitation, SR^M values at 40% saturation were 2.4 and 2.6 for $SEDDS_{SOLUTOL}$ and $SEDDS_{CREL}$, respectively. The corresponding values were 2.8 and 4.3 for $SEDDS_{CRH40}$ and $SEDDS_{T80}$,both of which showed evidence of drug precipitation (Figure 4.3). These observations suggest that lower SR^M values ($\leq$2.6) were associated with superior SEDDS performance. This provided an estimate of an apparent threshold SR^M, above which precipitation tended to occur, though this estimate was based on only half of the SEDDS used in the study. To probe the validity of the apparent threshold using a wider group of formulations, the fenofibrate loading in all SEDDS was adjusted so that a target SR^M of 2.6 would be attained. This SR^M value was selected for further study based on the results in Figure 4.3, which shows that the highest SR^M value that could be tolerated before significant drug precipitation occurred was 2.6 (i.e., $SEDDS_{CREL}$).

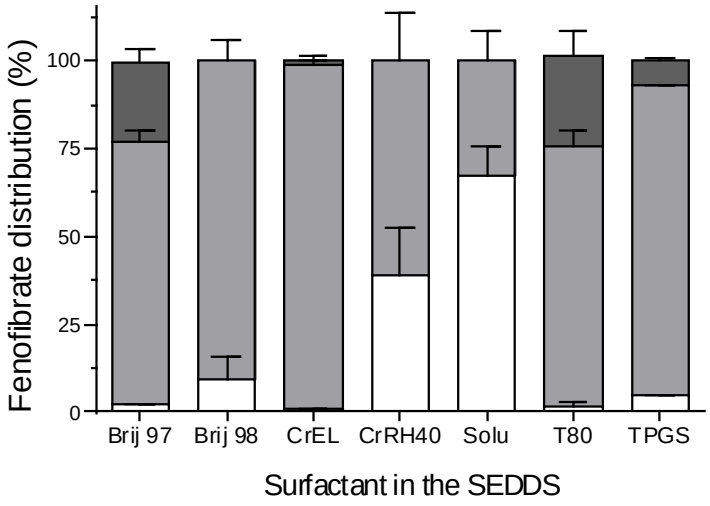


Figure 4.4: The performance of the seven SEDDS containing a fenofibrate load each of which generated a target SR^M of 2.6. The absolute fenofibrate loadings at this SR^M value were; 30.4 mg/g ($SEDDS_{BRIJ97}$), 31.2 mg/g ($SEDDS_{BRIJ98}$), 38.6 mg/g ($SEDDS_{CREL}$), 35.7 mg/g ($SEDDS_{CRH40}$), 41.6 mg/g ($SEDDS_{SOLUTOL}$), 23.9 mg/g ($SEDDS_{T80}$) and 38.5 mg/g ($SEDDS_{TPGS}$). The legend for Figure 4.2 contains details of the layout of this figure. Values are expressed as means (n =3) ± SD.

The effect of standardizing SR^M in all SEDDS on fenofibrate fate during digestion is shown in Figure 4.4 (which includes the $SEDDS_{CREL}$ results reproduced from Figure 4.3). The absolute fenofibrate loadings for each SEDDS are shown in the figure legend. Due to differences in fenofibrate solubility in the AP_{DIGEST} (i.e., the values in Table 4.3), the drug loading in respective SEDDS were varied in order to achieve the constant SR^M. Absolute drug loadings ranged from 23.9 mg/g ($SEDDS_{T80}$) up to 41.6 mg/g ($SEDDS_{SOLUTOL}$). With the exception of $SEDDS_{SOLUTOL}$ and $SEDDS_{CRH40}$, which showed evidence of significant drug precipitation, the different SEDDS maintained the majority of fenofibrate in a solubilized form, with little or no evidence of precipitation. In summary, standardizing the fenofibrate load in SEDDS to achieve a target SR^M of 2.6, led to formulations that were able to maintain drug in a supersaturated state in 5 of the 7 formulations.

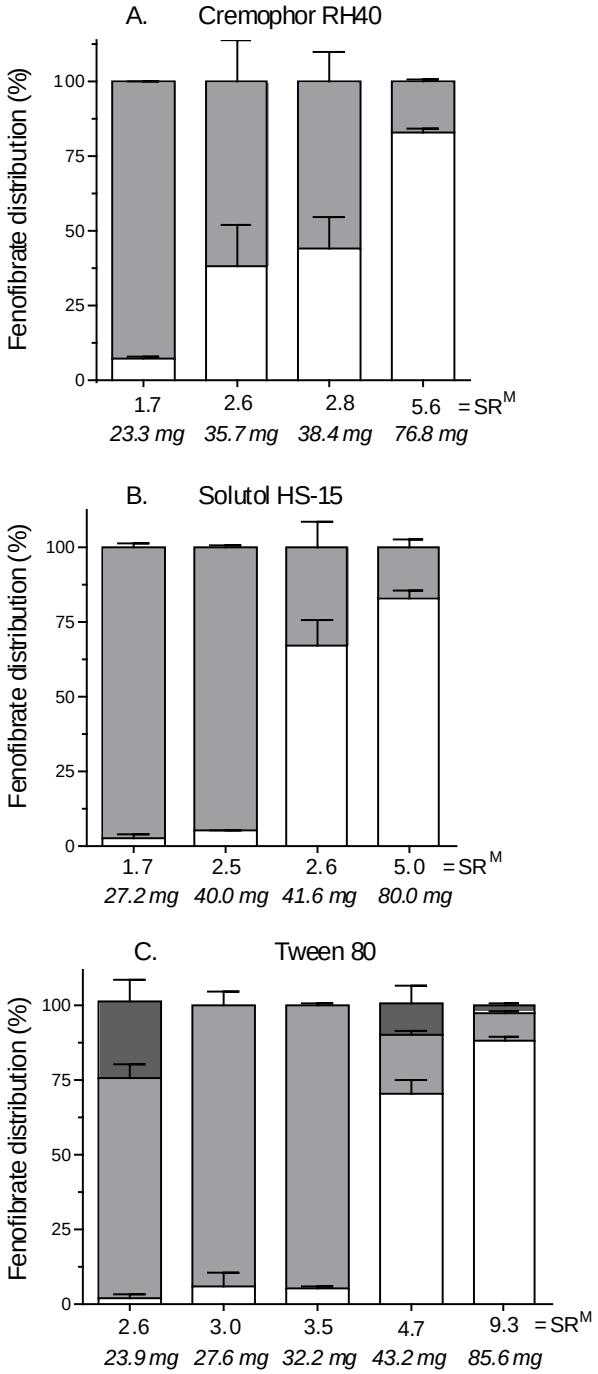


Figure 4.5**:** Fenofibrate distribution across the various digestion phases following 30 min *in vitro* digestion of $SEDDS_{CRH40}$ (A), $SEDDS_{SOLUTOL}$ (B) and $SEDDS_{T80}$ (C) with respect to SR^M (and absolute drug load). To allow comparison, certain results are duplicated from Figure 4.3 and Figure 4.4. The legend for Figure 4.2 contains details of the layout of this figure. Values are expressed as means (n =3) ± SD.

Figure 4.5 compares performance of $SEDDS_{CRH40}$ (Figure 4.5A), $SEDDS_{SOLUTOL}$ (Figure 4.5B) and $SEDDS_{T80}$ (Figure 4.5C) designed to generate a range of SR^M during digestion of the formulations. For each SEDDS, an increase in SR^M results from an increase in fenofibrate load, the details of which are shown in Figure 4.5 to aid the interpretation of the results. Performance of $SEDDS_{SOLUTOL}$ and $SEDDS_{T80}$ were compared because fenofibrate showed the highest and lowest solubilities in the respective $AP_{DIGESTS}$ of these formulations (400 µg/ml for $SEDDS_{SOLUTOL}$ and 230 µg/ml for $SEDDS_{T80}$), thereby capturing the widest range in absolute loadings required to achieve a particular SR^M. For example, at SR^M 2.6, the fenofibrate load was 41.6 mg/g for of $SEDDS_{SOLUTOL}$ but only 23.9 mg/g for of $SEDDS_{T80}$. Fenofibrate solubility in the AP_{DIGEST} from $SEDDS_{CRH40}$was intermediate to that of $SEDDS_{SOLUTOL}$ and of $SEDDS_{T80}$.

At 80% saturation, SR^M values for fenofibrate in each of the formulations were 5.0 for $SEDDS_{SOLUTOL}$, 5.6 for $SEDDS_{CRH40}$ and 9.3 for $SEDDS_{T80}$. The increase in SR^M across these formulations reflects the respective decrease in fenofibrate solubility in their $AP_{DIGESTS}$. $SEDDS_{CRH40}$ maintained the majority of fenofibrate in a solubilized but supersaturated state at SR^M 1.7, but there was evidence of increased drug precipitation at $SR^M \geq 2.6$ (Figure 4.5A). The SR^M threshold for $SEDDS_{SOLUTOL}$ was comparable at >2.5, above which there was significant drug precipitation (Figure 4.5B). In contrast, a higher SR^M threshold of >3.5 was required for precipitation from $SEDDS_{T80}$ (Figure 4.5C).

The highest absolute fenofibrate loading in $SEDDS_{T80}$ that resisted precipitation was 32.2 mg/g (SR^M 3.5) while $SEDDS_{SOLUTOL}$ was able to support a slightly higher loading of 40 mg/g (at SR^M 2.5) but not 41.6mg/g (at SR^M 2.6) . Therefore, the higher threshold SR^M value of 3.5 required to promote precipitation from $SEDDS_{T80}$ was not consistent with increased

drug load. This is better illustrated in Figure 4.6, which summarizes the performance of all seven SEDDS with respect to absolute mass of fenofibrate in the formulation.

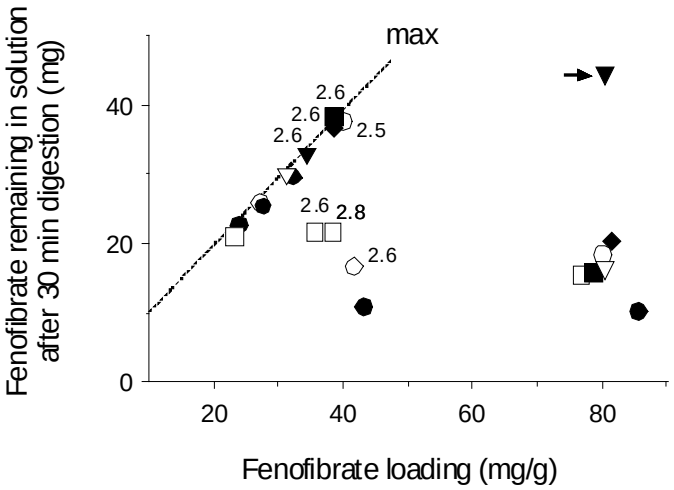


Figure 4.6: Mass of fenofibrate remaining in solution following 30 min *in vitro* digestion of the SEDDS plotted against absolute fenofibrate loading(mg/g) in the formulation. Symbols represent: ▼ $SEDDS_{BR97}$, ▽ $SEDDS_{BR98}$, ■ $SEDDS_{CREL}$, □ $SEDDS_{CRH40}$, ● $SEDDS_{T80}$, ○ $SEDDS_{SOLUTOL}$ and ◆ $SEDDS_{TPGS}$. Values plotted on the y-axis are derived from the dissolved drug concentration at 30 min multiplied by test volume and normalized to 1 g of SEDDS. The dashed line represents the maximum amount of drug in solution in the absence of precipitation. Selected SR^M values are also shown. The arrow refers to $SEDDS_{BR97}$ which was partially digested and formed a large oil phase (see text).

Performance in Figure 4.6 is compared by plotting the mass of fenofibrate that remains in solution (i.e., drug in the AP_{DIGEST} plus oil phase) following 30 min digestion against the dose. The dashed line denotes a 1:1 relationship between fenofibrate in the formulation and fenofibrate in solution, i.e. those points that lie on this dotted line represent absence of precipitation on digestion. Between ~20 - 35 mg/g fenofibrate loadings, all of the SEDDS lie

on the dotted line. However, there was considerable variability in performance for SEDDS containing between ~35 – 40 mg/g fenofibrate, with some SEDDS exhibiting extensive precipitation (e.g., $SEDDS_{CRH40}$) and others showing no such precipitation (e.g., $SEDDS_{SOLUTOL}$). This variability in SEDDS performance coincided with the generation of SR^M values that were ~2.5/2.6, or >3.5 in the case of $SEDDS_{T80}$. Further increases in fenofibrate loading produced higher values of SR^M, greater propensity for precipitation, and therefore, reduced variability between formulations. $SEDDS_{BR97}$, which contained fenofibrate at 80% saturation (see arrow in Figure 4.6) was the only exception at this high drug loading. The lower amount of precipitation in this case can be attributed to the reduced digestibility of this formulation (and presence of an oil phase). Figure 4.6 shows how increasing drug loading can be detrimental to the performance of the formulation, and how more sustained supersaturation is possible at lower drug loadings.

4.3.7 Microscopic analysis of the digestion pellet viewed under cross-polarized light

Figure 4.7 shows micrographs of the pellet formed following the digestion of $SEDDS_{T80}$ containing either (A) danazol or (B) fenofibrate. Pellets were viewed under cross-polarized light. The pellets formed from the danazol-containing $SEDDS_{T80}$ showed evidence of birefringence at 5 and 30 min digestion. Birefringent patterns were similar at the two time points, and since there was negligible precipitation of danazol in this case (see Figure 4.2A), the birefringence most likely originated from lamellar structures formed by calcium soaps of the fatty acids (Corkery, 2004).

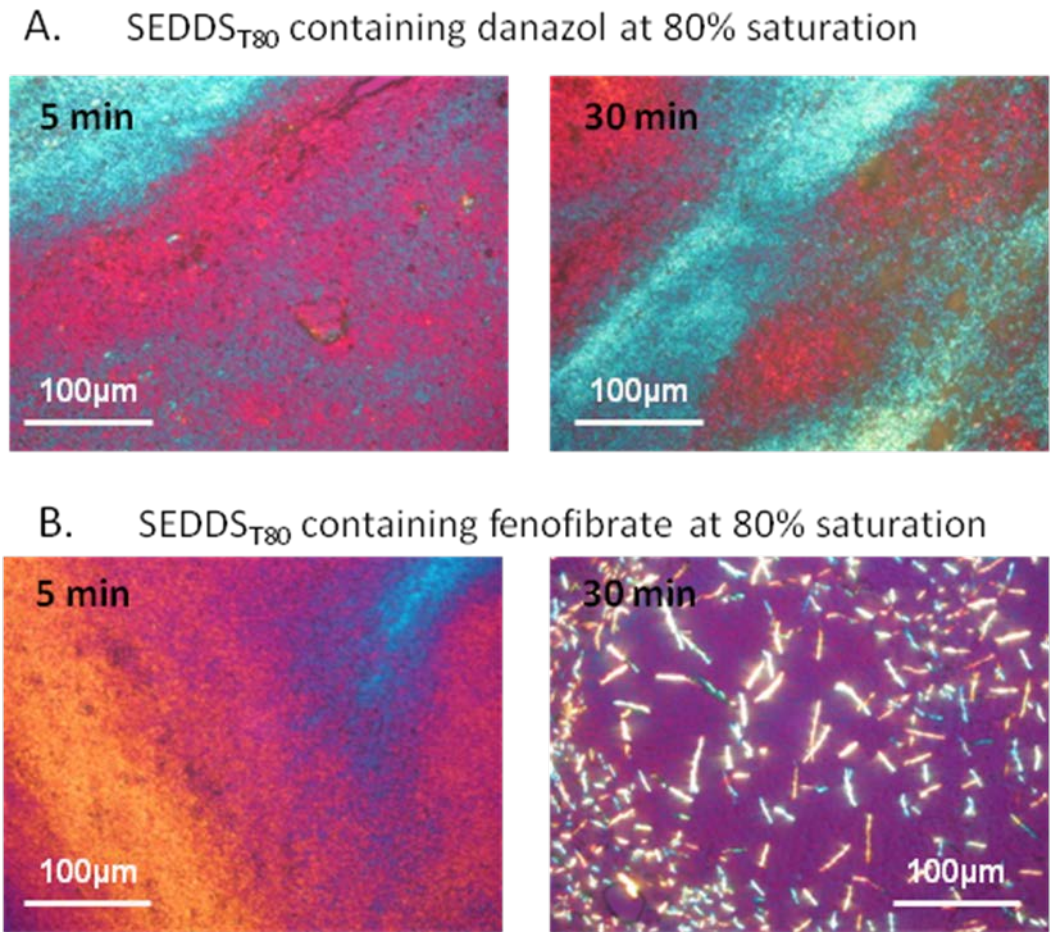


Figure 4.7: Micrographs of the pellet formed following digestion (5 or 30 min) of the SEDDS$_{T80}$. The formulation contained either A. danazol or B. fenofibrate at the 80% saturation level. Images were captured under cross-polarized light.

The pellet formed from the fenofibrate-containing SEDDS$_{T80}$ showed a similar birefringent pattern at 5 min, however the pellet after 30 minutes digestion was populated by imperfect rod-like crystal particles, indicating that the fenofibrate precipitate was crystalline. Fenofibrate is known to exist in two polymorphic forms (Di Martino et al., 2000). As the starting fenofibrate material consisted primarily of plate-like crystals (not shown), it is

possible that the fenofibrate precipitate in the digestion experiments consisted of the metastable polymorph.

4.4 Discussion

Self-emulsifying drug delivery systems (SEDDS) consisting of oil(s) and hydrophilic components such as surfactants(s) and co-solvent(s) often exhibit reduced solubilization of drugs following dispersion in aqueous fluids or following digestion of the lipid and/or surfactant (Pouton, 2006a, Williams et al., 2012c, Anby et al., 2012e, Porter et al., 2007b, Thomas et al., 2012a). *In vitro* dispersion and digestion tests, that determine if any loss of solubilization is sufficient to promote drug precipitation, are therefore useful to formulators for prediction of SEDDS performance. The emergence of a slowly dissolving precipitate *in vitro* is often predictive of decreased drug absorption *in vivo* (Anby et al., 2012e, Porter et al., 2004c, Han et al., 2009b, Dahan and Hoffman, 2006a, Gao et al., 2009).

In our previous study (Devraj et al., 2012c), a SEDDS of Type IIIA (according to the LFCS (Pouton, 2006a, Pouton, 2000a)), containing the drug fenofibrate, was shown to resist precipitation during *in vitro* dispersion testing, but showed a marked decrease in solubilization capacity on digestion, which in turn resulted in extensive crystallization of the drug. The aim of the present study was to better understand the factors that determine the performance of SEDDS during digestion and to inform formulators of SEDDS about the critical parameters that determine the fate of incorporated drug. The investigation involved seven different SEDDS, differing only in the identity of the nonionic surfactant used. Incorporation of danazol or fenofibrate revealed that the fate of the drug during digestion was predominantly governed by the maximum degree of supersaturation generated (SR^M), a parameter that describes the supersaturation pressure that occurs in the system (Williams et

al., 2012c). SR^M and other closely related parameters have previously been used to explain the risk of precipitation following digestion of range of different danazol-containing lipid formulations (Williams et al., 2012c, Devraj et al., 2012c). In the present study, the utility of SR^M to identify the threshold above which performance of lipid-based formulations becomes more variable is further highlighted.

SEDDS containing danazol at loadings equivalent to 80% of the respective equilibrium solubility in the formulations (denoted the '80% saturation level') showed no evidence of drug precipitation during 30 min digestion (Figure 4.2). Determination of danazol solubility in digested drug-free SEDDS, revealed that SR^M values generated by digestion of danazol formulations were less than ≤3.1. This finding was in general agreement with our previous work (Anby et al., 2012e, Williams et al., 2012c). In the latter studies when SR^M values > ~2.5 we observed precipitation during digestion of a range of lipid formulations containing danazol. Further insights into the relationship between SR^M and the performance of SEDDS containing danazol was limited in the present study by the low solubility of danazol in anhydrous formulations. The addition of a co-solvent such as ethanol to the SEDDS is a common approach to increase drug solubility in the formulation (Williams et al., In press, Pouton and Porter, 2008b). Co-solvents however contribute little to solubilization post-dispersion or post-digestion of the SEDDS and therefore often result in increased SR^M and promote danazol precipitation (Cuine et al., 2007b).

In contrast fenofibrate solubility in the anhydrous SEDDS was very high (>95 mg/g), >5-fold higher than the respective danazol solubility. Fenofibrate solubility in the digested formulations (i.e., the AP_{DIGEST}), was on average only 2-fold higher, and due to this disproportional change in solubility in the anhydrous and digested formulation, SR^M values

produced on digestion of SEDDS incorporated with fenofibrate at 80% saturation were >5.0 compared with ≤3.1 for the equivalent danazol-containing SEDDS. These higher SR^M values and the attendant increase in precipitation pressure explain the extensive drug crystallization during digestion of all seven fenofibrate-containing SEDDS (Figure 4.2B and Figure 4.7B).

In an attempt to better discriminate between the SEDDS formulations, the fenofibrate loading was lowered in an effort to decrease the precipitation pressure in the digestion test. The fenofibrate load was initially decreased by half to 40% saturation (thereby also halving SR^M), and this approach led to a clear reduction in precipitation from $SEDDS_{CREL}$ and $SEDDS_{SOLUTOL}$, but not $SEDDS_{CRH40}$or $SEDDS_{T80}$. It was proposed that, at 40% fenofibrate saturation, the higher SR^M values produced on digestion of $SEDDS_{CRH40}$ and $SEDDS_{T80}$ explained this difference in performance. Therefore, to normalize the effects of digestion on each formulation, the fenofibrate load was adjusted to achieve a SR^M of 2.6 (Figure 4.4), which represented the highest SR^M that did not result in precipitation at 40% saturation (Figure 4.3). Of the seven SEDDS investigated, five maintained practically the entire mass of drug in a solubilized (and supersaturated) state during the digestion test. Further tests to probe the performance of SEDDS at specific fenofibrate loadings (Figure 4.5) revealed that, with exception of $SEDDS_{T80}$, all SEDDS showed evidence of significant drug precipitation above a threshold SR^M of 2.5 - 2.6. The performance of SEDDS containing fenofibrate and a diverse range of surfactants could be explained by a single parameter, namely SR^M. The possibility that there exists a threshold supersaturation ratio, above which supersaturation cannot be maintained during the digestion tests, correlates with previous studies that evaluated danazol supersaturation resulting from digestion of SEDDS formulations containing medium-chain lipids (Williams et al., 2012c, Anby et al., 2012e) and lipid-free co-solvent/surfactant formulations (Williams et al., 2012c). The latter studies identified a

threshold SR^M value for danazol of approximately 2.5, which is very close to the threshold SR^M value for fenofibrate identified in the present study. Thus, the concept of a threshold SR^M value, that predicts the point at which drug precipitation becomes more prevalent *in vitro*, has now been shown to be applicable to a range of lipid formulations and two quite different drugs, fenofibrate being much more lipophilic than danazol. In practice the precise threshold value will be formulation-specific, and we do not wish to suggest that there is a threshold SR^M which is common to all formulations of all drugs. Nevertheless this and recent studies (Anby et al., 2012c, Williams et al., 2012a), suggest that formulators should be wary if their formulations generate $SR^M > 3$. For fenofibrate in this study only the Tween 80 formulation tolerated $SR^M > 3$ and we attribute that to the lower solvent capacity of this formulation for fenofibrate.

The observation that the threshold SR^M value may be largely formulation and drug-independent suggests that the capacity of lipid formulations to support drug for the duration of the digestion test (30 min in this study) may be explained by classical nucleation theory (CNT). The rate of nucleation (J) is defined in terms free energy change (ΔG^*) associated with the formation of a spherical nucleus of critical size:

$$J = K_0 \exp\left(\frac{-\Delta G^*}{k_B T}\right) \qquad \text{Equation 4}$$

Where T is temperature, k_B is the Boltzmann constant and K_0 is a kinetic coefficient. ΔG^* is the thermodynamic barrier to nucleation, below which small nuclei form but rapidly decompose. ΔG^* is commonly defined as:

$$\Delta G^{*} = \frac{16\pi\gamma^{3}(V_{m}/N_{A})}{3(k_{B}T\ln S)^{2}}$$ Equation 5

Where V_M is the molar volume of the solute, N_A is Avogadro's number, S is the degree of supersaturation and γ is the interfacial energy between the emerging new surface (i.e., the nuclei) and the bulk solution (James, 1985a, Turnbull and Fisher, 1949). This equation implies that increasing supersaturation leads to an exponential decrease in ΔG*, and in accordance with Eq. 4, an exponential increase in nucleation rate (J) (Vekilov, 2010).

The degree of supersaturation at which ΔG* is practically zero and nucleation occurs spontaneously has been termed the 'critical supersaturation' (Kashchiev and van Rosmalen, 2003). When a system is below the critical supersaturation, the higher ΔG* results in a slower rate of nucleation and enhanced metastability, such that periods of supersaturation are prolonged (Kashchiev and van Rosmalen, 2003, Lindfors et al., 2008). It is possible that the threshold supersaturation levels identified in the present studies and in previous work (Williams et al., 2012c, Anby et al., 2012e), that appear to predict the fate of supersaturated drug during the digestion of a lipid formulation, may mark the critical point at which the barrier to nucleation becomes negligible, allowing drug to precipitate readily.

The performance of a lipid formulation is expected to be most variable at or near the critical supersaturation, since only minor changes in drug loading and/or drug solubility in the digested formulation will determine whether a system is above (i.e., showing precipitation) or below (i.e., showing no precipitation) the critical point. Such variability was evident in the present study and is clearly illustrated in Figure 4.6 in the ~35 mg/g – 40 mg/g fenofibrate loading range. SR^M values within this range of fenofibrate load vary from 2.5 to just 2.8, yet performance varied from no precipitation (e.g., $SEDDS_{CREL}$) to considerable precipitation

(e.g., $SEDDS_{CRH40}$). The impact of using different surfactants on the performance of SEDDS was therefore most pronounced close to this threshold. Polymer precipitation inhibitors that slow the rate of nucleation or crystal growth may also be most effective in these instances, and may increase maximum degree of supersaturation that may be maintained (Anby et al., 2012e, Warren et al., 2011, Bevernage et al., 2011).

Of the seven SEDDS investigated, $SEDDS_{T80}$ was the only formulation that was able to support supersaturation above SR^M 2.6 (i.e., the threshold SR^M value for all other formulations). While this may suggest that Tween® 80 possesses a greater capacity to support supersaturation than the other surfactants investigated, this scenario was considered unlikely since sorbitan-fatty acid monoesters in Tween® 80 are readily hydrolyzed by pancreatic enzymes (such as carboxyl ester hydrolase) when investigated using *in vitro* digestion models (Christiansen et al., 2010a, Devraj et al., 2012c, Cuine et al., 2008b, Bakala N'Goma et al., 2012a). Alternatively, the capacity for $SEDDS_{T80}$ to support higher SR^M may be explained by the lower solvent capacity of this formulation. The concentration of fenofibrate required to generate SR^M = 2.6 in $SEDDS_{T80}$ was only 23.9 mg/g, whereas the other formulations contained between 30.4 and 38.5 mg/g. The fenofibrate concentrations at each particular SR^M were lower, which would be expected to reduce the collision frequency and nucleation rate.

Figure 4.8 illustrates how differences in solubilization capacity can lead to marked differences in concentration at equal degrees of supersaturation; SEDDS that exhibit the lowest and highest solubilization capacity post-digestion, namely $SEDDS_{T80}$ and $SEDDS_{SOLTUOL}$, are shown. The gradient of each slope represents the inverse of the solubilization capacity (230 µg/ml and 400 µg/ml for $SEDDS_{T80}$ and $SEDDS_{SOLTUOL}$, respectively) and the symbols capture instances where performance is characterized by negligible (closed symbols) or

extensive drug precipitation (open symbols). For $SEDDS_{SOLUTOL}$, the change from no precipitation to extensive precipitation occurs between SR^M 2.5 and 2.6, and at an AP_{MAX} of ~1000 µg/ml. However, due to the lower solubilization capacity of $SEDDS_{T80}$ after digestion, equivalent SR^M values of 2.5-2.6 are associated with a much lower AP_{MAX} of ~600µg/ml.

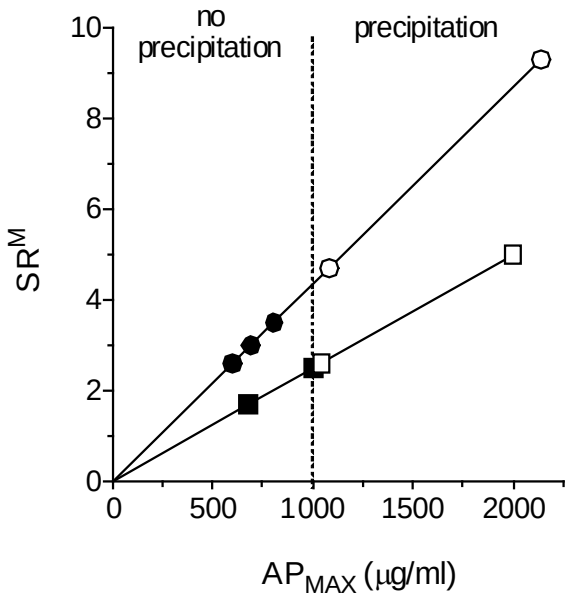


Figure 4.8: Linear plots of SR^M versus theoretical AP_{MAX} of fenofibrate after digestion of $SEDDS_{SOLUTOL}$ (squares) and $SEDDS_{T80}$ (circles). The theoretical AP_{MAX} is the concentration in the aqueous phase that would occur if no precipitation occurred. The vertical dotted line crosses the x-axis at a fenofibrate concentration of 1000 µg/ml. Open symbols to the right of the dotted line are indicative of incidences of extensive drug precipitation (see Figure 4.5B and Figure 4.5C).

The importance of absolute concentration to nucleation rate (J) is captured by the pre-exponential term in Equation 4, K_0, which is defined in Equation 6 as the number of molecules per unit volume, N_0 multiplied by the frequency at which the critical nuclei transform into crystals, V_0 (Boistelle and Astier, 1988):

$$K_0 = N_0 \cdot V_0 \qquad \text{Equation 6}$$

According to Equation 6, K_0 will increase as a system becomes increasingly concentrated and the number of collisions between supersaturated molecules and forming nuclei increases. Therefore, at a particular supersaturation, the lower fenofibrate concentration present in $SEDDS_{T80}$ implies that K_0 was lower, which will have resulted in a lower rate of nucleation (relative to other SEDDS at the same degree of supersaturation). This provides a possible explanation for the slightly higher capacity for $SEDDS_{T80}$ to support supersaturation. Bearing in mind potential differences in collision frequency, deviation from an apparent common SR^M threshold may be anticipated if formulations in a screening exercise show a wide range in solvent capacities after digestion. Also, while a SR^M threshold of approximately 2.5–2.6 appears to be valid for most danazol (Williams et al., 2012c, Anby et al., 2012e) and fenofibrate formulations, further work will be necessary to determine whether the threshold SR^M values discussed here are representative of common values that can be applied to a more diverse range of drug molecules.

As supersaturation can provide a driver for enhanced absorption (via increases in thermodynamic activity) (Gao et al., 2009), the design of SEDDS showing a capacity to maintain high drug concentrations in the supersaturated state during dispersion and digestion remains a goal for lipid formulation development. The use of measures of *in vitro* performance such as SR^M discussed in this study provide a mechanistic understanding of the

performance of lipid-based formulations that are likely to be of great value to scientists engaged in their development.

4.5 Conclusions

The loss of solubilization capacity resulting from dispersion and digestion of lipid-based formulations, such as the Type IIIA SEDDS investigated in this study, and other lipid formulations, can lead to metastable supersaturated systems that may promote drug absorption. However, as supersaturation may also promote drug precipitation, identification of the threshold level of supersaturation, beyond which extensive precipitation occurs, is necessary to ensure that the positive effects of supersaturation on drug absorption can be fully exploited. Here, the influence of maximum supersaturation ratio generated on digestion (SR^M) on the performance of seven different SEDDS, each containing either danazol or fenofibrate was evaluated. Despite differences in digestibility and solubilization capacity, the comparatively low SR^M values generated on digestion of SEDDS containing danazol, resulted in negligible precipitation, and therefore no significant differences in performance. In contrast, digestion of SEDDS containing fenofibrate generally led to higher SR^M values and above a threshold SR^M between 2 and 3 led to extensive drug crystallization. Performance of each SEDDS was variable close to a threshold SR^M value of 2.6. Below this value, SEDDS formulations maintained the bulk of the mass of drug in a solubilized form during dispersion and digestion. This study indicates that determination of the solubility of drug in each digested formulation, and the use of this data to calculate the value of SR^M for each formulation, is a simple and powerful tool for formulation scientists. Values of SR^M determined *in vitro* are unlikely to be a true reflection of the maximum SR^M encountered *in vivo*, nor can they predict the influence of the dynamic process of drug absorption on maintenance of supersaturation in the gut lumen. In our view the in vitro digestion test probably overestimates the likelihood of precipitation occurring *in vivo*. The 'rules of thumb' emerging from this and other recent *in vitro* studies can be used to take a conservative

approach to formulation if desired. The paper suggests that a simple *in vitro* test of solubility in a 'digested' formulation may be sufficient to identify the possibility of variable bioavailability caused by possible precipitation in the intestine. We suggest that formulators should adopt a strategy which includes an assessment of possible precipitation during digestion, to allow an informed assessment of risk to be taken into account when a final choice is made on which product to take forward into clinical development

.

Chapter 5: Summary, conclusions, and future directions

5.1 Summary and conclusions

Drugs incorporated in LBDDS and administered orally are expected to undergo transit within the GIT before being absorbed across the absorptive surface, predominantly enterocytes in the small intestine, and diffusing into the systemic circulation. Ideally, similar to any other carrier, drugs incorporated in LBDDS would be completely and efficiently absorbed and eventually elicit necessary pharmacological action. Broadly, various factors play a crucial role in achieving this end point *viz.* proper selection of excipients towards the design of the carrier, drug characteristics, the processes which the carrier system containing drug undergoes after peroral administration, physiological factors, and the patient-related or disease-related factors. Since the emergence of LBDDS a significant volume of literature has been published on the prediction of *in vivo* performance of PWSDs incorporated in LBDDSs. Until recently, the majority of these studies have determined the average particle size following dispersion in GI fluids as the potential indicator for *in vivo* performance (Pouton and Porter, 2008a, Gao et al., 1998a). Despite numerous academic studies to date, surprisingly few products have reached the market when weighed against their potential advantages (detailed in the introduction).

The common components of LBDDS being *lipids* and *surfactants,* many of which contain ester bonds, have been the subject of reports since the 1970s describing their digestion under the action of enzymes (Patton and Carey, 1979, Carey and Small, 1970, Carey et al., 1983, Gibson, 2007). The process of digestion, predominantly taking place in the small intestine, leads to significant changes in the chemical nature and properties of these components (Schick, 1977), and this has an impact on their suitability for incorporation into these systems. It was realized only in the recent past that the co-administered drug in these

systems is eventually solubilized in colloidal species formed, after initial dispersion and partial hydrolysis in gastric fluids into highly lipophilic coarse emulsion droplets, predominantly as a result of rapid hydrolysis of the esters groups within lipids and surfactants. This is the result of the secretion of pancreatic enzymes (including pancreatic lipase and others) after the arrival of these emulsion droplets in the small intestine from stomach (Fernandez et al., 2009b, Cuine et al., 2007a, Cuine et al., 2008a).

Drug absorption has been assumed to take place via passive diffusion of the free fraction of drug across the surface of the GIT. The drug is also present in solubilized form in colloidal species, and can partition between this and the free fraction. It is assumed that as the fraction of drug that is free is absorbed across GIT, a commensurate adjustment will cause it to be replenished from the solubilized portion of these colloidal species, maintaining a dynamic equilibrium and creating the flux and driving force for absorption across the absorptive surface. As, the nature and the solubilization capacity of the colloidal species formed during these processes changes dramatically as a function of time during GI transit, the fate of the drug incorporated in these systems during dispersion/emulsification process and subsequent digestion has been the focus of several studies using an *in vitro* digestion model (Porter et al., 2007a, Cuine et al., 2008a, Mohsin et al., 2009b, Thomas et al., 2012b, Tan et al., 2012b, Anby et al., 2012c).

However, the major drive for the studies conducted in the recent years has been to understand the significance of the potential loss of solubilization capacity of LBDDS during processes such as *dispersion (in gastric fluids)* (Pouton and Porter, 2008a, Chiang et al., 2011a, Pouton, 2006b) and *digestion (primarily taking place in small intestine)* (Cuine et al., 2008a, Anby et al., 2012c, Williams et al., 2012a, Devraj et al., 2013a, Kaukonen et al.,

2004b, Kaukonen et al., 2004a, Larsen et al., 2011a, Porter et al., 2004a, Porter et al., 2004b). These processes are expected to occur in the GIT potentially resulting in a condition of supersaturation (wherein drug is present in concentrations exceeding its equilibrium solubility in the intestinal environment). Supersaturation, has the advantage of increasing the absorption due to the attainment of enhanced absorptive flux and simultaneously has the disadvantage, due to its thermodynamically unstable nature, in leading to drug crystallization and eventually the risk of drug precipitation. Supersaturated drug concentrations have previously been reported to enable drug absorption from LBDDS as well as amorphous solid dispersions (Brouwers et al., 2009, Gao et al., 2009, Anby et al., 2012c). However, supersaturation ultimately causing drug precipitation in vitro may not be an indicator of poor drug performance. It has been demonstrated previously that there can be an enhanced *in vivo* bioavailability, even when precipitation is observed during *in vitro* studies, and the *in vivo* effect has been attributed to the formation of fast dissolving non-crystalline drug precipitates (Thomas et al., 2012b, Anby et al., 2012a). However, the majority of the studies reported in the literature have demonstrated poor *in* vivo performance of LBDDS upon evidence of precipitation of drug from these systems during *in vitro* evaluation (Anby et al., 2012c, Cuine et al., 2008a, Cuine et al., 2007a, Dahan and Hoffman, 2006b, Han et al., 2009a, Porter et al., 2004a, Porter et al., 2004b).

Prediction of in vivo performance of LBDDS from in vitro studies has been a challenge to scientists at research and development level as, despite having plenty of studies conducted and numerous literature available, the formulator even today still needs a standard generalized protocol to be followed to start working on a given candidate drug to be delivered orally via a LBDDS. Hence, rational formulation design with a systematic and simplistic approach of

handling is mandatory to prevent unnecessary and enduring effort, wastage of materials, time, and money during preclinical stage of drug development.

Recently, the focus of study in our laboratories (Devraj et al., 2013c, Devraj et al., 2013a, Williams et al., 2012a, Anby et al., 2012c) has been the impact of *extent of supersaturation* attained following dispersion and digestion as a consequence of loss of solubilization capacity on the performance of LBDDS. Increased supersaturation and thermodynamic instability has been previously reported to be the cause for extensive crystallization (James, 1985b, Turnbull, 1949).

In this thesis, Chapter 3: deals with investigation of a series of closely related SEDDS *viz.* Type II and Type IIIA defined as per LFCS (Pouton, 2006b, Pouton, 2000b), containing fenofibrate as model drug, to determine the factors influencing the performance of these systems during *in vitro* dispersion and digestion tests. The results were interpreted based on the level/extent of supersaturation attained during these *in vitro* processes to gain an insight into formulation performance and to establish guidelines for formulators. Precisely, emphasis was placed on the effects of *lipid composition (long-chain vs. medium-chain)* and the *surfactant type* (*hydrophilic vs. lipophilic)* on the solubilization properties of these formulations during dispersion and digestion.

Ideally, Type II LBDDS should contain no water-miscible components (Pouton, 2006b) and do not lose solvent capacity towards the incorporated drug after dispersion, thereby retaining drug completely in solubilized form. Previous studies conducted in our laboratory demonstrated partitioning of small proportion of excipients such as the non-ionic surfactant, Tween®85 from Type II formulations resulting in lowered solubilization capacity of the lipid-based formulation towards the incorporated hydrophobic drug (Mohsin et al., 2009b). Given

the polymeric nature of ethoxylated surfactants it would be expected that a proportion of the molecules in Tween 85 would be water-soluble, even though the bulk of the excipient is insoluble in water. In this thesis, we observed a similar effect however, the extent of this reduction in solubilization capacity was considered to be small. In support to this, our Type II formulations did not generate any significant supersaturation and therefore concluded that, dispersion of Type II lipid-based formulations maintained practically all the drug in solubilized form over 4 days with hardly any precipitation. Moreover, this solubilization capacity was retained despite the use of formulations containing drug at 80% of its equilibrium solubility in the anhydrous formulations.

Dispersion studies carried out on fenofibrate-containing Type IIIA formulations have shown a contrasting effect (compared to Type II formulations), typically resulting in a marked reduction in solubilization capacity and *higher degrees* of supersaturation. We have attributed this to the presence of a hydrophilic surfactant, Tween®80 which shows a greater affinity (than Tween®85) for aqueous media and hence might be expected to lose a greater proportion of its bulk solubilization properties on dispersion. Previously, in agreement with the present studies, more hydrophilic formulations such as Type IIIB and type IV have been reported to undergo a dramatic loss of solubilization capacity for the incorporated drug after in vitro dispersion, resulting in much higher degrees of supersaturation and eventually major loss of drug due to precipitation (Williams et al., 2012a, Mohsin et al., 2009b, Pouton, 2006b).

Our dispersion studies conducted on Type IIIA formulations further revealed that higher supersaturation ratios (>3) are required to promote rapid drug precipitation. With fenofibrate the maximum supersaturation ratios were all below 3, and although these represented a

significant loss of solubilization capacity, low proportions of drug were lost as precipitate during the initial 4 hours of the dispersion studies (see Chapter 3: , Figure 3.7C).

One method for generation of higher supersaturation ratios is by increasing the drug load of formulations. Our dispersion study was carried out at a relatively high fenofibrate load, equivalent to 80% saturation and yet we observed quite low degrees of supersaturation. We attributed this to be due to the presence of high proportion of lipid in Type IIIA formulations (50% in this study) which would be expected to result in lipid-rich colloidal species. Thus despite containing water-miscible excipients such as Tween®80, the Type IIIA formulation still retains quite good solvent capacity on dispersion.

From the dispersion studies conducted on Type IIIA formulations containing 50% lipids and described above, we have demonstrated that as long as the formulation is not hydrophilic enough, the precipitation of a highly lipophilic drug, fenofibrate will be limited from these formulations. In fact there would be very little precipitation in the stomach before the formulation is emptied into the intestine, and plenty of time for absorption to take place in the intestine. Hence, we concluded that for fenofibrate-containing formulations, the design of both, Type II and Type IIIA lipid-based formulations should be focused on the solubilization capacity of the excipients in the digested state rather than dispersion.

A dramatic loss of solvent capacity, and consequent precipitation of drug, was observed during the subsequent process of digestion. The digestion products are more hydrophilic and they fail to supplement the bile salt/phospholipid solubilizing phase (Kossena et al., 2003a). Significant loss in solubilization capacity (Chapter 3: , Figure 3.9 and

Table 3.2) was observed during the digestion studies for 7 out of the 8 formulations studied.

Substitution of long-chain lipids by medium-chain lipids, in amounts as little as 15% total lipid, in both Type II and Type IIIA formulations reduced the solubilization capacity significantly, resulting in loss of drug as precipitate.

Utilization of long-chain instead of medium-chain triglycerides in lipid-based formulations prevents the achievement of sudden and higher degrees of supersaturation and consequently reduces the risk of precipitation (Kossena et al., 2003a). The present digestion studies in Chapter 3: have indicated that this approach alone will not work for all drugs. For fenofibrate, various other strategies needs to be explored to prevent drug precipitation from formulations, such as lowering the drug load (Williams et al., 2012a), employing polymer-based precipitation inhibitors (Anby et al., 2012c), and/or by the proper selection of surfactants (Cuine et al., 2008a).

Typical formulation surfactants such as Tween® surfactants have previously shown to be substrates for pancreatic enzymes (Bakala N'Goma et al., 2012b, Christiansen et al., 2010b, Cuine et al., 2008a, Fernandez et al., 2008a) and post-digestion they yield polar and less amphiphilic molecules which can lower the solubilization capacity of the lipid-based formulation (Cuine et al., 2008a). Our results (Chapter 3: , Figure 3.3 and Figure 3.4) obtained from digestion study of these surfactant solutions are in agreement to the above studies. Due to the fact that various surfactants included in lipid-based formulations could affect the overall solubilization capacity of the formulation after digestion, a thorough investigation of the surfactant effects on lipid-based formulations was thought to be essential, and hence this was the subject of our studies in Chapter 4: .

The critical factor identified from studies carried out in this thesis, (Devraj et al., 2013a, Devraj et al., 2013c) and also previous studies (Williams et al., 2012a) has been the maximum supersaturation ratio, SR^M.

The role of supersaturation, existence of a threshold SR^M, and the potential formulation factors such as the surfactants impacting the supersaturation and eventually the solubilization were all studied in Chapter 4: wherein Type IIIA long-chain formulations were chosen as the candidate formulations. This is due to the fact that these systems exhibit loss (owing to the presence of hydrophilic components) of solubilization capacity following dispersion and also post-digestion as demonstrated in Chapter 3: of this thesis and also elsewhere in the literature (Porter et al., 2007a, Thomas et al., 2012b, Anby et al., 2012c, Pouton, 2006b, Williams et al., 2012a, Devraj et al., 2013a). The rationale for the study presented as Chapter 4 was to investigate whether the fenofibrate formulations supported the existence of a threshold SR^M which indicated the likelihood of precipitation after in vitro digestion.

Seven different Type IIIA SEDDS were selected for this study. The formulations contained danazol at a load equivalent to 80% of its solubility in each anhydrous formulations, differing only with respect to the non-ionic surfactant used. The formulations showed no drug precipitation during in-vitro digestion and the SR^M values calculated were all noted to be ≤ 3.1. Previous work (Anby et al., 2012c, Williams et al., 2012a) from our laboratory also revealed a similar result with different formulations wherein the danazol precipitation from alternative formulations was observed only when the SR^M exceeded a value of ~2.5. The studies conducted here supported the previous work.

When the above study was carried out with fenofibrate, the results showed a completely contrasting effect. All seven formulations produced extensive drug precipitation after *in*

vitro digestion. Fenofibrate-containing formulations experienced a much higher supersaturation (SR^M >5.0) during their digestion and evidently this resulted in drug loss as precipitate.

A lowering of fenofibrate dose in 4 representative formulations out of the 7, halving the saturation from 80% to 40%, resulted in significant reduction of drug precipitation in two cases. These were the formulations prepared with Cremophor EL or Solutol HS15. The SR^M values for these 2 SEDDS were 2.6 and 2.4 respectively. The other 2 SEDDS, which still performed poorly, were those containing Cremophor RH40 and Tween 80. From this study at 40% saturation, we attributed this difference in performance of formulations to the higher SR^M values, 2.8 and 4.3, observed for formulations containing Cremophor RH40 and Tween80 respectively. A subsequent study carried out at a common SR^M =2.6 ,to normalize the supersaturation pressure caused by digestion, demonstrated that 5 out of the 7 SEDDS could maintain the entire mass of fenofibrate in solubilized state during digestion (Chapter 4: , Figure 4.4).

By adjusting the dose in order to generate SR^M values around 2.6 to monitor performance during digestion, we noted a threshold SR^M of 2.5-2.6 for fenofibrate, above which there was an extensive precipitation evident (Chapter 4: , Figure 4.5).

Studies carried out in this thesis with SEDDS prepared from *long-chain lipids* and with two different drugs, danazol and fenofibrate (Devraj et al., 2013c) were found to be in good correlation to previous studies conducted on *medium-chain lipids* (Anby et al., 2012c, Williams et al., 2012a) and *lipid-free co-solvent/surfactant* formulations (Williams et al., 2012a) containing model drug, danazol. Taken as a whole, the data generated from these studies suggest that the critical SR^M value for fenofibrate is close to 2.5 for a wide variety of

formulations, and a similar critical SR^M is evident for danazol. Chapter 4: , clearly shows that whether hydrophobic or lipophilic, the fate of drugs incorporated in lipid-based formulations, rely predominantly on the level of supersaturation seen during digestion. It can be concluded that SR^M is a simple and valuable tool in predicting the likelihood of precipitation towards their performance.

Dietary lipids upon arrival into the small intestinal region, post-dispersion in the stomach fluids, are acted upon by a variety of lipolytic enzymes, resulting in complete and efficient breakdown to yield digestion products (fatty acids, mono-glycerides/di-glycerides), prior to their eventual absorption across GIT with the aid of endogenous detergents such as bile salts and phospholipids (Hofmann, 1963). Each of these enzymes has a specific role. Pancreatic lipase in the presence of co-lipase (Erlanson-Albertsson, 1992b) acts mainly on triglycerides (Armand et al., 1996, Carriere et al., 1993), the other enzymes, carboxyl ester hydrolase, phospholipase A2, and the pancreatic-lipase related protein2 act on phospholipids, cholesteryl esters, and formulation surfactants (Bakala N'Goma et al., 2012b, Borgstrom, 1993b). Lipid-based formulations which are based on the dietary lipids (Charman et al., 1993, Charman et al., 1997, Crounse, 1961, Humberstone et al., 1996, Sunesen et al., 2005, Welling, 1996) and primarily noted as carriers for PWSDs due to their various advantages (Porter et al., 2008a, Porter et al., 2007a, Larsen et al., 2008, Hauss, 2007b) are expected to undergo a *completion of digestion* in vivo in a similar fashion to the dietary lipids as a first step towards an efficient absorption of incorporated drugs. In vitro evaluation technique such as the in vitro digestion test (section 1.4) employing in vitro digestion models (Devraj et al., 2013b, Williams et al., 2012e, Sek et al., 2002a, Anby et al., 2012c, Larsen et al., 2011a, Taillardat et al., 2007a, Tan et al., 2011b, Thomas et al., 2012b) have been used extensively in the last few years in studying the digestion process of lipid-based formulations. The majority of these studies

have failed to achieve a completion of lipolysis and it has been suggested that this may be a prime reason behind the lack of *IVIVC.*

Studies conducted in this thesis (Chapter 2:) addressed this issue of non-completion of lipolysis and developed the *in vitro* lipolysis model with a particular attention towards achievement of sink condition by driving the lipolysis to completion.

Calcium was selected as a tool (keeping other variables constant) to promote completion of lipolysis The significance of this approach was evaluated by determining the solubility of model drugs fenofibrate and danazol in the aqueous phase samples of lipid digests obtained during lipolysis.

The rate and extent of lipolysis of LCT increased as the bile concentration was increased. This created a sink allowing sequestration of degradation products and achieved good digestion. However, bile alone could not force digestion of LCT to completion (only ~75%) (Chapter Chapter 2: , Figure 2.2).

Increase in calcium concentration progressively increased the extent of LCT digestion and achieved completion, as reported by others (Christensen et al., 2004b, Hwang et al., 2009, Zangenberg et al., 2001a) creating an efficient sink condition *in vitro* (Chapter 2: , Figure 2.3 and Table 2.1).

The standard bile concentration (set at 5mM in this study) was chosen to reflect fasted state intestinal contents. At 5mM bile reduced the positive effects of calcium on rate and extent of digestion, as demonstrated previously by others ((Hofmann and Mysels, 1992, Lindahl et al., 1997, Persson et al., 2006)

The influence of calcium on lipolysis was thought to be due to FA removal, forming calcium soaps. In addition calcium increased pancreatic lipase activity directly, as LCT digestion rate

increased by >6-fold on the addition of 5mM calcium compared to the rate in the absence of calcium.

Solubilization capacity of the digests for drug was markedly decreased at high calcium concentration (40mM was used in this study to push digestion to completion). Danazol solubility was less structure dependent and simply a function of total BS+lipid digestion products. Fenofibrate solubility was found to be species dependent and at low bile conditions (5mM) evidenced high vesicular content increased the extent of fenofibrate solubilization. Upon raising calcium to 40mM, solubilization fell dramatically. At high bile conditions, total fenofibrate solubility was unchanged and reliance on vesicles for solubilization capacity was reduced, presumably compensated by the higher concentration of mixed micelles.

Overall, the data obtained from our results (Chapter 2:) indicates that high levels of calcium can bring about a completion of lipolysis, resulting in the complete digestion of the lipidic vehicle to its constituent MG(s) and FA(s). However, this digestion does not provide any enhanced solubilization of the drug in the AP digests. Instead the solvent capacity of the digest is reduced, presumably because fatty acids and bile components are all sequestered as soaps in a crystalline phase.

5.2 Future directions

Work presented in Chapter 2: was carried out using simple lipid solutions (e.g., LCT) which are representative of LFCS type I formulations. These formulations, which are very similar to dietary lipids, require to be completely digested in the small intestine to liberate their digestion products and allow presentation of poorly water-soluble lipophilic drugs in colloidal solution. Prediction of performance of formulations and determination of their fate *in vivo* has been extensively reported and reviewed in this thesis (section 1.4.1). A major limitation of the existing in vitro digestion models has been attributed to their inefficiency in simulation of the achievement of completion of digestion, a situation that is expected to occur *in vivo.* The work conducted in this thesis suggests that this deficiency cannot be overcome simply by increasing bile and/or calcium concentration. The major consequence of this deficiency is the inability of the digestion model to predict the fate of drugs from Type 1 formulations, resulting in poor *IVIVC*.

Although the results (in Chapter 2:) obtained are revealing , it would be useful to carry out the similar study under *dynamic* conditions that persist *in vivo* wherein the model drug is dissolved at any of the desired proportion (e.g., 80% or 50% saturated solubility) in the formulation to gain an insight into the behavior of solubility of drugs in the aqueous/solubilized phase under these conditions. The lipolysis process is highly dynamic, produces different lipolytic products, which upon interaction with endogenous BS/PL form different colloidal species which would be expected to have varied solubilization capacities towards different drugs. Therefore, a characterization of the colloidal species that form during the lipolysis process and the accompanied enhancement in the tested drug solubilization provided under circumstances where *lipolysis goes to completion* should be investigated.

Characterization may involve, determining the nature of colloidal species formed *viz.* simple micelles, mixed micelles, and vesicles etc. The size and zeta-potential of the AP digests would also yield useful information.

Further, from the studies carried out in Chapter 2: , we have shown that the solubilization capacity of the digests towards model drugs (fenofibrate and danazol) was markedly decreased at high calcium (40mM used in this study to push digestion) and that this was evident at both the conditions of bile when danazol was present as the model drug, and only at lower conditions of bile when fenofibrate was the model drug. However, this dramatic reduction in fenofibrate solubility as seen in the other cases above was observed to be 'protected' resulting in marked attenuation of drug solubility in the presence of elevated bile salt concentrations (Chapter 2: , Figure 2.5 and Figure 2.6).

We concluded that possibly, the danazol solubility could be less structure dependent and simply a function of total BS+lipid digestion products and fenofibrate solubility to be highly species dependent (Kleberg et al., 2010) with greater fenofibrate solubilization achieved in the presence of lipid rich vesicles when compared to micelles (presumable, as a result of the higher lipid solubility of fenofibrate when compared to danazol). At high bile conditions (20mM), the total fenofibrate solubility was observed to be unchanged and reliance on vesicles for solubilization capacity reduced (and that of micelles increased) as opposed to the conditions of low bile (5mM, representative of fasted state) and also with another drug, danazol at both the conditions of bile. Therefore the impact of reduction in lipid content on addition of 40mM calcium was also reduced and drop in solubilization attenuated. This result was consistent with previous studies (Kleberg et al., 2010) therefore requires a detailed colloidal species characterization and eventually the precise effects of high calcium.

Further, a direction worthwhile studying and which to date has hardly been reported in the literature is to explore the *oil/aqueous phase volume ratio* to attain completion of lipolysis and achievement of sink conditions during *in vitro* lipolysis. Ideally such a model needs to be evaluated for its efficiency utilizing model drugs and the optimum conditions selected for the appropriate prediction of *in vivo* performance.

Modeling the fate of drugs on digestion of Type 1 formulations may require a genuine sink which can simulate absorption to be provided, to overcome the drawbacks of the closed system. This will be a challenging objective but potentially could be achieved by using an organic phase as a sink.

In addition to the usage of "high lipid" content (50%) in the studies carried out in Chapter 3: towards the attenuation of drug precipitation of formulations, one needs to employ other strategies such as, *lowering of drug load* (Williams et al., 2012a), *usage of polymer-based precipitation inhibitors* (Anby et al., 2012c) towards achieving the stabilization of supersaturation post dispersion and digestion in GI fluids, a condition reflective of potential drug crystallization.

Further we have shown that the precipitation of the drug, fenofibrate from long-chain Type IIIA formulations could be explained by a single parameter, SR^M as, the possibility that there exists a threshold supersaturation ratio, above which supersaturation could not be maintained during the digestion tests correlated well with previous studies that evaluated danazol (unlike fenofibrate used in our studies) supersaturation using SEDDS formulations containing medium-chain lipids (Anby et al., 2012c, Williams et al., 2012a) and lipid-free cosolvent/surfactant formulations (Williams et al., 2012a). The latter studies identified a threshold SR^M value for danazol of approximately 2.5, which was very close to the threshold SR^M value for fenofibrate identified from our study carried out in Chapter 4: .

A thorough understanding of the occurrence of threshold SR^M is required as, despite various surfactants (seven different used in our study) and various formulation lipids employed elsewhere (Anby et al., 2012c, Williams et al., 2012a), the SR^M values did not experience any drastic differences implicating the effects could probably be due to a common component. Danazol with a log p value of 4.5 was common in all these studies. Hence, future experiments may further investigate this phenomenon with different drugs with varying lipophilicity including any other hydrophobic drug having a log p value similar to that of danazol.

It would be valuable in the future to explore the predictive potential of the in vitro studies conducted here by carrying out bioavailability studies using the same formulations. This would ideally be conducted in humans but, given the cost of clinical studies, bioavailability studies in dogs would be a more practical prospect. At this stage there are few dog studies which aim to test the extent of IVIVC using LBDDS. There is a need for studies using a range of drugs with different physicochemical properties, to investigate whether our in vitro digestion studies are able to predict precipitation in the intestine in vivo. In vivo, factors such as the membrane permeability of the drug may also play a significant role in determining bioavailability. Several cross over studies will be required using several drugs before it will be possible to relate the quality of the IVIVC to the physicochemical properties of the drug. Collecting such a database will take some time and commitment but it will advance substantially the science underlying the formulations of LBDDS.

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Appendix A: Published Version of Paper 1

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In vitro digestion testing of lipid-based delivery systems: Calcium ions combine with fatty acids liberated from triglyceride rich lipid solutions to form soaps and reduce the solubilization capacity of colloidal digestion products

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Lipolysis

ABSTRACT

In vitro digestion testing is of practical importance to predict the fate of drugs administered in lipid-based delivery systems. Calcium ions are often added to digestion media to increase the extent of digestion of long-chain triglycerides (LCTs), but the effects they have on phase behaviour of the products of digestion, and consequent drug solubilization, are not well understood. This study investigates the effect of calcium and bile salt concentrations on the rate and extent of *in vitro* digestion of soybean oil, as well as the solubilizing capacity of the digestion products for two poorly water-soluble drugs, fenofibrate and danazol. In the presence of higher concentrations of calcium ions, the solubilization capacities of the digests were reduced for both drugs. This effect is attributed to the formation of insoluble calcium soaps, visible as precipitates during the digestions. This reduces the availability of liberated fatty acids to form mixed micelles and vesicles, thereby reducing drug solubilization. The use of high calcium concentrations does indeed force *in vitro* digestion of LCTs but may overestimate the extent of drug precipitation that occurs within the intestinal lumen.

1. Introduction

While a number of formulation and non-formulation strategies have been developed to address the increasing number of new chemical entities that demonstrate oral absorption limited by low aqueous solubility (Williams et al., 2013), approaches that utilize drug pre-dissolved in a lipid vehicle remain popular. The basis for using lipids stems from a number of studies that noted improved absorption and bioavailability of a poorly water-soluble drug (PWSD) following co-administration with a lipid-rich meal (Charman et al., 1993, 1997; Crounse, 1961; Humberstone et al., 1996; Sunesen et al., 2005; Welling, 1996). In a broad sense, lipid-based drug delivery systems (LBDDS) therefore aim to harness the often positive effect of dietary lipids on oral drug absorption (Hauss, 2007; Larsen et al., 2008; Porter et al., 2007, 2008) by circumventing drug dissolution, which in the case of PWSD is often slow and potentially limits the rate and extent of drug absorption, and by increasing the solubilization reservoir in the GI fluids (Cuine et al., 2007; Kleberg et al., 2010; Porter et al., 2004, 2007).

There are many different types of LBDDS and these may be discriminated on the basis of their composition and properties following interaction with endogenous GI fluids. In an effort to facilitate this discrimination, Pouton proposed the Lipid Formulation Classification System (LFCS) (Pouton, 2000, 2006), which classifies LBDDS into five discrete groups (Type I, II, IIIA, IIIB and IV) according to the proportion of oil, lipophilic surfactant, hydrophilic surfactant and cosolvent in the formulation. Type I and II formulations represent the most lipophilic formulations and form coarse and highly turbid emulsions on dispersion in aqueous fluids. Digestion of the dispersed oil phase promotes *in vivo* performance since this forces drug to partition from the poorly dispersed oil droplet phase into more solubilized colloidal phases. In contrast, it is generally well recognized that digestion is not essential to the performance of Type IIIA/B and Type IV systems as they form finer (*i.e.*, nanosized) emulsions and/or micellar phase systems in the GI tract (Pouton, 2006). However, as LBDDS enter the small intestine, digestion of formulation components is inevitable and may significantly impact the subsequent formulation behaviour. Assessment of all types of

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LBDDS during *in vitro* digestion tests is therefore necessary for a complete understanding of formulation performance.

Pancreatic lipase is the main enzyme involved in the digestion of triglycerides (Armand et al., 1996; Carriere et al., 1993). Pancreatic lipase is an interfacial enzyme that, in the presence of a co-lipase 'anchor', will effectively bind to the hydrophobic surface of an oil droplet (Erlanson-Albertsson, 1992). Other enzymes present in the small intestine and thought to play a role in lipid digestion include carboxyl ester hydrolaze (CEH), phospholipase A_2 and pancreatic lipase-related protein 2 (PLRP 2). However, these enzymes are believed to contribute more to the hydrolysis of phospholipids, cholesterol esters and formulation surfactants rather than triglyceride (Bakala N'Goma et al., 2012; Borgstrom, 1993). Pancreatic lipase hydrolyses molecules of triglyceride (TG) to yield two molecules of fatty acids (FA) and a molecule of 2-monoglyceride (2-MG). Further hydrolysis of 2-MG is limited by the regiospecificity of pancreatic lipase towards positions 1 and 3 of the TG molecule (Carriere et al., 1997), although CEH and PLRP 2 may also hydrolyze 2-MG to yield a third FA and glycerol (Bakala N'Goma et al., 2012). However, the activity of CEH in porcine pancreatin extract – the source of pancreatic lipase commonly used in *in vitro* digestion models – has not been determined, and PLRP 2 has not yet been identified in this extract (de Caro et al., 2008). Alternatively, 2-MG may undergo slow isomerization to the relatively less lipase-stable 1-MG to allow further hydrolysis to yield a third FA and glycerol, although this process is usually limited *in vitro* (Mattson and Volpenhein, 1964). The extent of MG hydrolysis to FA and glycerol *in vitro* is therefore unknown. Natural detergents in the small intestine *i.e.*, bile salts and phospholipids (secreted along with pancreatic lipase from the gall bladder in response to lipids entering the small intestine) form mixed micelles that shuttle the products of lipid digestion from the site of production (*i.e.*, the oil:water interface) to the site of absorption (*i.e.*, the enterocyte membrane) (Hofmann, 1963).

In vitro digestion tests are designed to simulate the above described digestion processes so that the fate of drug may be monitored as the physical and chemical nature of LBDDS change. It is also customary to relate this outcome to the extent of formulation digestion. Detailed descriptions of *in vitro* digestion models have been already provided elsewhere (Sek et al., 2002; Williams et al., 2012a). Following digestion, drug that is solubilized within a colloidal aqueous phase digest (containing micelles and vesicles) is expected to be in rapid equilibrium with drug in free solution providing a reservoir of drug that is highly available for absorption (Boyd et al., 2003; Porter et al., 2007). In contrast, drug dissolved into the LBDDS that subsequently precipitates during digestion (collecting within the pellet phase) is thought to represent drug that is poorly available for absorption since re-dissolution is required, and the dissolution of solid PWSD is usually poor unless the drug forms a more rapidly dissolving amorphous precipitate (Sassene et al., 2010). One of the experimental complexities of *in vitro* digestion models is the difficulty in achieving complete digestion of the lipid substrate. This issue is particularly the case for highly lipophilic, long-chain lipid formulations (Williams et al., 2012b), and stems from the fact that most *in vitro* digestion models are 'closed' systems. As such, the absence of a sink for removal of digestion products (such as that provided *in vivo* by FA and MG absorption) causes a progressive increase in the concentration of lipid digestion products which, depending on the solubilization capacity of the digestion medium for the digestion products, ultimately results in accumulation of the lipid digestion products at the oil droplet surface, suppressing further digestion of the remaining oil phase (Brockerhoff, 1968; Brockerhoff and Jensen, 1974; Fave et al., 2004; Scow et al., 1979). Since this phenomenon is attenuated *in vivo* by absorption, strategies to 'force' lipid digestion to completion *in vitro* have been sought, and the most common approaches include an increase in the bile salt reservoir (Li et al., 2011) and the addition of a FA complexant, *e.g.* calcium ions either within the digestion media or *via* continuous addition (Alvarez and Stella, 1989; MacGregor et al., 1997; Patton and Carey, 1979; Patton et al., 1984; Zangenberg et al., 2001a,b). The effect of increasing calcium concentration on the phase behaviour of digested lipids and the resultant impact on drug solubilization, however, is not well understood. The first aim of the current study was therefore to determine whether increasing calcium concentration could be used to push the *in vitro* digestion of a model long-chain triglyceride (LCT) to completion. The second aim was to probe the solubilization capacity of the digests formed by this approach. In the current studies, soybean oil was chosen as a model long-chain lipid substrate, and fenofibrate and danazol were employed as model PWSD (Fig. 1) in order to represent two types of drug candidates for formulation in LBDDS, that is, a highly lipophilic drug (fenofibrate) and a hydrophobic but less lipophilic (lipid soluble) drug (danazol).

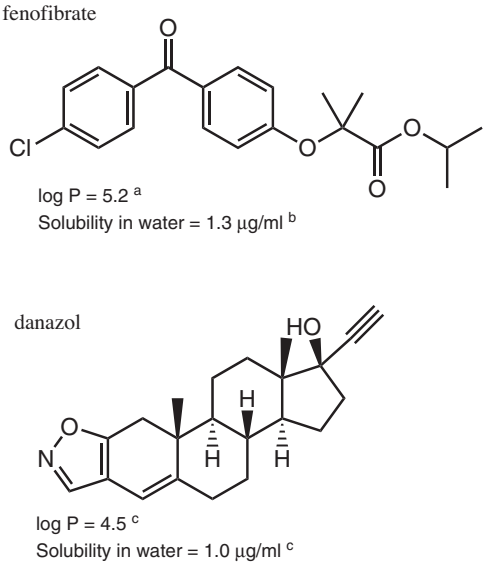

Fig. 1. The chemical structure of model drugs fenofibrate and danazol investigated in this study. [a]Munoz et al. (1994); [b]Sheu et al. (1994) and [c]Bakatselou et al. (1991).

2. Materials and methods

2.1. Materials

Danazol was obtained from Sterling Pharmaceuticals Pty Ltd. (Sydney, Australia). Fenofibrate, soybean oil (the long-chain triglyceride), sodium taurodeoxycholate > 95% (NaTDC), pancreatin extract (from porcine pancreas, P7545, 8× USP specifications activity), calcium chloride dihydrate, Tris-maleate, and the lipid digestion inhibitor 4-bromophenylboronic acid were purchased from Sigma–Aldrich Co. (St. Louis, MO). Lecithin (*ca.* 99.2% egg-phosphatidylcholine (PC), Lipoid E PCS) was purchased from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). 1.0 M sodium hydroxide (Univol) was purchased from Ajax Finechem Pty Ltd. (Sydney, Australia) and was diluted with water (Milli-Q water purification system, Millipore, Bedford, MA) to achieve a 0.2 M titration solution. Methanol and chloroform used in this work were HPLC grade from Merck (Melbourne, Australia).

2.2. In vitro digestion experiments

In vitro digestion experiments were performed as previously described (Sek et al., 2002). Briefly, 250 ± 5 mg of soybean oil was directly weighed into a water jacketed-glass reaction vessel

(T = 37 °C) and dispersed in 9 ml aqueous digestion buffer (pH 7.5) consisting of 50 mM Tris-maleate, 150 mM sodium chloride and 0, 5, 20 and 40 mM calcium chloride dihydrate, supplemented with 0, 5, 20 and 100 mM bile salt and 0, 1.25, 5 and 25 mM PC. The range in bile salt conditions were chosen to represent the widest range of potential solubilization conditions of the intestine, ranging from 'extreme fasted' (*i.e.*, 0 mM bile salt and 0 mM PC) to 'extreme fed' conditions (*i.e.*, 100 mM bile salt and 25 mM PC). In each test, a bile salt:PC ratio of 4:1 was employed, which is the ratio secreted in human bile (Carey and Small, 1978; Scherste, 1973). Dispersion of the LCT was provided *via* a magnetic stirrer, and over 10 min vigorous mixing, the LCT formed a coarse emulsion. During this dispersion phase, the pH was adjusted to pH 7.5 ± 0.05 with 1.0 M NaOH/HCl. Digestion was initiated by the addition of 1 ml pancreatin, containing 10,000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per ml of digestion medium. The pancreatin extract was prepared fresh on each day of testing from 1 g of pancreatin powder thoroughly mixed in 5 ml digestion buffer (in the absence of bile salt or PC). To minimize loss of enzyme activity, the prepared pancreatin extract was stored on ice prior to use. The mixture was then centrifuged (1600 × *g*, 5 °C, Eppendorf 5408R, Eppendorf AG) for 15 min, then the supernatant was recovered and the pH adjusted to pH 7.5 ± 0.1 with 5.0 M NaOH.

Digestion of the LCT was continuously monitored using a pH-stat automatic titration unit (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.2 M NaOH.

2.3. Extent of digestion in vitro

Digestion profiles were corrected for the background fatty acid (FA) released upon digestion of the bile salt-PC mixed micelles; this concentration was determined in separate experiments undertaken in the absence of LCT. This correction was particularly important in the present study as the concentration of PC used in the digestion medium varied from 0 mM to 25 mM (and therefore, would have a significant effect on the amount of total FA titrated).

For each mole of liberated FA, 1 mole of NaOH is titrated to neutralize the acid and maintain the pH at 7.5. Assuming that each mole of TG is digested into two moles of FA and 1 mole of 2-MG (it is widely recognized that the conversion [by isomerization] of 2-MG into 1-MG and eventually into a third FA and glycerol is restricted only to *in vivo* conditions (Mattson and Volpenhein, 1964) while the extent of 2-MG hydrolysis *in vitro* using porcine pancreatin extract is not known) the fraction FA released was calculated using Eq. (1):

$$\text{Extent of digestion } (\%) = \frac{(V_{NaOH} \times M_{NaOH}) \times MW_{TG}}{m_{TG} \times 2} \times 100 \qquad (1)$$

where M_{NaOH} is the molarity of the NaOH titrant, V_{NaOH} is the total volume of titrant consumed during titration, m_{TG} is the mass of the TG added to the digestion vessel and MW_{TG} is its molar mass.

2.4. Drug solubility in the aqueous colloidal phase

The equilibrium solubility of danazol and fenofibrate in the aqueous colloidal phase (AP_{DIGEST}) generated by the digestion of LCT was evaluated after 5, 30 or 60 min digestion (as described above). At these time points, 2× 4 ml samples were collected from the reaction vessel and immediately treated with digestion inhibitor (9 μl/ml of digestion medium of 0.5 M 4-bromophenylboronic acid in methanol) to arrest ongoing digestion. Samples were ultracentrifuged (400,000 × *g*, 37 °C, Optima XL-100K Ultracentrifuge, SW-60 swinging-bucket rotor, Beckman, Palo Alto, CA) in soft-walled polyallomer tubes (Beckman) for 30 min to separate the digestion phases. The sample tubes were pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the AP_{DIGEST} to ensure it was not contaminated with the undigested oil phase (Sek et al., 2002; Williams et al., 2012a). Crystalline drug was then added in excess to 4 ml AP_{DIGEST} before incubation at 37 °C in an orbital mixer. After 48 h, the mixtures were centrifuged (1600 × *g*) for 15 min and 100 μl of the supernatant was diluted with methanol before analysis for drug content by high performance liquid chromatography (HPLC), as described in Section 2.5.

The equilibrium solubility of both drugs were also determined in LCT-free digestion medium, containing (i) 5 mM bile salt/5 mM calcium, (ii) 5 mM bile salt/40 mM calcium, (iii) 100 mM bile salt/5 mM calcium and (iv) 100 mM bile salt/40 mM calcium (with PC at a concentration to provide a 4:1 bile salt:PC ratio). These drug/digestion media mixtures were equilibrated and analyzed for drug content as described above. Solubility was assessed with respect to equilibration time (*i.e.*, samples were removed at 1, 6, 24, 48, and 120 h).

Equilibrium solubility defined as being obtained when solubility values at two consecutive time points varied by less than 5%.

2.5. HPLC assays

HPLC analysis for danazol and fenofibrate were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, MA). The columns used for both drugs were reverse-phase C18 columns; a Waters Symmetry® column for danazol (150 mm × 3.9 mm, 5 μm, Waters Alliance Instruments) and a Phenomenex® Luna column for fenofibrate (C18 (2), 150 mm × 4.6 mm, 3 μm, Phenomenox, Torrence, CA). The mobile phase employed for both drugs consisted of methanol and water in a 75:25 (v/v) ratio pumped through the HPLC columns at 1 ml/min. The sample injection volume was 50 μl, with UV detection for danazol and fenofibrate at 288 nm and 286 nm, respectively. Standard solutions of danazol (0.1–5.0 μg/ml) and fenofibrate (0.1–5.0 μg/ml) were prepared by dilution of a concentrated 1 mg/ml stock solution of each drug with methanol. Linearity across the working concentrations of either drug was confirmed during each HPLC assay using standard measures of regression. All samples and standards were maintained at 10 °C and the column temperature maintained at 25 °C. The HPLC assay for danazol and fenofibrate was validated by replicate (n = 5) analyses of quality control samples at low (0.5 μg/ml) and high (5.0 μg/ml) concentrations and was found to be accurate to within ±10% of target and precise to within 10% CV.

3. Results

3.1. Effect of bile salt concentration

The effect of increasing bile salt concentration on the quantity of titratable FA produced during *in vitro* digestion of LCT is shown in Fig. 2. Titratable FA corresponds to FA released from the LCT in response to digestion by enzymes in the pancreatin extract. Total concentrations of FA titrated over 30 min and the calculated levels of LCT digestion (according to Eq. (1)) are summarized in Table 1. In the absence of calcium and bile salt (closed circles; Fig. 2A), there was a lag period of approximately 10 min before FA was detected. By the end of the digestion period (30 min), 5.6 ± 0.4 mM FA was titrated, which was similar to the FA concentration titrated when using 5 mM bile salt (7.1 ± 0.9 mM, open circles; Fig. 2A), however, in this case, no lag period was observed. In tests performed using 20 mM and 100 mM bile salt (closed and open triangles, respectively; Fig. 2A), the total concentration of FA titrated increased significantly to 19.2 ± 0.3 and 39.8 ± 2.2 mM, respectively. This increase in titrated FA at the higher bile salt concentrations was most pronounced during the first 10 min of the

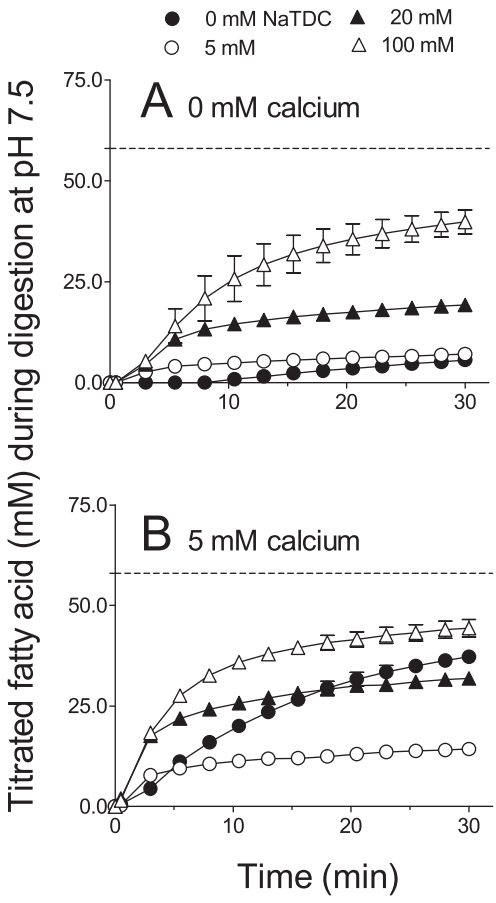

Fig. 2. Apparent titration of fatty acids (FA) during *in vitro* digestion of soybean oil (a long-chain triglyceride, LCT) in conditions of increasing bile salt (NaTDC) concentration. Digestion tests were performed at (A) 0 mM calcium and (B) 5 mM calcium. Digestion was initiated at $t = 0$ min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion of the LCT through the addition of 0.2 M NaOH. Values are expressed as means ($n = 3$) ± SD with background correction for the level of FA released in background digestion tests (no LCT). NaTDC; sodium taurodeoxycholate (the bile salt used in this study). The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. (1)).

Table 1
Summary of the effect of calcium concentration and bile salt concentration on the digestion of long-chain triglyceride (LCT).

Digestion condition	Fatty acid titrated over 30 min LCT digestion (mM)[a]	Extent of LCT digestion (%)[b]
0 mM calcium, with:		
0 mM bile salt	5.6 ± 1.4	9.8
5 mM	7.1 ± 0.9	12.4
20 mM	19.2 ± 0.3	33.4
100 mM	39.8 ± 2.2	69.3
5 mM calcium, with:		
0 mM bile salt	37.3 ± 1.2	64.9
5 mM	14.3 ± 5.2	24.0
20 mM	31.9 ± 1.0	55.5
100 mM	44.4 ± 2.2	77.3
20 mM calcium, with:		
0 mM bile salt	52.3 ± 1.9	91.1
5 mM	26.7 ± 0.1	46.4
20 mM	41.9 ± 0.6	73.0
40 mM calcium, with:		
0 mM bile salt	60.9 ± 1.7	106.1
5 mM	36.1 ± 1.7	60.0
20 mM	51.9 ± 1.6	87.5

[a] Total fatty acid concentration titrated at pH 7.5.
[b] Calculated using Eq. (1).

digestion tests. The FA titration rate during the remainder of the experiments was, however, generally independent of bile salt concentration and was also much slower.

The dashed horizontal line in Fig. 2 denotes the theoretical concentration of FA when the extent of LCT digestion is 100% (according to Eq. (1)). In the absence of calcium, the extent of digestion was ~10% at 0 and 5 mM bile salt, and 33.4% and 69.3% at 20 and 100 mM bile salt, respectively (Table 1).

The results of LCT digestion tests performed in digestion media containing 0–100 mM bile salt, but supplemented with 5 mM calcium, are shown in Fig. 2B. Direct comparison of titration profiles in Fig. 2A to those in Fig. 2B therefore reveals the effect of calcium (5 mM) on LCT digestion. Using the cumulative concentration of titrated FA over 30 min and Eq. (1), the LCT digestion extent at 0 mM bile salt increased from 10% in the absence of calcium, to 64% with 5 mM calcium. At 5 mM bile salt, digestion also increased with the addition of calcium, but to a lesser extent (*i.e.*, from 12% to 24%). With 20 mM and 100 mM bile salt, the addition of 5 mM calcium had no effect on extent digestion.

The addition of 5 mM calcium to the bile salt-free digestion medium led to faster rate of FA titration (closed circles; Fig. 2A and B). The total FA concentration in this condition of no bile salt and 5 mM calcium was 37.3 ± 1.2 mM. This exceeded the FA concentrations obtained when 5 mM and 20 mM bile salt was added, suggesting that the addition of bile salt (≤20 mM) depressed the effects of calcium on LCT digestion.

The highest extent of LCT digestion achieved was 77%, and was at the highest tested bile salt concentration (100 mM) with 5 mM calcium. This was slightly higher than the highest extent of digestion obtained in the absence of calcium (69%; Table 1). Therefore, despite increasing bile salt to concentrations far-exceeding those in the human small intestine (in both fasted and fed states (Kleberg et al., 2010)), it was not possible to achieve the complete digestion of the LCT *in vitro* when using 0 or 5 mM calcium.

3.2. Effect of calcium concentration

The effect of increasing calcium concentration on titration of FA during *in vitro* digestion of the LCT is shown in Fig. 3. Tests were performed in digestion media containing either no (Fig. 3A), 5 (Fig. 3B) or 20 mM bile salt (Fig. 3C), with the latter two conditions mimicking typical fasted and fed bile salt concentrations, respectively. Data obtained at 0 mM and 5 mM calcium is reproduced from Fig. 2 and is included to further illustrate the effect of calcium and bile salt concentration on digestion.

In the absence of bile salt (Fig. 3A), increasing calcium led to a progressive increase in titrated FA. Between 0 and 5 mM calcium, the amount of titrated FA at 30 min increased 6-fold. Further increases in calcium concentration to 20 and 40 mM led to higher titrated FA concentrations, although the effect of calcium on FA titration progressively diminished. Since the total extent of digestion at 30 min at 40 mM calcium reached 106%, it was clearly evident that the use of high calcium concentrations could lead to complete LCT digestion. Therefore, a gradual depletion of available substrate most likely explains the smaller effect of increasing calcium concentration >20 mM on total LCT digestion.

Increasing calcium also led to a progressive increase in titrated FA in the presence of 5 (Fig. 3B) and 20 mM bile salt (Fig. 3C). However, consistent with the previous section, the positive effects of calcium on digestion are attenuated by the bile salt. Therefore, in the presence of bile salt, it was not possible to achieve complete digestion of LCT through increasing calcium concentration. To further illustrate this depressive effect of bile salt on digestion, the total concentration of titrated FA (*i.e.* values at 30 min from Figs. 2 and 3) is plotted as a function of either bile salt or calcium concentration, as shown in Fig. 4A and B. Both plots illustrate the

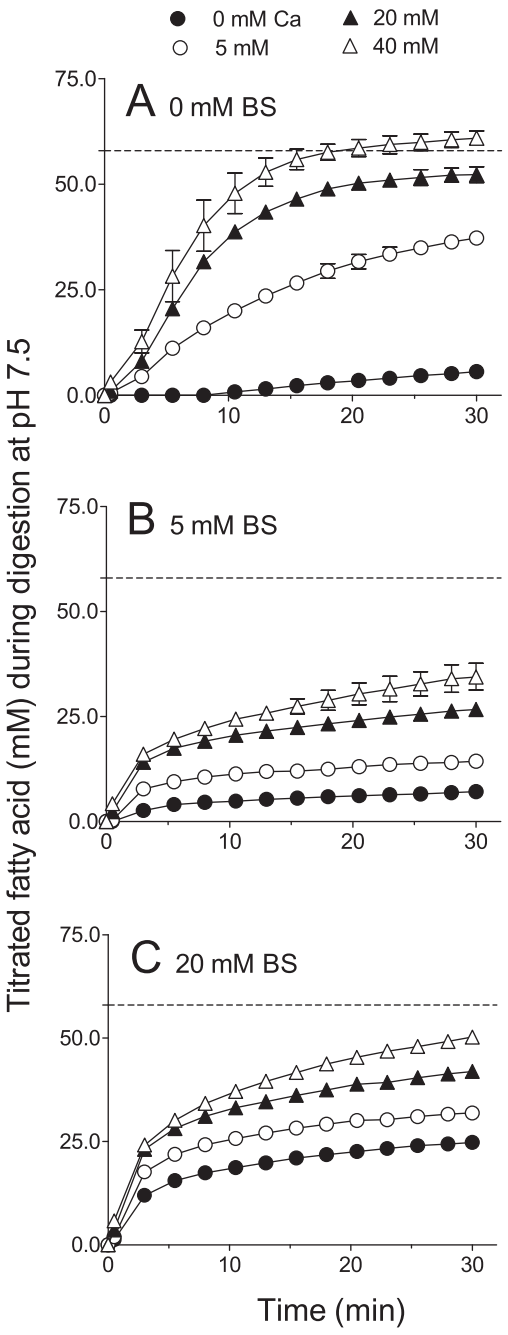

Fig. 3. Apparent titration of fatty acids during *in vitro* digestion of soybean oil (a long-chain triglyceride, LCT) in conditions of increasing calcium concentration. Digestion tests were performed at (A) 0 mM, (B) 5 mM and (C) 20 mM bile salt (NaTDC). Digestion was initiated at $t = 0$ min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion of the LCT through the addition of 0.2 M NaOH. Values are expressed as means ($n = 3$) ± SD with background correction for the level of fatty acid released in background digestion tests (no LCT). NaTDC; sodium taurodeoxycholate (the bile salt used in this study). The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. (1)).

depressant effect of bile salt on digestion and it is most marked at 5 mM bile salt.

3.3. Effect of calcium on the drug solubility in AP_{DIGEST}

To assess the effect of calcium on the solubilization capacity of the colloidal species produced on lipid digestion, the colloidal aqueous phase was isolated from digests following 5, 30 and 60 min of digestion (AP_{DIGEST_5min}, AP_{DIGEST_30min} and AP_{DIGEST_60min}) and incubated with excess danazol and fenofibrate and solubility assessed over a period of 48 h. Previous work by Kossena et al. has shown that the LCT lipid digestion products swell bile salt-phospholipid mixed micelles and vesicles which, in turn, increases the solubilization capacity of this bile salt-phospholipid phase (Kossena et al., 2005).

3.3.1. Low bile salt concentration (5 mM)

The upper panel in Fig. 5 plots digestion as a function of time, and reiterates the increase in LCT digestion in the presence of the higher calcium concentrations (*i.e.*, 40 mM) and the increase in digestion as a function of time. Data points highlighted at 5, 30 and 60 min digestion refer to the time points at which samples of the digest were removed for the solubility studies. The lower panels in Fig. 5 show the equilibrium solubilities of fenofibrate (bottom left) and danazol (bottom right) in these colloidal digests ($AP_{DIGESTS}$) isolated from either the low or high calcium conditions. To show the effect of calcium on solubility in the absence of LCT and its digestion products, the measured drug solubilities in the equivalent digestion medium (*i.e.*, no LCT) are also shown.

At 5 mM calcium, fenofibrate solubility in the AP_{DIGEST} progressively increased from 47.0 ± 1.4 μg/ml in AP_{DIGEST_5min} to 186.0 ± 20.3 μg/ml in AP_{DIGEST_60min} (lower left panel; Fig. 5). As fenofibrate solubility in the equivalent digestion medium (*i.e.*, in the absence of digestion products) was only 23 ± 2 μg/ml, the increase in solubility in the $AP_{DIGESTS}$ indicates that the digestion products were contributing significantly to drug solubilization. Accordingly, a degree of ongoing digestion between 5 and 60 min and the resulting higher concentration of digestion products in this phase provides a likely explanation for the increase in solubilization capacity of the AP_{DIGEST} with respect to digestion time.

In contrast, and despite evidence of ongoing digestion between 5 min and 60 min, fenofibrate solubility in $AP_{DIGESTS}$ formed in the presence of 40 mM calcium did not increase. In addition, the solubility values at this higher calcium concentration were generally lower than solubility values measured at the lower calcium concentration (except at 5 min post digestion), and were only marginally above the solubility in digestion medium (*i.e.*, no LCT). The data obtained at the two calcium concentrations therefore suggests that the solubilization capacity of the AP_{DIGEST} was not directly related to the extent of LCT digestion, since increasing calcium increased digestion, but did not always increase drug solubilization. Furthermore, as fenofibrate solubility in the digestion media was not affected by calcium concentration (23 ± 2 μg/ml and 23 ± 1 μg/ml at 5 and 40 mM calcium, respectively), the differences in fenofibrate solubility in the $AP_{DIGESTS}$ (seen in Fig. 5) could not be directly attributed to the higher concentration of calcium ions.

Consistent with the fenofibrate results, danazol solubility values in the AP_{DIGEST} were lower at 40 mM calcium compared with 5 mM calcium (lower right panel; Fig. 5). These danazol solubility values in the AP_{DIGEST} at 5 and 40 mM calcium were, however, below the solubility in simple digestion media (no LCT), suggesting that the presence of lipid digestion products had a limited impact on danazol solubility in model intestinal fluids at both high and low calcium concentrations. This was in contrast to fenofibrate, which showed a higher solubility in the AP_{DIGEST}. The difference in danazol solubility between the simple digestion medium and the AP_{DIGEST} was most pronounced at the higher calcium concentration. However, as seen in Fig. 5 (lower right panel), danazol solubility in digestion media alone (*i.e.*, no digestion products) was 16 ± 3 μg/ml at 5 mM calcium and 14 ± 1 μg/ml at 40 mM calcium. The solubility of danazol was, therefore, not directly affected by calcium, which is consistent with the fenofibrate results.

Of additional note was the appearance of the pellet phase during the solubility studies. The pellet phase of digestion forms a

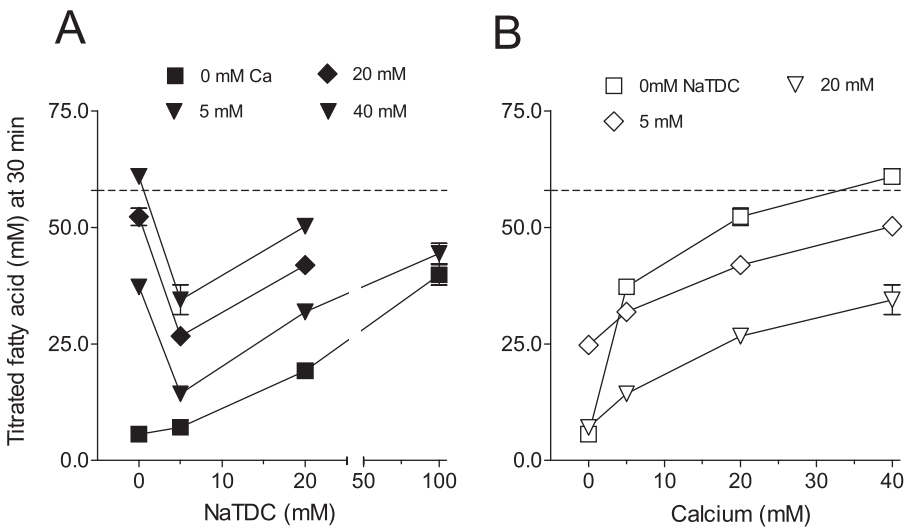

Fig. 4. Graphical summary of the effect of bile salt (NaTDC) and calcium concentration on the total concentration of fatty acid (FA) titrated over 30 min during the *in vitro* digestion of soybean oil (a long-chain triglyceride, LCT). (A) Effect of increasing NaTDC concentration on total titrated FA at four calcium concentrations. (B) Effect of increasing calcium concentration on total titrated FA at three NaTDC concentrations. Values are expressed as means ($n = 3$) ± SD. NaTDC; sodium taurodeoxycholate (the bile salt used in this study). The dashed horizontal line denotes the theoretical maximum FA concentration on complete digestion of the LCT substrate (according to Eq. (1)).

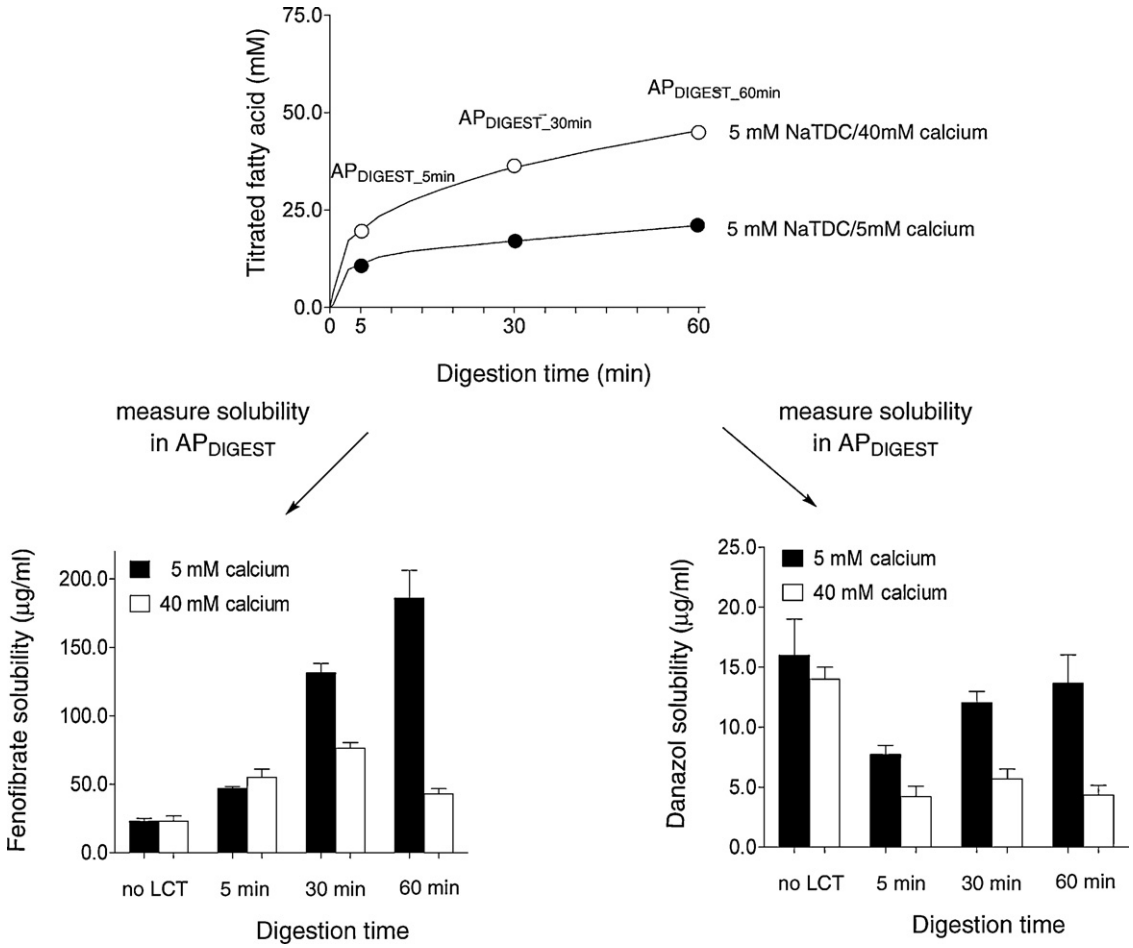

Fig. 5. Titrated fatty acid profiles describing the digestion of LCT in digestion media containing 5 mM bile salt/5 mM calcium (closed symbols) or 5 mM bile salt/40 mM calcium (open symbols) and the determination of fenofibrate (left) and danazol (right) equilibrium solubility in the AP_{DIGEST} isolated following LCT digestion for 5 min (AP_{DIGEST_5min}), 30 min (AP_{DIGEST_30min}), and 60 min (AP_{DIGEST_60min}). Solubility studies in the $AP_{DIGESTS}$ were performed at 37 °C over a 48 h equilibration period. The equilibrium solubility of fenofibrate and danazol in the digestion medium (no LCT) containing 5 mM bile salt/5 mM calcium and 5 mM bile salt/40 mM calcium are also shown.

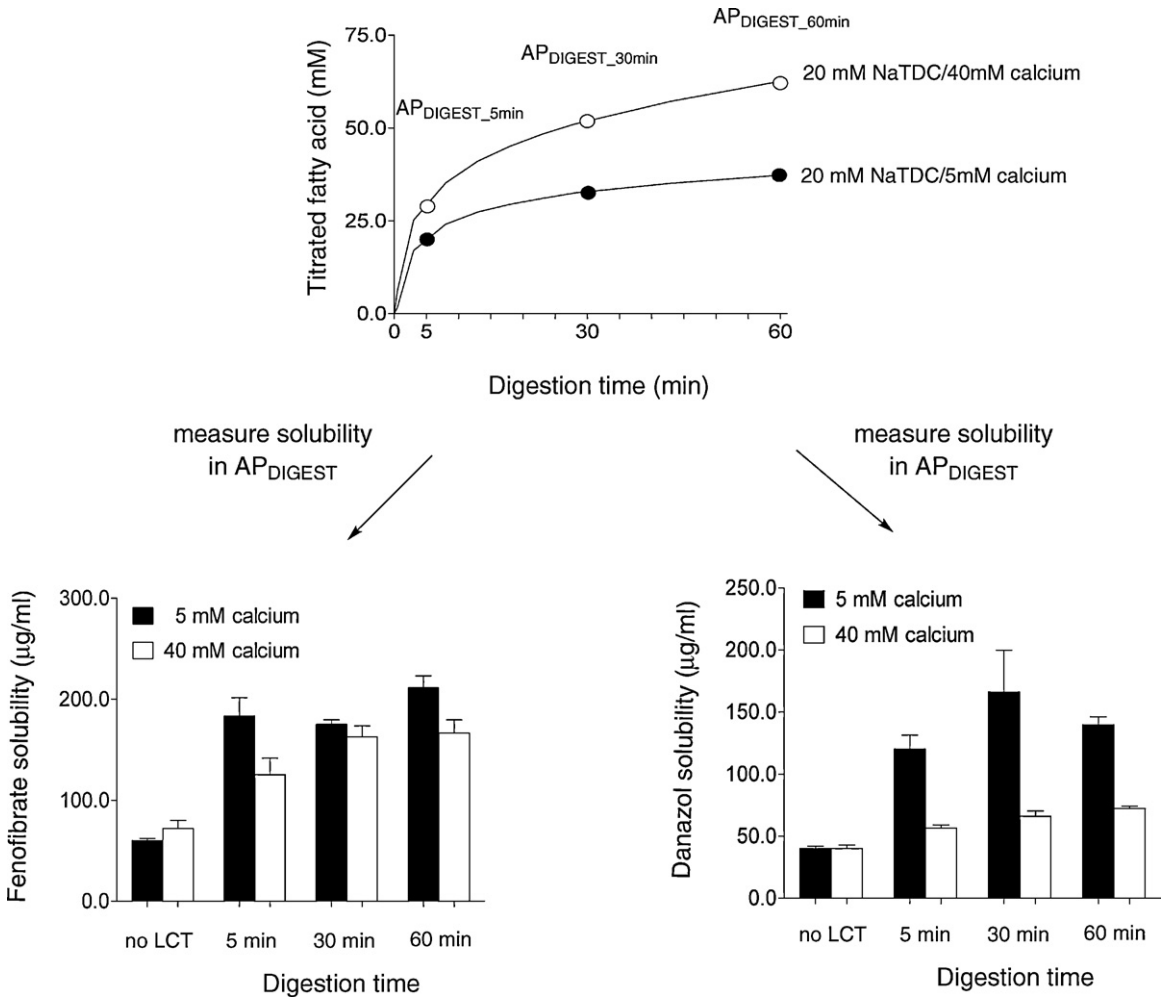

Fig. 6. Titrated fatty acid profiles describing the digestion of LCT in digestion media containing 20 mM bile salt/5 mM calcium (closed symbols) or 20 mM bile salt/40 mM calcium (open symbols) and the determination of fenofibrate (left) and danazol (right) equilibrium solubility in the AP_{DIGEST} isolated following LCT digestion for 5 min (AP_{DIGEST_5min}), 30 min (AP_{DIGEST_30min}), and 60 min (AP_{DIGEST_60min}). Solubility studies in the $AP_{DIGESTS}$ were performed at 37 °C over a 48 h equilibration period. The equilibrium solubility of fenofibrate and danazol in the digestion medium (no LCT) containing 20 mM bile salt/5 mM calcium and 20 mM bile salt/40 mM calcium are also shown.

sediment during ultracentrifugation and consists primarily of insoluble calcium soaps of fatty acid (Patton and Carey, 1979; Sek et al., 2002). In the present study, the pellet phase at the higher calcium concentration was notably larger compared with the pellet observed at the lower calcium concentration, suggesting the quantity of fatty acid calcium soaps in the presence of 40 mM calcium had increased.

3.3.2. *High bile salt concentration (20 mM)*

The results for aqueous phase solubilization at 20 mM bile salt are shown in Fig. 6 (c.f. Fig. 5) with the upper panel indicating that the extent of digestion increased with increasing bile salt and that data obtained at 40 mM calcium was higher compared to 5 mM calcium. Thus, both bile salt and calcium increased the extent of digestion. The drug solubility data, however, were more complex. For danazol in either the presence or absence of calcium, increasing bile salt concentrations led to much higher (~10-fold) drug solubilities than that obtained at 5 mM bile salt, presumably reflecting a combination of increasing bile salt concentrations, and increasing digestion leading to solubilization of larger quantities of lipid digestion products (and therefore an increase in drug solubilization capacity). At higher calcium (40 mM), although digestion was increased, drug solubilization decreased moderately (~2-fold), consistent with calcium removing fatty acids from the bile salt micelles *via* complexation thereby decreasing solubilization capacity.

In contrast, in the case of fenofibrate, a somewhat different trend was evident at differing bile salt concentrations. Firstly, increasing bile salt concentrations had a much less significant effect on increasing drug solubility in bile salt/lipid digestion product mixed micelles and fenofibrate solubility at 20 mM bile salt, at least at 5 mM calcium, was similar to that observed at 5 mM bile salt. Secondly, unlike the data described above at 5 mM bile salt, where the effect of raised calcium was dramatic and fenofibrate solubility dropped ~4-fold at 40 *versus* 5 mM calcium, the reduction in drug solubility at higher calcium concentrations was markedly attenuated in the presence of elevated bile salt concentrations (compare lower left panels in Figs. 5 and 6).

The reasons for the 'protective' effects of high bile salt concentrations in reducing the drop in solubilization capacity in the presence of higher calcium for fenofibrate, but not danazol, is not clear at this time. However, Kleberg et al. (2010), have shown previously that the solubility properties of fenofibrate are highly dependent on the nature of the colloidal structures formed by combinations of bile salt and lipid digestion products, with much greater fenofibrate solubilization achieved in the presence of lipid-rich vesicles when compared to micelles (presumably, as result of the higher lipid solubility of fenofibrate when compared to danazol). In contrast, danazol solubility was less structure dependent

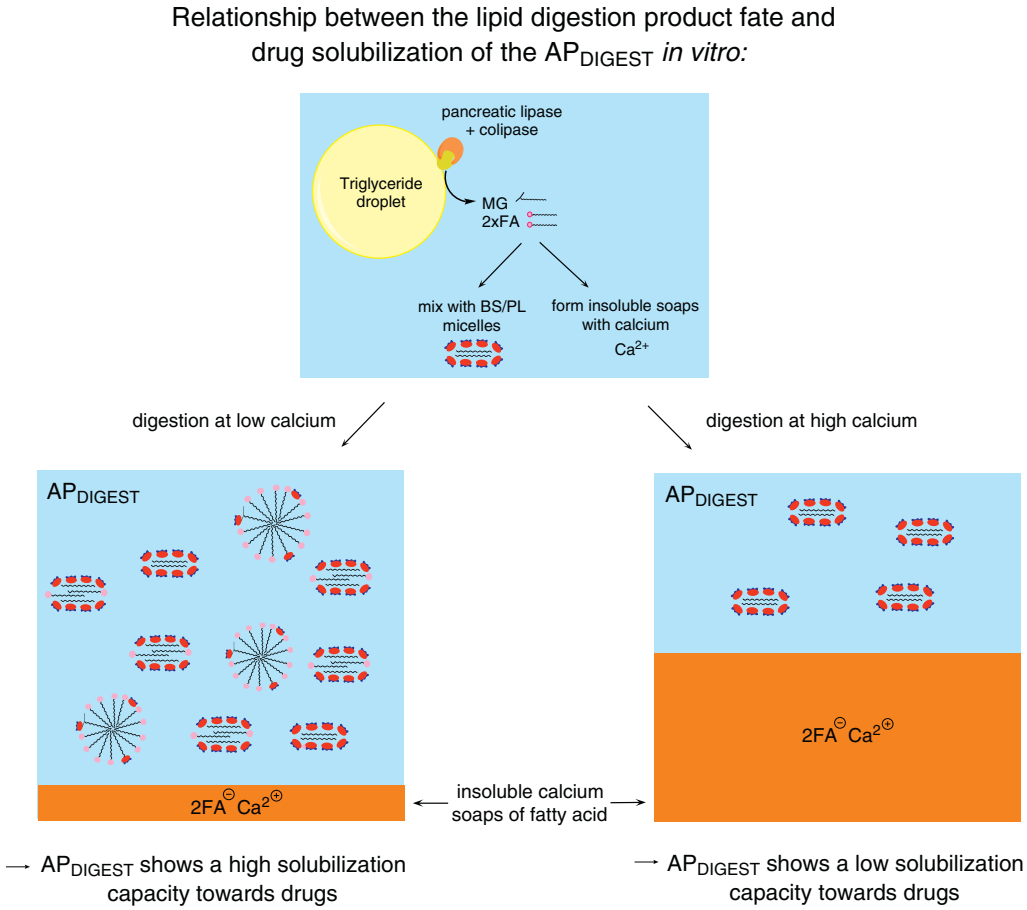

Fig. 7. Schematic to illustrate the general process of triglyceride digestion under the action of pancreatic lipase–colipase complex (upper panel) and the subsequent fate of digestion products (fatty acid (FA) and monoglyceride (MG)). Digestion of triglyceride in the intestine is mediated primarily *via* pancreatic lipase and its cofactor, colipase. Digestion of one LCT molecule liberates two molecules of FA and one molecule of monoglyceride. Molecules of FA may become incorporated into bile salt-phospholipid mixed micelles in the bulk aqueous phase. These colloids swell in size following incorporation of digestion products and contribute significantly to the solubilization capacity of the AP_{DIGEST}. However, liberated FA shows a tendency of forming insoluble soaps in the presence of calcium ions. FA are therefore not contributing to the solubilizing properties of the AP_{DIGEST}.

and simply a function of the total concentration of bile salt plus lipid digestion products. It is possible, therefore, that at low bile salt concentrations, fenofibrate solubility is highly dependent on the presence of lipid digestion products (in order to stimulate vesicle formation), and that increased calcium leads to a reduction in vesicular content, and therefore, a marked drop in solubilization capacity. In contrast, at higher bile salt concentration, total fenofibrate solubility is unchanged, but the reliance on vesicles for solubilization capacity is reduced (and that of micelles increased), and therefore, the impact of the reduction in lipid content on addition of 40 mM calcium is also reduced and the drop in drug solubilization attenuated. For danazol, where solubilization is more linearly dependent on total solubilizer concentration, the effects of calcium are less marked at low bile salt concentrations, but remain evident at high bile salt concentrations.

4. Discussion

In vitro digestion models are increasingly being used to assess the performance of lipid-based formulations (LBF) under conditions that mimic lipid digestion in the small intestine (Ahmed et al., 2012; Anby et al., 2012; Larsen et al., 2011; Taillardat et al., 2007; Tan et al., 2011; Thomas et al., 2012; Williams et al., 2012a,b). A potential disadvantage of the *in vitro* digestion model is that it is a 'closed' system: as the LBF undergoes enzymatic-mediated hydrolysis, the concentration of lipid digestion products (*i.e.*, FA and MG) progressively increases, and, in the absence of an appropriate sink (*in vivo*, these digestion products are effectively removed *via* lipid absorption), a build-up of digested lipids at the surface of an oil droplet surface can block further binding of the pancreatic lipase–colipase complex thereby limiting further digestion. This inhibition can complicate the interpretation of *in vitro* testing of highly oil-rich LBF since a large proportion of drug following 30–60 min digestion of a LBF may remain sequestered within a partially digested oil phase (Williams et al., 2012b).

By binding to FA and forming insoluble complexes, calcium ions can effectively strip digestion products from the oil droplet surface (Alvarez and Stella, 1989; Armand et al., 1992). While this property explains why high concentrations of calcium are often added to digestion tests to drive digestion of long-chain triglycerides (LCTs) and other lipid substrates (Christiansen et al., 2010; Hwang et al., 2009; Zangenberg et al., 2001a,b), the effects calcium ions have on phase behaviour and the solubilization properties of colloidal lipids are not well understood.

Soybean oil, a LCT widely used in lipid drug delivery, was used to represent an incompletely digested lipid substrate (at least, *in vitro*), and was digested under conditions of varying bile salt and calcium concentration (see Figs. 2 and 3). In the absence of calcium, increasing bile salt concentration led to a progressive increase in the rate and extent of LCT digestion (Table 1), consistent with previous reports of increased digestion of LC lipids with increasing bile salt (Bernback et al., 1990; Carey et al., 1983; MacGregor

et al., 1997; Williams et al., 2012b). Very high bile salt concentrations (*i.e.*, >20 mM), in excess of likely concentrations in the small intestine in both fasted and fed conditions (McConnell et al., 2008; Persson et al., 2006), were utilized in order to further examine the potential for increases in solubilization capacity of the aqueous phase, to increase the extent of digestion. However, digestion of the LCT by the end of the experiment was (at best) only 75% complete. An initial period of rapid digestion was observed in the presence of bile salt, but this was not sustained beyond 10 min. Digestion profiles were therefore 'biphasic' (Fig. 2). Similar profiles have been observed previously during *in vitro* digestion testing of LCT under the same (Han et al., 2009; Sek et al., 2002) and similar (Li et al., 2011) experimental conditions, and this slow and incomplete digestion of LCT is known to manifest through a mechanism that involves the accumulation of digestion products (*i.e.*, FA and MG) at the oil droplet surface limiting access of the lipase–colipase complex to the substrate (Brockerhoff and Jensen, 1974; Patton and Carey, 1979; Scow et al., 1979).

The fact that increasing bile salt concentration allowed greater LCT digestion suggests that the presence of a larger solubilization reservoir for digestion products allowed more effective interaction between the pancreatic lipase–colipase complex and the lipid substrate. A recent study by Williams et al. noted, however, that correlations between concentrations of titrated (*i.e.*, ionized) FA and total lipid digestion were not always apparent when bile salt concentration was increased (Williams et al., 2012b). Underpinning this effect was the partial ionization of long-chain FA at pH 6.5 (the experimental pH used by Williams et al.), and consequently, FA ionization that was highly sensitive to the changing solubilization conditions attained on increasing bile salt concentration. In the present study, however, a higher experimental pH (pH 7.5) was used to ensure more efficient titration of FA (Patton and Carey, 1981; Sek et al., 2001) and a lower sensitivity of FA to changes in the degree of ionization in the presence of bile salt was therefore expected. Furthermore, the effects of bile salt on FA ionization described by Williams et al. were most pronounced at low bile salt concentrations (*i.e.*, <5 mM), and became less evident at higher bile salt concentrations (*i.e.*, up 10 mM). The effects of bile salt on concentrations of titrated FA in the present study are therefore more likely to result from the continued digestion of the residual oil phase.

An alternative approach to the use of increasing bile salt concentrations, namely the use of higher calcium concentrations, was also taken to increase the extent of digestion of LCT. Calcium has been used previously to push the digestion of long-chain lipid substrates towards completion (Christensen et al., 2004; Hwang et al., 2009; Zangenberg et al., 2001a) and increasing calcium concentrations in the present study similarly led to increased digestion of the LCT (Fig. 3), such that digestion was complete at the highest calcium concentration (40 mM). Underpinning the pronounced effect of calcium on digestion is its capacity to allow pancreatic lipase to continually access the surface of the oil droplet by binding to and subsequently removing FA from this surface in the form of calcium soaps (Alvarez and Stella, 1989; Patton and Carey, 1979). Alternative roles of calcium include the capacity to reduce the surface charge of oil droplets (which may reduce the electrostatic repulsion between the enzyme and its substrate therefore promoting binding (Armand et al., 1992; Wickham et al., 1998)) and being required as a co-factor to activate pancreatic lipase (Alvarez and Stella, 1989; Kimura et al., 1982). However, studies by MacGregor et al. (1997) have shown that calcium effects on lipid digestion are most prominent for LC lipid substrates, and consequently, a general consensus that the role of calcium in promoting *in vitro* digestion primarily involves the efficient removal of digestion products from the oil droplet surface has emerged. In the present study, LCT digestion increased by >6-fold on the addition of 5 mM calcium (Table 1). Since the increase in digestion induced by addition of low calcium concentrations is too great to be explained solely through FA complexation, the calcium effect (at this low concentration) probably reflected both complexation and the capacity to increase pancreatic lipase activity directly (Alvarez and Stella, 1989; Kimura et al., 1982; Whayne and Felts, 1971).

While the use of increasing concentrations of calcium led to increases in lipid digestion and ultimately to complete LCT digestion (~106% at 40 mM calcium (Table 1)), the increases in digestion mediated by calcium were typically decreased by the presence of increasing concentration of bile salt. Thus, addition of 5 mM bile salt reduced the extent of lipid digestion by approximately 50% when compared to digestion in the absence of bile salt, at 5 mM, 20 mM and 40 mM calcium. This is in contrast to the situation in the absence of calcium when bile salt micelles increased digestion. Thus, the beneficial effects of calcium on LCT digestion were attenuated by bile salt, and most effectively attenuated at concentrations reflecting the conditions of the fasted small intestine (Hofmann and Mysels, 1992; Lindahl et al., 1997; Persson et al., 2006). The apparent interplay between bile salt/phospholipid micelle concentrations and calcium concentration on LCT digestion rate and extent was not a focus of the present study, and as such potential explanations for such behaviour are only briefly discussed. Nonetheless, these include the possibility that the activity of pancreatic lipase is decreased in the presence of micellar quantities of bile salts and phospholipids (Patton and Carey, 1981), that bile salt-phospholipid mixed-micelles provide an alternative binding site for pancreatic lipase (Patton and Carey, 1979, 1981), that phospholipids coat the oil droplet surface and therefore prevent lipase binding (Patton and Carey, 1981), and that solubilization and subsequent displacement of bound lipase at the surface of the oil droplet by bile salt-phospholipid micelles reduces enzyme activity (Bauer et al., 2005; Borgstroem et al., 1963). While increasing bile salt and PC concentrations therefore typically led to a net increase in LCT digestion in the absence of calcium, it is plausible that some inhibitory effects were simultaneously occurring and that these inhibitory effects became more apparent at the higher digestion levels seen under higher calcium concentrations. Furthermore, the inhibitory effects of bile salt and phospholipid mediated at the surface of the oil droplet are thought to be mitigated by colipase, since the pancreatic lipase–colipase complex shows an enhanced binding affinity towards hydrophobic surfaces (Bezzine et al., 1999; Patton et al., 1978). Since colipase is present within the crude porcine pancreatin extract used in the present study (Patton et al., 1978), these inhibitory affects may have been reduced.

Aside from effects on lipase, conjugated bile acids have been shown to interact with and form salts with calcium ions (Gu et al., 1992; Hofmann and Mysels, 1992; Jones et al., 1986). There are also reports of decreasing bile salt concentration with the continuous titration of calcium during *in vitro* lipid digestion studies (Christensen et al., 2004; Zangenberg et al., 2001b). The combined use of calcium with bile salts, and the subsequent formation of poorly soluble calcium salts of bile acid, may therefore decrease the available concentration of both species. However, the aforementioned studies that associated decreasing bile salt concentration with increasing calcium utilized a crude bile salt extract (porcine derived) containing a mixture of glycine- and taurine-conjugated bile salts. In contrast, taurodeoxycholate was the only bile salt used in the present study. Since calcium salts of taurine-conjugated bile acids are much more soluble than those glycine-conjugated bile acids (Gu et al., 1992; Hofmann and Mysels, 1992; Jones et al., 1986), bile salt precipitation in the present study was less likely.

In some industries, it is common to use calcium ions to achieve higher concentrations of MG during enzymatic hydrolysis of oils, where build-up of FA in the absence of calcium is usually limiting to the MG yield (Hwang et al., 2009). From a drug delivery perspective,

the fate of both FA and MG are important since they are known to contribute to the solubilization capacity of the intestinal milieu (Charman et al., 1993; Kossena et al., 2005; Porter et al., 2007). Indeed, several recent studies have discussed the link between solubilization properties of a digested lipid formulation and the risk of drug precipitation (Anby et al., 2012; Porter et al., 2011; Williams et al., 2012b). We have therefore used the measured drug solubility in the digests to probe the solubilization capacity of the phase. Digests were formed under varying bile salt and calcium conditions to determine whether drug solubilization was related to the extent of lipid digestion.

The solubility results (Figs. 5 and 6) compare the solubilization capacity of $AP_{DIGESTS}$, formed under conditions of varying bile salt and calcium concentration, towards the model PWSDs and show that the solubilization capacity of the digests is markedly decreased at high calcium concentrations. The lower solubilization capacity of the digests formed under higher calcium concentration was in spite of a higher extent of digestion. While conventional understanding of lipid digestion reasons that increasing the digestion of LC lipids will lead to an enrichment of the colloidal aqueous phase with lipid digestion products (and an increased solubilization capacity of this phase), the results of the present study show that such enrichment does not occur at high calcium. Instead of enhancing the solubilization capacity of the bile salt-phospholipid phase, the presence of calcium ions leads to the greater part of liberated FA from LCT forming insoluble complexes, and as described schematically in Fig. 7, an AP_{DIGEST} depleted of digestion products.

Since the solubility of danazol and fenofibrate in simple bile salt–phosphatidylcholine media was not influenced by the concentration of calcium ions, the potential for calcium induced bile acid precipitation (Gu et al., 1992; Hofmann and Mysels, 1992) to affect drug solubility in the present study is small. Furthermore, the reduction in solubilization capacity of the AP_{DIGEST} at the high calcium concentration was evident both at 5 mM and 20 mM bile salt concentrations, and as described earlier, bile salt precipitation is likely to be more prevalent with glycine-conjugated bile salts (rather than taurine-conjugate used here).

The implications of these findings are that the use of high calcium concentrations during *in vitro* testing of LBDDS does promote digestion, however the sink phase produced by calcium may be biased towards digestion products only. As a result, the use of high calcium concentrations creates an imbalance between the solubilization capacity of a digesting lipid formulation (by depleting the colloidal phases of digestion products) and the solubilized drug concentration (which is not affected directly by calcium), with the eventual outcome being an increase in the likelihood of drug precipitation. The use of high calcium concentrations may therefore overestimate the extent drug precipitation that may occur *in vivo* in the intestine, and in turn, potentially underestimate *in vivo* performance of the LBDDS. Conditions that provide sink conditions for digestion products and drug (*i.e.*, those mimicking the absorptive membrane in the GI tract) may avoid the imbalance provided by a preferential sink such as calcium.

5. Conclusions

In vitro digestion models are increasingly being utilized to predict the *in vivo* performance of lipid-based drug delivery systems (LBDDS). While some models have in the past utilized calcium ions to push the lipid digestion process to completion, the consequence of such an approach on drug solubilization has not been investigated. Consistent with the literature, we have showed that increasing calcium is a much more effective approach to promoting LCT digestion when compared with increasing bile salt concentration. However, the much lower solubilities of danazol and fenofibrate in the digests formed under conditions of high calcium (40 mM in this study) indicate that this use of calcium (for the purposes of pushing digestion to completion) is to the detriment of drug solubilization. This is due to calcium selectively removing fatty acids from solution (by precipitating fatty acid calcium soaps), leading to an effective decrease in the concentration of fatty acids that are involved in micellar and vesicular drug solubilization. Therefore, the implication for *in vitro* testing of LBBDS is that the use of high calcium concentrations to promote digestion may exaggerate the decrease in solubilization capacity of the lipid formulation as it digests and, in turn, overestimate the extent of drug precipitation that occurs in the intestine.

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Appendix B: Published Version of Paper 2

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In vitro assessment of drug-free and fenofibrate-containing lipid formulations using dispersion and digestion testing gives detailed insights into the likely fate of formulations in the intestine

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ABSTRACT

The solubilizing properties of lipid-based formulations (LBFs) can change dramatically following dispersion and digestion of the formulation components. This study investigated the performance of self-emulsifying LBFs consisting of four different long-chain (LC)/medium-chain (MC) lipid blends formulated with the lipophilic drug fenofibrate and either a water-insoluble surfactant polysorbate 85 (Tween® 85) or its more hydrophilic relative, polysorbate 80 (Tween® 80). These components allowed closely related Type II and IIIA LBFs of fenofibrate to be evaluated during *in vitro* dispersion and *in vitro* digestion testing. Initial assessment of the solvent capacity of drug-free LBFs during dispersion and digestion revealed that the solubility of fenofibrate was more dependent on the surfactant type rather than lipid composition. Type II LBFs in the dispersed state were generally better at solubilizing fenofibrate than equivalent Type IIIA LBFs, regardless of lipid composition. However, even when high drug loadings were used, supersaturation/drug precipitation after dispersion of Type II or Type IIIA LBFs was only moderate. In contrast, digestion of both Type II and IIIA LBFs led to much higher levels of drug supersaturation, and this resulted in drug precipitation. After digestion the ability of each LBF to maintain drug in a solubilized state was highly dependent on lipid composition as well as the choice of surfactant. Notably, MC lipids exhibited very good solubilizing properties in the dispersed state, but resulted in a higher degree of supersaturation on digestion, leading to higher susceptibility to drug precipitation. This study showed that replacing LC lipids with MC lipids in Type II and IIIA LBF, in the proportions used here has little effect on fenofibrate solubilization during dispersion, but is likely to promote supersaturation on digestion. Without careful consideration of drug loading and choice of surfactant in Type II/IIIA MC lipid formulations, there is a high risk of precipitation of drug in the intestine.

1. Introduction

It is well recognized that low solubility and/or a slow dissolution rate can be severely limiting to drug absorption from the gastro-intestinal (GI) tract. Unfortunately, an increasing number of molecules in drug discovery and development exhibit these hydrophobic characteristics (Williams et al., 2013). However, lipid-based formulations (LBFs), particularly those administered as liquid-filled capsules, present a formulation strategy through which the oral bioavailability of poorly water-soluble drugs (PWSD) may be improved (Charman et al., 1992; Hauss, 2007; Porter et al., 2004b, 2007). The primary mechanism through which LBFs enhance drug absorption is that the drug is pre-dissolved in the lipid formulation, eliminating the dissolution required by solid phase delivery systems. LBFs can therefore provide a high concentration of solubilized drug in the GI lumen, which enhances the rate and extent of drug absorption (Charman et al., 1992; Porter et al., 2008, 2007). Depending on the LBF composition and its location within the GI tract, the co-administered drug is likely to be solubilized within many different colloidal species, ranging from emulsified oil droplets in the stomach to smaller and less lipophilic micellar phases, which result from the digestion of lipids from the formulation in the small intestine. The digestive processing of a LBF is a consequence of the natural way in which the GI tract responds to the presence of lipids. Therefore an understanding of the fate of the drug during the dispersion/emulsification process and subsequent digestion of its lipid-based vehicle is necessary

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in order to fully predict the *in vivo* performance of a LBF (Porter et al., 2007).

During dispersion of a LBF, hydrophilic formulation components are likely to partition into the aqueous GI fluids. This will decrease the solubilizing capacity of the formulation for hydrophobic drug, in turn creating the risk of drug precipitation in instances where drug concentrations exceed solubilizing capacity (i.e., supersaturation). Precipitation is undesirable as this regeneration of the solid-state re-introduces the need for the drug to dissolve prior to absorption. The second critical process is digestion of the LBF, which occurs primarily in the small intestine (Bakala N'Goma et al., 2012). Both lipid components and surfactants containing ester groups from the LBF and are highly susceptible to enzymatic hydrolysis. Since hydrolysis will significantly alter their physicochemical properties, *in vitro* digestion models are widely used to better understand the *in vivo* performance of LBFs (Anby et al., 2012; Cuine et al., 2008; Dahan and Hoffman, 2008; Sassene et al., 2010; Tan et al., 2012; Thomas et al., 2012). Efforts to establish standardized digestion conditions are also underway in an initiative supported by the LFCS Consortium (Williams et al., 2012a,b).

To model digestion *in vitro*, the LBF is dispersed in a medium that is representative of the contents of the upper small intestine, and digestion is then initiated by the addition of a porcine-derived pancreatic extract containing pancreatic lipase and other pancreatic enzymes. An immediate descriptor of LBF performance is the rate and extent of LBF digestion, determined from the rate of addition of sodium hydroxide required to maintain a designated pH, to correct for the effect of liberated fatty acid which decreases the pH of the medium. Samples may be removed at intervals during the digestion test, and centrifuged to allow separation of three distinct phases, namely; a pellet phase consisting of insoluble calcium soaps of fatty acid; an aqueous colloidal phase consisting of amphiphilic digestion products, bile salt and phospholipids; and an oily phase containing a mixture of incompletely digested lipid and more lipophilic digestion products. To determine the solubilization capacity of the digested formulation, excess crystalline drug may be mixed with the aqueous colloidal phase formed by digestion of a drug-free LBF. Alternatively, if a drug is included in the LBF prior to digestion, the distribution of drug within the three phases produced during digestion can be quantified to evaluate the effect of digestion on the fate of an incorporated drug.

Several studies have correlated evidence of drug precipitation during *in vitro* dispersion/digestion with decreased *in vivo* bioavailability (Anby et al., 2012; Cuine et al., 2007, 2008; Dahan and Hoffman, 2006; Han et al., 2009; Porter et al., 2004a,b), and while there are some examples to the contrary (i.e., examples when formulations have demonstrated *in vivo* performance that surpassed expectations based on *in vitro* studies), these could be explained by the formation of a fast-dissolving non-crystalline drug precipitate (Thomas et al., 2012), or dose-dependency in first-pass drug metabolism (Anby et al., in preparation). Most of the published studies indicate that LBFs that result in drug precipitation *in vitro* are likely to have poorer *in vivo* performance when compared to more robust LBFs.

The aim of the present work was to explore a series of closely related formulations of fenofibrate to determine the factors that affect the performance of LBFs in *in vitro* dispersion and digestion tests, with a particular emphasis on drug solubilization/precipitation. This approach is an ideal way to identify formulations that might be expected to perform well or poorly *in vivo*, and will facilitate selection of a limited number of formulations to take forward into *in vivo* studies, to explore whether *in vitro-in vivo* correlation can be achieved using *in vitro* digestion testing. The LBFs investigated were all Type II or Type IIIA, as defined by the Lipid Formulation Classification System (LFCS) (Pouton, 2000, 2006). A minimum number of excipients were used so that LBFs were closely related in term of chemical composition. Type II formulations are highly lipophilic formulations that form turbid emulsions on dispersion in aqueous fluids, typically with a mean droplet diameter in the range 250–3000 nm. Type IIIA formulations may contain the same lipid composition as those in Type II, but in addition contain hydrophilic components such as a water-miscible surfactant or cosolvent. The incorporation of these additional hydrophilic components is associated with improved dispersion and the potential of forming ultrafine (i.e., nanosized) emulsions with aqueous media. The solubilizing properties of a series of Types II and IIIA LBFs toward the model drug fenofibrate (Fig. 1) was investigated. With a clinical dose in excess of 100 mg (Keating and Croom, 2007) and low aqueous solubility (Vogt et al., 2008), fenofibrate is a BCS class II compound. Fenofibrate, however, exhibits a high solubility in lipids (>75 mg/g), and together, this low aqueous solubility/high lipid solubility combination is characteristic of the most ideal candidates for oral lipid drug delivery. The study was divided into two parts; the solubilization studies in Part 1 providing the necessary data to allow the subsequent digestion studies of fenofibrate LBFs in Part 2 to be interpreted in terms of drug supersaturation.

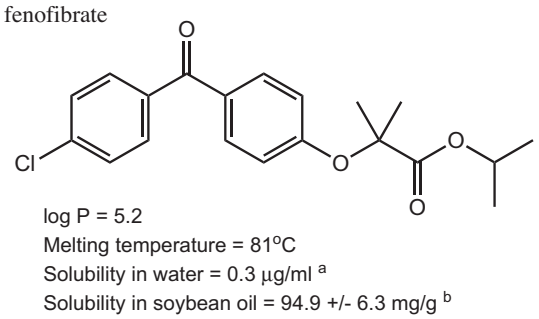

Fig. 1. The chemical structure and properties of the model drug, fenofibrate. (a) Vogt et al. (2008); (b) this work.

2. Materials and methods

2.1. Materials

Fenofibrate, soybean oil (a long-chain triglyceride), polyoxyethylene (20)-sorbitan monooleate (polysorbate 80, Tween® 80, HLB 15), polyoxyethylene (20)-sorbitan trioleate (polysorbate 85, Tween® 85, HLB 11), sodium taurodeoxycholate >95% (NaTDC), pancreatin extract (from porcine pancreas, P7545, 8 × USP specifications activity), calcium chloride dihydrate, Tris–maleate, and the lipid digestion inhibitor 4-bromophenylboronic acid (4-BPB) were purchased from Sigma–Aldrich Co. (St. Louis, MO). Miglyol® 812 (a medium-chain triglyceride) and Imwitor® 988 (a blend of medium-chain mono- and diglycerides) were supplied by Sasol Germany GmbH (Werk Witten, Witten-Germany). Maisine™ 35-1 (a blend of long-chain mono-, di and some triglyceride) was supplied by Gattefosse (Saint-Priest, France). Lecithin (ca. 99.2% egg-phosphatidylcholine (PC), Lipoid E PCS) was purchased from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). 1.0 M sodium hydroxide (Univol) was purchased from Ajax Finechem Pty Ltd. (Sydney, Australia) and was diluted with water (Milli-Q water purification system, Millipore, Bedford, MA) to achieve a 0.6 M titration solution. Methanol and chloroform used in this work were HPLC grade from Merck (Melbourne, Australia).

2.2. Lipid formulations

The composition of the eight lipid-based formulations (LBFs) investigated in this study are shown in Table 1. Each LBF contained

Table 1
The composition of the Type II and IIIA LBFs investigated in this study.

Lipid blend	Formulation component (% w/w)					
	Soybean oil (LCT)	Miglyol® 812 (MCT)	Maisine™ 35-1 (LCM)	Imwitor® 988 (MCM)	Tween® 85	Tween® 80
Type II						
LCT/LCM	35%	–	15%	–	50%	–
LCT/MCM	35%	–	–	15%	50%	–
MCT/LCM	–	35%	15%	–	50%	–
MCT/MCM[a]	–	35%	–	15%	50%	–
Type IIIA						
LCT/LCM	35%	–	15%	–	–	50%
LCT/MCM	35%	–	–	15%	–	50%
MCT/LCM	–	35%	15%	–	–	50%
MCT/MCM[a]	–	35%	–	15%	–	50%

[a] The properties of these Type II and IIIA LBF on dispersion have also been investigated by Mohsin et al. (2009).

50% lipid and 50% surfactant. The lipid component in the LBFs consisted of 35% long-chain triglyceride (LCT; soybean oil) or medium-chain triglyceride (MCT; Miglyol® 812), and 15% long-chain glyceride mixture (LCMix; Maisine™ 35-1) or medium-chain glyceride mixture (MCMix; Imwitor® 988). As seen in Table 1, LBFs contained either all LC lipids, all MC lipids, or a blend of LC and MC lipids. For Type II LBFs, the water-insoluble surfactant Tween®85 was used. Type IIIA LBFs used the more hydrophilic Tween® 80 surfactant.

2.3. Part 1: Fenofibrate solubility assessment

2.3.1. Anhydrous excipients and investigated LBFs

Crystalline fenofibrate was added in excess to glass sample tubes containing 3 g of each of the anhydrous excipients and LBFs shown in Table 1. Drug–excipient/LBF slurries were vortex-mixed and then incubated at 37 °C in an orbital mixer (Ratek Instruments, Melbourne, Australia) to provide continuous mixing during the equilibration period. At 24 h intervals over 6 days, a 0.5 g sample was removed and centrifuged (Eppendorf 5408R, Eppendorf AG, Hamburg, Germany) at 1600*g* for 15 min at 37 °C. Centrifugation separated the samples into a solid pellet phase and a particle free supernatant. Accurately weighed samples were removed from the supernatant, transferred to 5 ml volumetric flasks and made up to volume with chloroform:methanol (2:1 v/v). Aliquots (50–100 μl) were subsequently diluted >100-fold with methanol. Analysis of fenofibrate content in all cases was conducted using a UV spectrophotometer (Cecil CE 3021; Cecil Instruments, Ltd., UK) measuring absorbance at 286 nm, with the exception of fenofibrate/Tween® samples, which were analyzed for fenofibrate content by HPLC (Section 2.7). Equilibrium solubility in the anhydrous excipients and LBFs was defined as the value attained when consecutive solubility values differed by <5%.

2.3.2. Dispersed and digested surfactants Tween® 85 and Tween® 80

The solubilization capacity of the surfactants Tween® 80 and Tween® 85 post-dispersion and post-digestion was determined by equilibrium solubility measurements. A series of Tween® 85/Tween® 80 surfactant solutions (0.5%, 1.25% and 2.5% w/v) were prepared in a digestion medium (pH 7.5, 50 mM Tris–maleate, 150 mM sodium chloride, 5 mM calcium chloride dihydrate, 5 mM NaTDC and 1.25 mM PC). Fenofibrate was added in excess to 10 ml samples of the dispersed surfactant preparations and subsequently incubated at 37 °C in an orbital mixer (Ratek Instruments). After a 48 h period of equilibration, 1 ml samples were removed and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min to separate suspended drug from the solutions or colloidal dispersions of surfactants. A 50–100 μl aliquot of the homogenous supernatant was diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see Section 2.7).

To assess the solubilization capacity of the surfactants (at 1.25%) post-digestion, *in vitro* digestion tests were carried out using a method described previously (Devraj et al., 2013; Sek et al., 2002). In brief, 0.125 g of surfactant was dispersed in 9 ml digestion medium (pH 7.5, see above) for 10 min followed by the addition of 1 ml pancreatin containing 10,000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per ml of digestion medium. Digestion of the surfactant was continuously monitored using a pH-stat automatic titration unit (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.6 M NaOH. We chose to use 5 mM NaTDC and 1.25 mM PC deliberately to represent the fasted state. This is because drugs such as fenofibrate are absorbed best in the fed state and suffer from low bioavailability when administered to a fasted stomach. Our intention is to use the *in vitro* tests to examine the likely fate of drugs in the fasted intestine.

After 30 min digestion, 2 × 4 ml samples were collected from the reaction vessel and digestion quenched using the lipid digestion inhibitor 4-BPB (0.5 M in methanol, 9 μl/ml of sample). Samples were then ultracentrifuged (400,000*g*, 37 °C, Optima XL-100 K Ultracentrifuge, SW-60 swinging-bucket rotor, Beckman, Palo Alto, CA) in soft-walled polyallomer tubes (Beckman) for 30 min to separate each digestion sample into a colloidal aqueous phase (AP_{DIGEST}) and a pellet phase. Fenofibrate was added in excess to 2 ml samples of each AP_{DIGEST} and its solubility determined using the equilibration/sampling method described above for the dispersed surfactant solutions.

2.3.3. Dispersed and digested LBFs

The solubilization capacity of the LBFs shown in Table 1 post-dispersion and post-digestion was determined by equilibrium solubility measurements. The principles described in Section 2.3.2 were used, however the more complex phase behavior of the LBFs post-dispersion and post-digestion required a variations in the methods, which are described below.

In the dispersion tests, 1 g of LBF was weighed into 100 ml volumetric flasks and made up to volume with water. Flasks were subsequently incubated at 37 °C in an orbital mixer (Ratek Instruments). After mixing for 30 min to allow complete dispersion of the LBF, 3 × 10 ml aliquots were removed, mixed with excess fenofibrate and incubated at 37 °C in an orbital mixer (Ratek Instruments). At intervals (i.e., 24 and 48 h), 1 ml samples were removed and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min. In instances where the centrifugation process resulted in the phase-separation of an oily cream phase from the aqueous

phase (which was typical for the Type II LBFs containing LC lipids), this cream was gently redispersed with the bulk of the aqueous phase using an adjustable pipette (so as to not disturb any pellet phase). A 50–100 μl aliquot of the homogenous supernatant was removed and subsequently diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see Section 2.7).

In the digestion experiments, 0.25 g of LBF was digested in 9 ml digestion medium and 1 ml pancreatin, using the method described in Section 2.3.2. Digestion samples (2 × 4 ml) were removed after 30 min and separated by ultracentrifugation (also as described in Section 2.3.2) to separate the digestion phases, namely a poorly dispersed oil phase (in the case of the more lipophilic LBF), a colloidal aqueous phase (AP_{DIGEST}) and a pellet phase. Sample tubes were pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the AP_{DIGEST}. This approach was essential to ensure that an oil phase (which collected at the top of the sample) was not carried over into the AP_{DIGEST}. Fenofibrate was added in excess to 3 ml AP_{DIGEST}, and equilibrated for 48 h, during which 1 ml samples were removed at intervals (i.e., 4, 8, 24 and 48 h), centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min. A 50–100 μl aliquot of each homogenous supernatant was diluted >10-fold with methanol before analysis of fenofibrate content by HPLC (see Section 2.7). Equilibrium solubility in the dispersed/digested LBFs was defined as the value attained when consecutive solubility values differed by <5%.

2.4. Part 2: In vitro assessment of fenofibrate-containing LBF

2.4.1. Drug incorporation

All LBFs in Part 2 of this study were loaded with fenofibrate at 80% of its equilibrium solubility in the respective anhydrous formulation (determined method described in Section 2.3.1 and results presented in Section 3.1.1). The required mass of fenofibrate was weighed directly into clean screw-top glass vials and drug-free LBF was added up to the target mass loading. Vials were sealed, vortex-mixed and incubated at 37 °C for at least 24 h prior to testing.

The fenofibrate content in the formulation was verified (in triplicate) on the day of testing using the sampling procedure (without centrifugation) described in Section 2.3.1 before the fenofibrate content was determined by HPLC (Section 2.7).

2.4.2. In vitro dispersion testing

Dispersion testing of fenofibrate containing LBF was conducted in accordance with the method described in Section 2.3.3: The use of the same methods allowed direct comparison of the solubility results (Part 1) to the evaluation during dynamic studies (Part 2), where 1 g of LBF containing fenofibrate at 80% solubility was dispersed in 100 ml water, and incubated at 37 °C in an orbital mixer (Ratek Instruments). To measure the concentration of fenofibrate that remained solubilized in these tests, 1 ml samples were withdrawn at intervals (0.5, 1, 2, 4, 8, 16, 24, 32, 48, 72, and 96 h) and centrifuged (Eppendorf 5408R, Eppendorf AG) at 1600*g* for 15 min to sediment any drug precipitate. Consistent with Section 2.3.2, in instances where the centrifugation process resulted in the phase-separation of an oily cream phase from the aqueous phase, this cream was gently redispersed with the bulk of the aqueous using an adjustable pipette so as to not disturb any pellet phase. A 100 μl aliquot of the homogenous dispersion was diluted >10-fold before analysis of fenofibrate content by HPLC (see Section 2.7.

2.4.3. In vitro digestion testing

In vitro digestion experiments were performed in accordance with to the method described in Section 2.3.3, where 0.25 g LBF containing fenofibrate at 80% solubility was digested for 30 min in 9 ml digestion medium (plus 1 ml pancreatin). In these experiments, the fenofibrate concentration across each of the digestion phases in the centrifuged samples was determined using the following method. Firstly, the oil phase in the digestion samples was carefully aspirated using an adjustable pipette and transferred to a 10 ml volumetric flask, followed by 50 μl of 1 M HCl and chloroform–methanol mixture (2:1 v/v) up to volume. The sample tubes were then pierced near the bottom using a 5 ml syringe-23G needle assembly to extract the AP_{DIGEST}. Finally, to remove the pellet, the polyallomer tube was cut just above the mass of the pellet phase and suspended in 100 μl chloroform–methanol mixture (2:1 v/v). The pellet was then transferred to a 5 ml volumetric flask followed by 50 μl of 1 M HCl and chloroform–methanol mixture (2:1 v/v) up to volume. Each of the recovered phases was further diluted >10-fold in methanol prior to HPLC analysis (see Section 2.7) to determine the fenofibrate content in individual phases.

In certain digestion experiments, it was necessary to remove more than two 4 ml samples. In these tests, experiments were scaled up to 0.5 g of LBF and 18 ml digestion medium, prior to the addition of 2 ml pancreatin, as described above.

2.5. Extent of surfactant and LBF digestion

Digestion profiles were corrected for the background fatty acid released upon digestion of the bile salt/phospholipid mixed-micelles; this concentration was determined in separate experiments undertaken in the absence of surfactant/LBFs. The total concentration of fatty acid titrated over 30 min (corrected for the background fatty acid) was compared to the theoretical quantity of fatty acid that could be liberated if the surfactants/LBFs were completely hydrolyzed to provide an estimation of the extent of digestion using Eq. (1). In-line with previous work (Cuine et al., 2008), it was assumed that on digestion of lipids, one triglyceride molecule released two fatty acid molecules, and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a single fatty acid molecule. In the case of the surfactants, it is assumed that all fatty acids are available for hydrolysis, which is consistent with previous work.

$$\text{Extent (\%) of digestion} = \frac{\text{titrated fatty acid (mmoles)}}{\text{Theoretical maximum titratable fatty acid (mmoles)}} \times 100 \quad (1)$$

2.6. Supersaturation

Solubility values in the dispersed and digested LBFs determined in Part 1 of this study were used to calculate the supersaturation ratio (SR) during dispersion/digestion testing of fenofibrate-containing LBFs in Part 2 via Eqs. (2a) and (2b).During dispersion:

$$SR = \frac{\text{Fenofibrate dissolved (mg)}}{\text{Fenofibrate solubility in dispersed LBF (mg)}} \quad (2a)$$

During digestion:

$$SR = \frac{\text{Fenofibrate dissolved in } AP_{DIGEST} \text{ (mg)}}{\text{Fenofibrate solubility in } AP_{DIGEST} \text{ (mg)}} \quad (2b)$$

Eqs. (3a) and (3b) were used to calculate the maximum supersaturation ratio (SR^M), which is the ratio between the fenofibrate dose in the LBF (maximum theoretical concentration of solubilized drug in the absence of any drug precipitation) and drug solubility in dispersed or digested (i.e., AP_{DIGEST}) LBF:

During dispersion:

$$SR^M = \frac{\text{Fenofibrate dose (mg)}}{\text{Fenofibrate solubility in dispersed LBF (mg)}} \quad (3a)$$

During digestion:

$$SR^{M} = \frac{\text{Fenofibrate dose (mg)}}{\text{Fenofibrate solubility in } AP_{DIGEST} \text{ (mg)}} \quad (3b)$$

Each of the values used to calculate SR^{M} values during dispersion and digestion are shown in Table 2.

2.7. HPLC assay

HPLC analysis for fenofibrate were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, MA), with a Phenomenex® Luna column (C18 (2), 150 × 4.6 mm, 3 μm, Phenomenex, Torrence, CA). The mobile phase consisted of methanol and water in a 75:25 v/v ratio pumped in isocratic mode through the HPLC column at 1 ml/min. The sample injection volume was 50 μl, with UV detection at 286 nm. All samples and standards were maintained at 10 °C and the column temperature maintained at 25 °C.

2.8. Polarized light microscopy

A Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters was used to analyse selected dispersion/digestion pellets containing fenofibrate. Following centrifugation, the pellet was carefully removed from the sample tube and placed on a microscope slide. Samples were analyzed under normal light and cross-polarized light at 20× magnification, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan). Pellets were isolated and analyzed in the manner described above on the same day.

3. Results

3.1. Part 1: Fenofibrate solubility studies

3.1.1. Anhydrous excipients and surfactants

Fig. 2 shows the equilibrium solubility of fenofibrate in the excipients used in this study. The lowest solubility of 94.9 ± 6.3 mg/g was evident in the LCT, soybean oil. In contrast, the highest solubility of 137.0 ± 6.2 mg/g was evident in the MCT, Miglyol® 812. Consistent with this solubility dependence on lipid chain length, fenofibrate solubility in the mixed LC glyceride, Maisine™ 35-1 (110.9 ± 4.7 mg/g) was lower than the solubility in the equivalent mixed MC glyceride, Imwitor® 988 (133.4 ± 4.0 mg/g). The investigated surfactants were Tween® 80 (polyoxyethylene sorbitan monooleate) and the more lipophilic Tween® 85 (polyoxyethylene sorbitan trioleate) with fenofibrate solubility in the surfactants was highly comparable at 99.0 ± 2.4 mg/g and 102.1 ± 3.3 mg/g, respectively.

Table 2
Maximum supersaturation ratios (SR^{M}) for fenofibrate produced by dispersion and digestion of Type II and IIIA LBFs.

Formulation	Fenofibrate dose (mg) in 1 g LBF[a]	Fenofibrate solubility (mg) in 1 g LBF following		SR^{M}	
		Dispersion[b]	Digestion (AP_{DIGEST})[c]	Dispersion[d]	Digestion[e]
Type II					
LCT/LCM	80.3	80.7	7.6	1.0	10.6*
LCT/MCM	87.8	72.0	6.0	1.2	14.6*
MCT/LCM	101.3	97.9	5.2	1.0	19.5*
MCT/MCM	82.0	96.6	2.4	0.8	34.2
Type IIIA					
LCT/LCM	85.1	48.5	9.2	1.8	9.3*
LCT/MCM	92.4	29.6	4.0	3.1	23.1
MCT/LCM	102.4	68.5	3.6	1.5	28.4
MCT/MCM	88.7	72.1	2.4	1.2	37.0

[A] 80% of the solubility in anhydrous LBFs; values in Fig. 5A.
[b] 1 in 100 dilution; values in Fig. 5B.
[c] 1 in 40 dilution; values in Fig. 5C.
[d] Fenofibrate dose in the LBF divided by solubility in the dispersed LBF (i.e., Eq. (3a)).
[e] Fenofibrate dose in the LBF divided by solubility in the LBF AP_{DIGEST} (i.e., Eq. (3b)).
* SR^{M} values are overestimated since an oil-phase evident on digestion meant that the solubility in the AP_{DIGEST} does not reflect the entire solubilization capacity of the digested LBF.

3.1.2. Dispersed and digested surfactant solutions

Fig. 3 shows the solubilization capacity of dispersed and digested Tween® 80 and Tween® 85 surfactants for fenofibrate. To allow a direct comparison with the solubility of fenofibrate in the anhydrous surfactants (taken from Fig. 2), solubilities are expressed as the mass of fenofibrate dissolved by 1 g of dispersed/digested surfactant. Surfactant concentrations of 0.5% w/v and 1.25% w/v represent the concentration present during the LBF dispersion studies (i.e., 1 g of the LBFs shown in Table 1 dispersed in 100 ml) and digestion studies (i.e., 1 g of the LBFs shown in Table 1 digested in 40 ml), respectively. To enable direct comparison of the solubilization capacity of dispersed and digested surfactants, solubility studies were performed in the digestion medium (containing bile salt and phospholipid). However additional studies confirmed that solubility trends were not dependent on the presence of these endogenous solubilizers (see Supplementary information).

As expected, the fenofibrate solubility in Tween® 80 and Tween® 85 decreased with dilution in the aqueous medium. In the dispersed state, Tween® 85 exhibited a higher solubilization capacity than Tween® 80, This higher affinity of a lipophilic drug for a more hydrophobic surfactant is consistent with previous work (Alvarez-Nunez and Yalkowsky, 2000; Yalkowsky, 1999).

A direct comparison of the solubility in the dispersed and digested 1.25% surfactant solutions firstly reveals that digestion lowered the solubilization capacity of both surfactants. Secondly, as there was a more marked effect of digestion on the solubility in Tween® 85 (a 3.2-fold decrease), the difference in solvent capacity between Tween® 85 and Tween® 80 was less pronounced after digestion. These observations correlate well with the digestion profiles for the two surfactants, shown in Fig. 4. Titratable fatty acid in this figure corresponds to fatty acid released from the surfactant in response to digestion of surfactant esters by pancreatic enzymes (Bakala N'Goma et al., 2012; Cuine et al., 2008; Fernandez

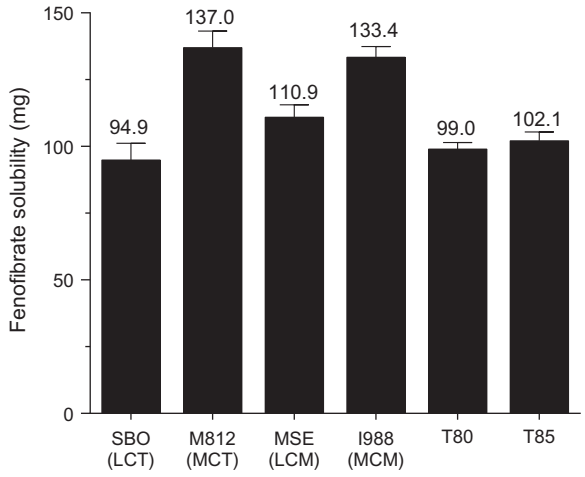

Fig. 2. Fenofibrate equilibrium solubilities in the anhydrous lipid excipients at 37 °C. SBO – soybean oil; M812 – Miglyol® 812; MSE – Maisine™ 35-1; I988 – Imwitor® 988; T80 – Tween® 80; T85 – Tween® 85. Mean ± SD (n = 3).

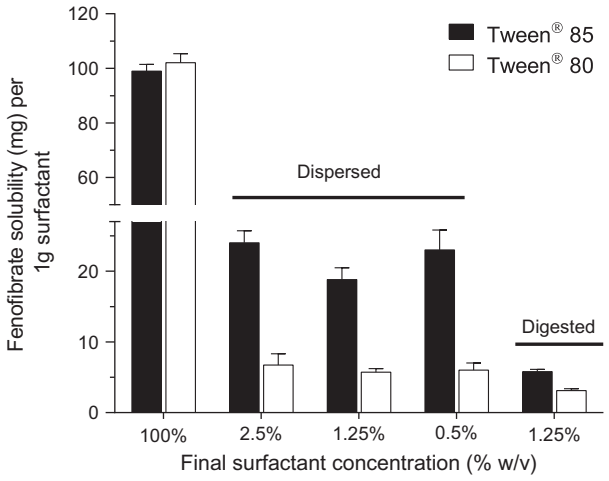

Fig. 3. The effect of dispersion and digestion on the solubilization capacity of 1 g of Tween® 85 (black bars) and 1 g Tween® 80 (white bars) for fenofibrate. Solubility is expressed as mass of fenofibrate dissolved by 1 g of surfactant, undiluted and at three different dilutions and after digestion (as described in methods). Mean ± 1 SD (n = 3).

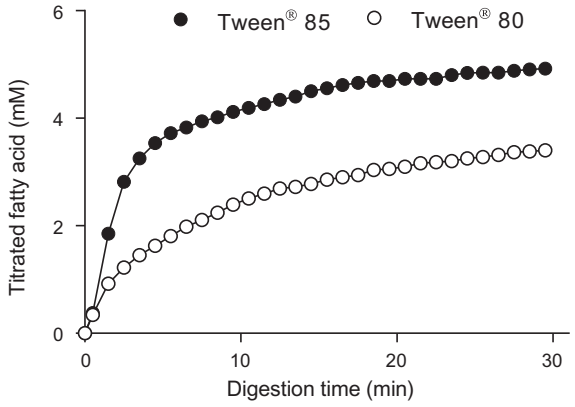

Fig. 4. Apparent titration of fatty acids during *in vitro* digestion of Tween® 85 and Tween® 80. Digestion was initiated at t = 0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during digestion. Data were corrected for the level of fatty acid released in digestion tests in the absence of surfactant. Mean ± 1 SD (n = 3).

et al., 2007). The concentration of fatty acid released from Tween® 85 after 30 min digestion was 1.4-fold higher than Tween® 80. This was expected since Tween® 85 is a polyethoxylated sorbitan tri-ester, and therefore, contains more fatty acid esters (per unit mass) than Tween® 80. An approximation of the extent of digestion of these surfactants (assuming that each Tween® 80 molecule releases one fatty acid molecule, and Tween® 85 potentially releases three fatty acid molecules) was 35.6% for Tween® 80 and 24.1% for Tween® 85.

The results therefore show that the lower solubilization capacity of a surfactant following dispersion may be further reduced by digestion (in-line with previous work (Cuine et al., 2008)). It was during dispersion that the greatest differences in solubilization by Tween® 85 and Tween® 85 were evident.

3.1.3. Anhydrous, dispersed and digested LBFs

Fig. 5 shows the equilibrium solubility of fenofibrate in 1 g of each LBF in the anhydrous form (Fig. 5A), following dispersion in water (Fig. 5B) and following 30 min digestion (Fig. 5C).

Fenofibrate solubility in the anhydrous LBFs ranged from 72 mg/mg to 126 mg/g. For both Type II and IIIA LBF, the highest solubilities were attained using the MCT/LCM lipid blend. This most likely reflects the higher fenofibrate solubility in MCT over the other lipids (Fig. 2). However, the lowest solubility was also evident in LBFs containing MCT (i.e., the MCT/MCM formulations), highlighting the complexity of attempting to relate solubility in LBFs based on solubilities of the individual excipients (Williams et al., 2012b). The solubility differences within in each of the four lipid Type II/IIIA LBFs pairs was insignificant (Fig. 5A), which is consistent with the observation that fenofibrate solubilities in anhydrous Tween® 80 and Tween® 85 were similar (i.e., Fig. 2).

The solubility of fenofibrate in 1 g LBF that was dispersed in 100 ml water is shown in Fig. 5B. A comparison of solubility values in Fig. 5A to those in Fig. 5B firstly reveals that dispersion of the LBFs lowered their solubilization capacity for fenofibrate. As dispersed Type II LBFs could solubilize between 75 mg and 96 mg of fenofibrate, and Type IIIA LBFs between 40 mg and 70 mg fenofibrate, it is also apparent that the decrease in LBF solubilization on dispersion was most marked for the Type IIIA LBFs. As the only difference between Type II and IIIA LBFs was the surfactant, this lower solubilization capacity of dispersed Type IIIA can be attributed to the lower solubilization capacity of dispersed Tween® 80. This is consistent with solubility results using individual surfactants, shown in Fig. 3, and with previous studies by Mohsin et al. (2009) and Williams et al. (2012a), both of which reported a lower solubilization capacity of hydrophilic LBFs/excipients on dilution.

For both Type II and IIIA LBFs, those containing the highest proportion of MC lipid, namely MCT/LCM and MCT/MCM, exhibited the highest solubilization capacity in the dispersed state. Variation in solubility across the four lipid blends was more marked in the case of the Type IIIA LBFs suggesting that the solubilizing properties of the lipids were more important when using the hydrophilic surfactant Tween® 80. The fact that surfactants demonstrate a higher solubility in water compared to lipids meant overall that it was the nature of the surfactant that most strongly affected solubilization of a dispersed LBF (rather than the composition of the lipids).

Fig. 5C shows the solubility of fenofibrate in the aqueous colloidal phase (AP_{DIGEST}) obtained following 30 min digestion (values are normalized to 1 g LBF in 40 ml to allow comparison). To aid in the interpretation of the results, the digestion profiles for each LBF are shown in Fig. 6. Profiles in the upper panels plot concentrations of titrated fatty acid, while those in the lower panels plot the % digestion of the LBFs (calculated using Eq. (1)). The fenofibrate solubilities in digested LBFs were lower than those in the dispersed state (i.e., compare white bars in Fig. 5B to those in Fig. 5C). This decrease in solubilization was observed despite the use of a higher LBF concentration in the digestion tests (i.e., 2.5% w/v, compared with only 1% during dispersion) and, consistent with previous work (Williams et al., 2012a), digestion of LBF-containing MC lipids led to a more marked decrease in solubilization. For example, the Type IIIA MCT/MCM was capable of solubilizing 72 mg fenofibrate in a dispersed state, but after 30 min digestion, solubilized only 2.4 mg fenofibrate, representing a >30-fold decrease in solubility. Therefore, though it had the highest solubilization capacity on dispersion, the Type IIIA MCT/MCM formulation exhibited the lowest solubilizing capacity following digestion.

Digestion of the Type II MCT/MCM formulation led to a 40-fold decrease in solubilization capacity, further illustrating the lower solubilizing properties of digested MC lipids. Despite differences in surfactant, there was no difference in the solubilization capacity of digested Type II and IIIA LBF containing MCT/MCM lipids.

The use of the fenofibrate solubility in the AP_{DIGEST} to assess the solubilization capacity of other digested Type II LBFs was complicated by the formation of an oil phase during centrifugation of

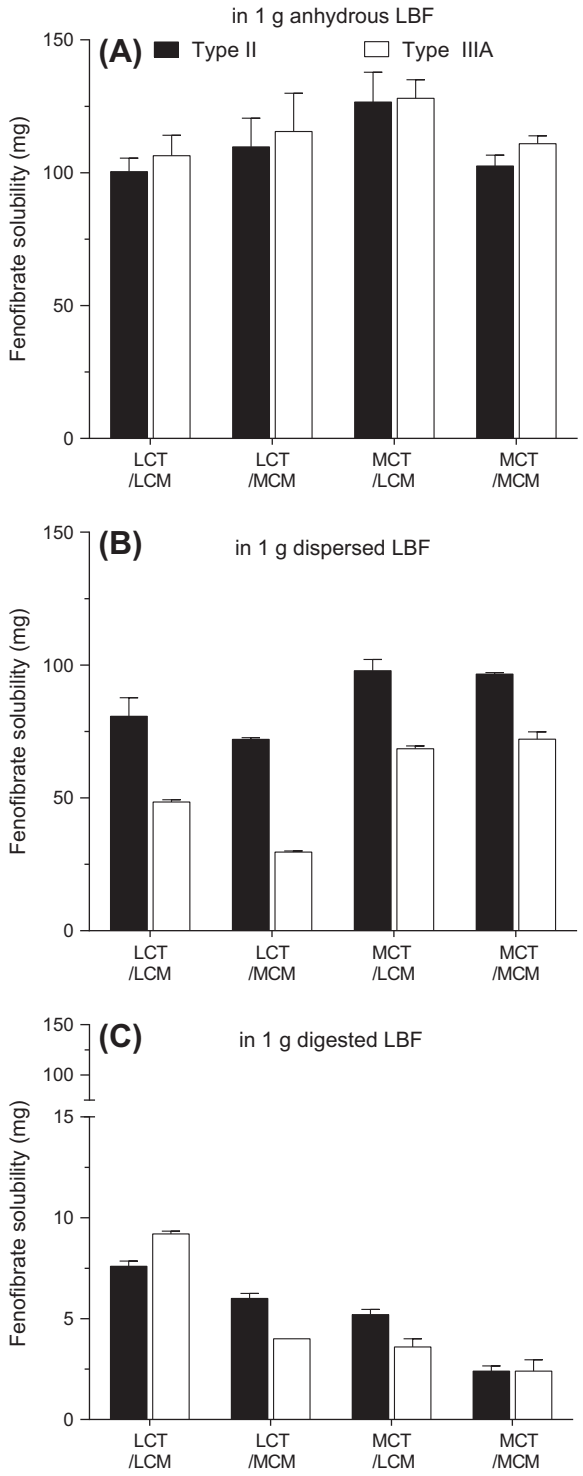

Fig. 5. Fenofibrate solubilities at 37 °C in the eight LBFs in the anhydrous, diluted, or digested forms. (A) Solubilities in anhydrous LBFs. (B) Solubilities in 1 g LBFs after dispersion in 100 ml. (C) Solubilities in 1 g digested LBFs (in 40 ml). Mean ± SD ($n = 3$). Type II LBFs contained Tween® 85, Type IIIA contained Tween® 80 (see Table 1).

the respective digestion samples. As this oil phase is expected to include a mixture of undigested triglyceride and highly lipophilic digestion products such as diglyceride, monoglyceride and any protonated fatty acids (Sek et al., 2002; Williams et al., 2012a,b), the greater prevalence of an oil phase in the case of the Type II LBFs over Type IIIA LBFs (only the Type IIIA LCT/LCM formulation formed a very small oil phase) can be attributed to the lower extent of digestion of Type II LBFs (see lower panels in Fig. 6). This results in a greater amount of undigested triglyceride, and differences in the capacity of each surfactant phase to solubilize lipid. As these phase-separated lipids will exhibit a high capacity to solubilize fenofibrate, measured solubilities in Type II $AP_{DIGESTS}$ are not representative of the solubilization capacity of the completely digested LBF, which, in turn, prevents a direct comparison of solubilization capacities of digested Type II LBFs (which form an oil phase) with their Type IIIA equivalents.

The solubilities in the Type II $AP_{DIGESTS}$ shown in Fig. 5C nevertheless reveal that increasing MC lipid content in the formulation progressively decreased the solubilization capacity of this phase and that the solubilities in the Type II and III $AP_{DIGESTS}$ were similar, despite the evidence of an oil phase in the case of the Type II LBF. The decreasing solubility of fenofibrate in digested LBFs with increasing MC lipid was consistent for both Type II and IIIA LBF, and also coincided with increasing digestion of the LBF (particularly the Type II LBF).

3.2. Part 2: Evaluation of drug-containing LBFs

3.2.1. In vitro dispersion testing

The results of *in vitro* dispersion testing of Type II and IIIA LBFs containing fenofibrate at a concentration equivalent to 80% of the equilibrium solubility in the formulation (i.e., 80% of the values in Fig. 5A), are shown in Fig. 7. For Type II LBFs (Fig. 7A and B), with the exception of the Type II LCT/MCM formulation, practically all of the drug remained in a solubilized state for the entire 96 h. In the case of the Type II LCT/MCM, ~82% drug remained solubilized, indicating that almost 20% of the dose had precipitated on dispersion. Precipitation did not occur to a significant extent during the first 4 h, and the large standard deviation bars indicate that precipitation was variable. There was a greater propensity for precipitation in the case of equivalent (but more hydrophilic) Type IIIA LBFs (Fig. 7C and D). For all formulations apart from the Type IIIA MCT/MCM, this precipitation commenced within the first 4 h, and was more prevalent after 4 h.

Fig. 8 shows the absolute mass of fenofibrate that remained in the solubilized form at selected time points during dispersion of Type II (Fig. 8A) and Type IIIA (Fig. 8B) LBFs. Having measured the solubilization capacity of the dispersed LBFs in Part 1 of this study (i.e., data included in Fig. 5B and shown in Fig. 8 by the dashed horizontal line), it is possible to evaluate the performance of respective LBFs in terms of the degree of supersaturation generated during their dispersion and digestion. The supersaturation ratio (SR) can be calculated at any of the sample time points using Eq. (2). The parameter SR^M describes the maximum level of supersaturation in the absence of drug precipitation (Eq. (3)), and can be utilized to describe the driving force of drug precipitation (Anby et al., 2012; Williams et al., 2012a). Calculated SR^M values for dispersion of the LBFs are shown in Table 2.

The results show that for all Type II LBFs, except the Type II LCT/MCM formulation, dispersion did not lead to supersaturation ($SR^M \leqslant 1$, Table 2). In other words, the dose utilized was not high enough to exceed the solubilization capacity of the dispersed formulation, and the lack of supersaturation in turn explains the lack of drug precipitation (Fig. 7A, B and Fig. 8A). The lower solubilization capacity of the dispersed Type II LCT/MCM formulation was responsible for the generation of supersaturation (SR^M 1.2). During the extended dispersion study, this modest degree of supersaturation was sufficient to cause precipitation.

The SR^M values for Type IIIA LBFs were 1.8 (LCT/LCM), 3.1 (LCT/MCM), 1.5 (MCT/LCM) and 1.2 (MCT/MCM) (Table 2). Type IIIA LBFs therefore produced higher SR^M values compared to equivalent Type II LBFs because the more hydrophilic Type IIIA LBFs have lower solubilization capacities on dispersion (illustrated clearly in

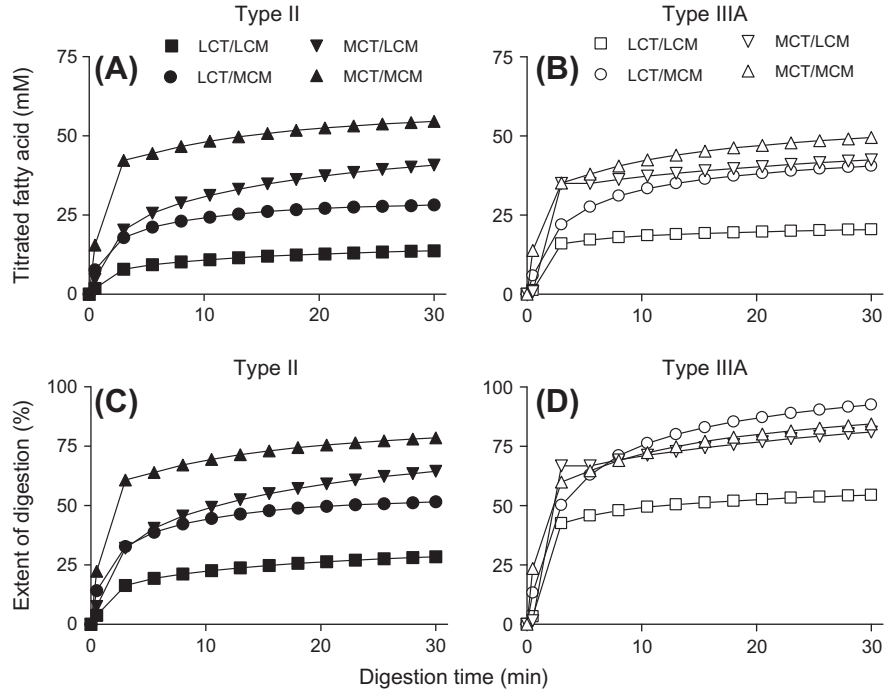

Fig. 6. *In vitro* digestion of the eight LBFs. (A and B) Apparent titration of fatty acids released during *in vitro* digestion of Type II and IIIA LBFs. Data were corrected for the level of fatty acid released in digestion tests in the absence of LBF. (C and D) Data are plotted as estimated% LBF digestion, calculated using Eq. (1).

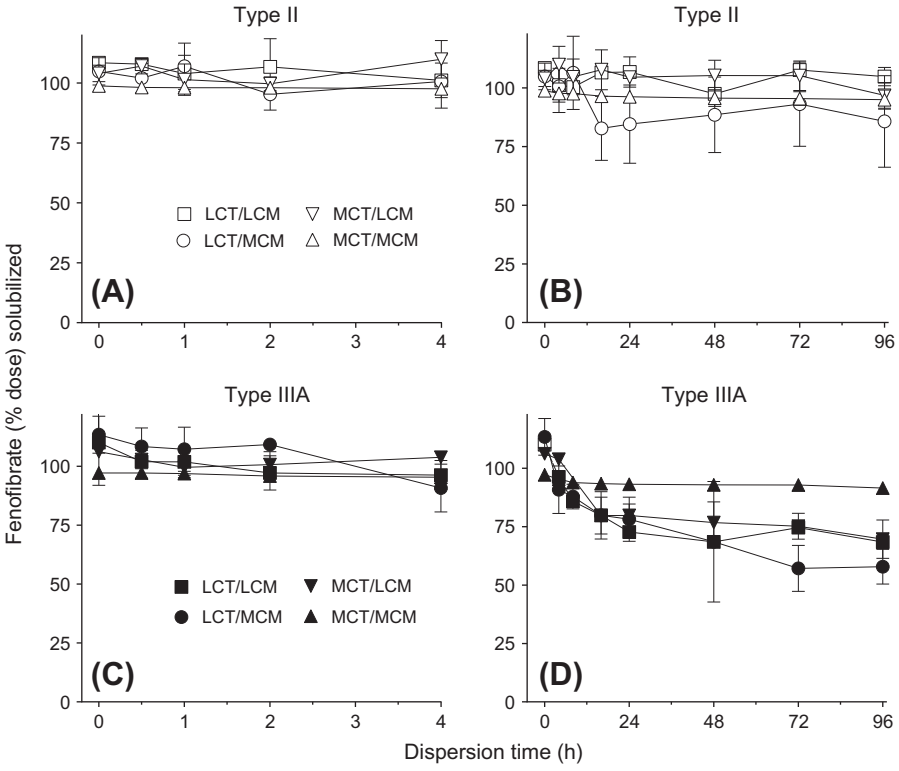

Fig. 7. Percentage of the fenofibrate dose solubilized in the aqueous phase during *in vitro* dispersion of LBFs. (A and B) Type II LBFs; (C and D) Type IIIA LBFs. (A and C) 4 h period; (B and D) 96 h period. Means ± SD (n = 3). Each LBF contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

Fig. 5B and tabulated in Table 2). Type IIIA LBFs that generated the highest SR^M values (LCT/LCM and MCT/LCM) were the first to show evidence of precipitation (by 4 h). In accordance with the higher SR^M values, the extent of precipitation was also greatest for these two formulations. Of the Type IIIA formulations, the MCT/MCM variant generated the lowest SR^M value, and as a consequence resulted in the least amount of precipitation.

In summary the results of the *in vitro* dispersion tests revealed that Type II LBFs out-performed Type IIIA equivalents, and that this observation was explained by the degree of supersaturation

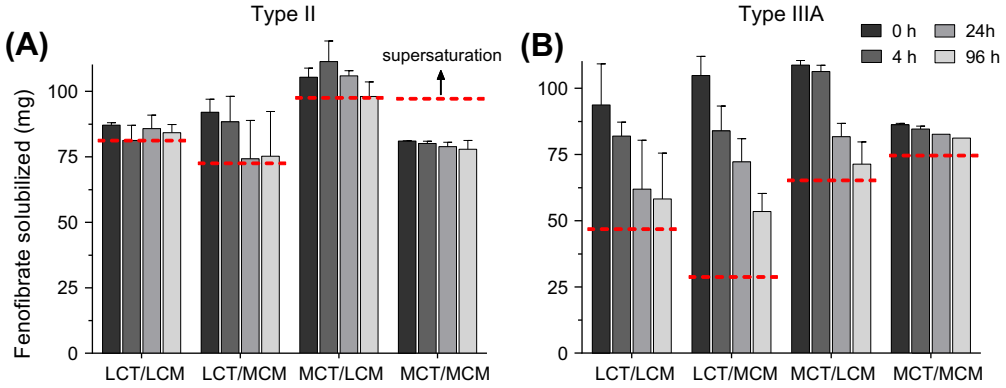

Fig. 8. Mass of fenofibrate solubilized as a function of time during *in vitro* dispersion. (A) Type II LBFs, (B) Type IIIA LBFs. Mean ± SD (n = 3). Each LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation. The dashed horizontal lines denote the solubilization capacities of the dispersed LBFs. Values above this line indicate supersaturation.

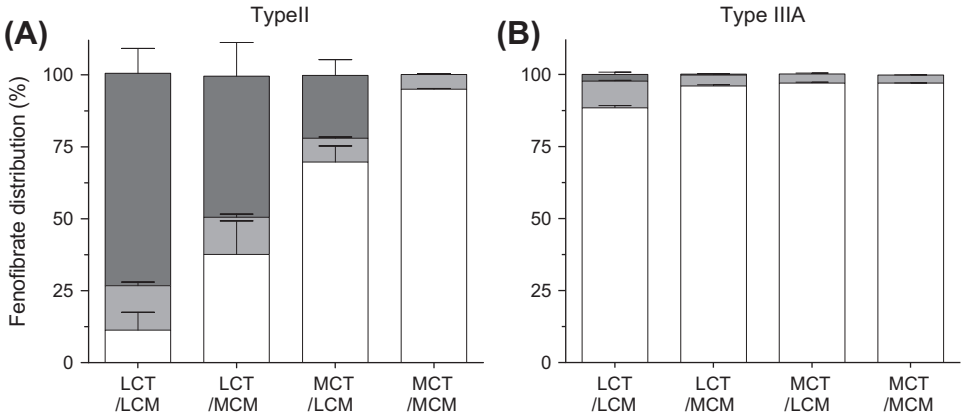

Fig. 9. The effect of *in vitro* digestion on the fate of fenofibrate in (A) Type II and (B) Type IIIA lipid formulations. Box-plots show the distribution of fenofibrate across the three phases assayed: a poorly dispersing oil phase (dark shaded bars); colloidal aqueous phase, AP_{DIGEST}, (light shaded bars); and pellet phase (white bars). Mean ± SD (n = 3). LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

obtained following dilution of the formulation. Since performance differences within the Type II/IIIA LBFs classes were small, the performance of these particular LBFs on dispersion were therefore most dependent on the nature of the surfactant and not the composition of the lipid component.

3.2.2. *In vitro digestion testing*

The effect of 30 min *in vitro* digestion on fenofibrate solubilization by Type II and IIIA LBFs is summarized in Fig. 9. The results are presented as the % of the drug dose contained in each of the isolated digestion phases, namely; an oil phase, the colloidal AP_{DIGEST} and the pellet phase (containing any precipitated drug).

Decreasing the quantity of LC lipid in the Type II LBFs resulted in decreases in the % of fenofibrate in the oil phase (Fig. 9A). This directly correlated (r^2 = 0.9798) with the extent of digestion for respective Type II LBFs (calculated via Eq. (1), and shown in Fig. 6C). Coincidently, the decreased quantity of fenofibrate in the oil phase, caused by reducing LC lipid/increasing MC lipid, also correlated with an increase in drug precipitation, which increased from 11.3 ± 6.2% in the case of the LCT/LCM formulation to 95.0 ± 0.3% for the MCT/MCM formulation. The increased likelihood of drug precipitation from MC over LC lipid formulations on digestion is consistent with previous work (Dahan and Hoffman, 2006; Porter et al., 2004a; Williams et al., 2012a). However, the present study also revealed that by substituting as little as 15% LC lipid for MC lipid (i.e., comparing LCT/LCM to LCT/MCM formulations in Fig. 9A) a formulator could inadvertently produce a poorer formulation that results in a marked increase in precipitation (~3-fold in this case). In contrast the substitution of 15% MC lipid for LC lipid (i.e., comparing MCT/MCM to MCT/LCM formulations in Fig. 9A) can have the opposite effect of decreasing drug precipitation. Therefore, despite increasing the proportion of MC lipids in the LBF to increase digestibility and to increase the lipid concentration in the AP_{DIGEST}, the fenofibrate concentrations in the respective $AP_{DIGESTS}$ were highest when LBFs contained the most LC lipid (250.4 ± 18.9 μg/ml (LCT/LCM) and 248.5 ± 33.5 μg/ml (LCT/MCM) compared with 182.3 ± 16.1 μg/ml (MCT/LCM) and 83.6 ± 3.2 μg/ml (MCT/MCM)).

For all Type IIIA LBFs, 30 min digestion caused >80% of the incorporated fenofibrate to precipitate (Fig. 9B). Differences in performance after 30 min between the different Type IIIA LBF were negligible, however the removal of samples at earlier timepoints during digestion, 5 and 15 min, revealed that drug precipitation during digestion of the LBF containing only LC lipid was slower compared with the MC lipid counterpart (Fig. 10). Indeed, in the case of the LCT/LCM Type IIIA LBF, ~50% of the incorporated dose had precipitated after 5 min of digestion (Fig. 10A), whereas for the MCT/MCM formulation (Fig. 10B), >90% of the dose had precipitated in this time. The use of LC lipids therefore offered some resistance to precipitation, though after 15 min, the small differences in the performance of Type IIIA LBFs were negligible.

Analogous to the dispersion study, the theoretical maximum supersaturation ratio (SR^M) achieved on digestion was calculated using Eq. (3a), producing the values shown in Table 2. SR^M values

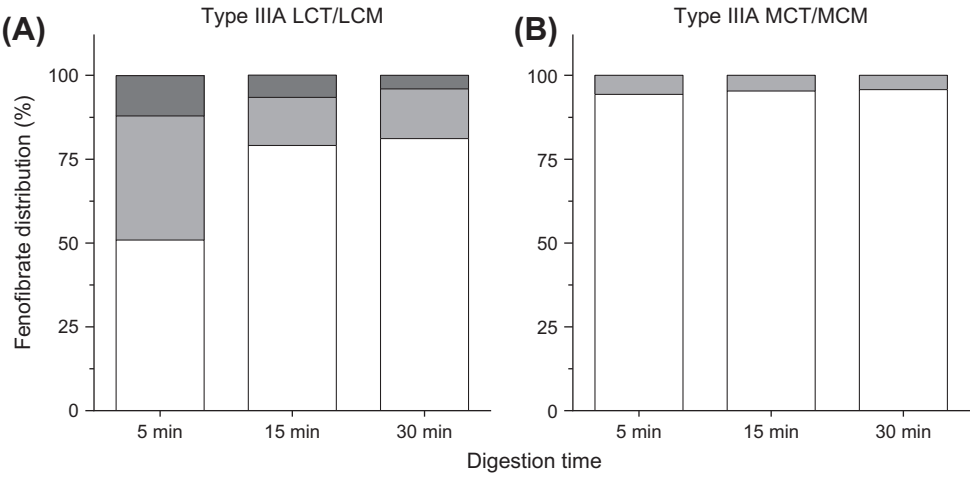

Fig. 10. The effect of *in vitro* digestion time on the fate of fenofibrate in (A) Type IIIA LCT/LCM and (B) Type IIIA MCT/MCM LBFs. Box-plots show the distribution of fenofibrate across the three phases assayed: a poorly dispersing oil phase (dark shaded bars); colloidal aqueous phase, AP_{DIGEST}, (light shaded bars); and pellet phase (white bars). LBFs contained a fenofibrate load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation.

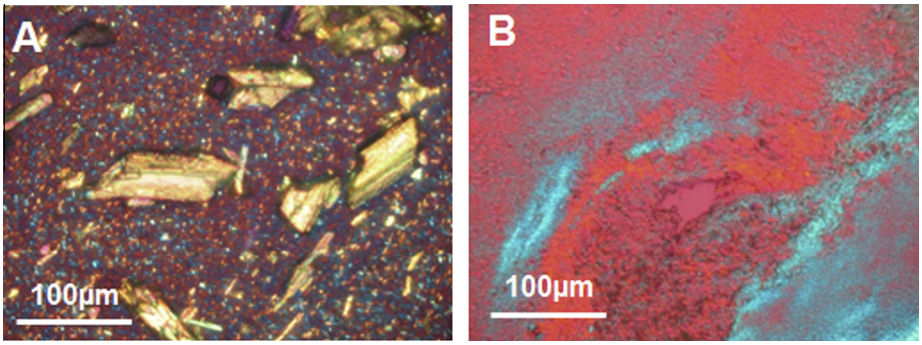

Fig. 11. Micrographs of the pellet phase following digestion of the Type IIIA LCT/MCM formulation. (A and B) Pellet phase after 30 min digestion. In (A) the formulation contained fenofibrate at 80% saturation. In (B), the formulation did not contain fenofibrate.

were typically higher for Type IIIA LBFs compared with Type II LBFs, and typically increase with decreasing LC lipid/increasing MC lipid in the formulation. It is also apparent that SR^M values produced on digestion were much higher than those produced by dispersion. As respective LBFs tested in dispersion and digestion experiments (in Part 2) contained the same fenofibrate dose, this difference in SR^M can be ascribed to the lower solubilization capacity of the digested formulations (previously described in Section 3.1.3). For example, dispersion of the Type II MCT/MCM formulation did not lead to supersaturation (SR^M 0.8), however the marked decrease in solubilization of this LBF on digestion led to a greater than 40-fold increase in SR^M (Table 2), and in turn, extensive drug precipitation. Similar, albeit less pronounced, increases in SR^M were apparent for all other LBFs. Direct comparisons of SR^M attained on dispersion and digestion were complicated in situations where LBFs formed an oil phase on digestion (denoted by * values in Table 2). As discussed in Section 3.1.3, the solubilization capacity of the digested LBF in these cases is underestimated (and therefore, SR^M values are overestimated) because a proportion of the formulation is sequestered in the oil phase. As shown in Fig. 9A, this oil phase contributes significantly to drug solubilization. Nonetheless, SR^M values for Type II LBFs are lower in all cases than those produced on digestion of Type IIIIA counterparts.

3.2.3. Assessment of fenofibrate precipitate under cross polarized light

Fig. 11 shows micrographs of the pellet phase from samples removed following digestion of the Type IIIA LCM/MCM formulation containing fenofibrate (Fig. 11A) and the drug-free equivalent Type IIIA LCM/MCM formulation (Fig. 11C). Pellets were viewed under cross-polarized light, with evidence of birefringence used to identify areas of crystallinity. Pellets containing precipitated fenofibrate (Fig 11A) show clear evidence of crystals confirming that the drug had precipitated out in a crystalline form.

4. Discussion

In vitro testing of lipid-based formulations (LBFs) is increasingly focused on the need to monitor the fate of incorporated drug during critical events that occur in the gastro-intestinal (GI) tract, namely dispersion of the formulation in aqueous fluids and subsequent digestion of the formulation components. This focus stems from the fact that dispersion and digestion can dramatically alter physicochemical properties of a LBF and, in turn, trigger drug precipitation. Incidences of precipitation will decrease the concentration of solubilized drug and, where the precipitate consists of a slow dissolving crystalline solid, formulations with a higher tendency to precipitate are expected to result in lower bioavailability *in vivo*.

In the present study LBFs that were either Type II or IIIA according to the Lipid Formulation Classification System (LFCS) were evaluated in *in vitro* dispersion and *in vitro* digestion tests. Both Type II/IIIA LBFs may be described as self-emulsifying drug delivery systems (SEDDS), yet due to differences in the amount of hydrophilicity excipients, Type II/IIIA LBFs can show marked

differences in emulsification and drug solubilization properties following dispersion and digestion. The tendancy of Type IIIA LBFs to produce ultrafine dispersions, has resulted in these formulations being the more favored option within industry. It has been assumed that fine particles would promote rapid absorption, though the interplay between this and their fate during digestion has not been studies in detail.

The aim of the present work was to discern the effects of lipid composition and surfactant type on the solubilization properties of Type II and IIIA LBFs during dispersion and digestion. This study follows on from work described by Mohsin et al. (2009), and more recently by Anby et al. (2012) and Williams et al. (2012a), with each showing that the degree of supersaturation attained on dispersion and/or digestion of a LBF can be used to explain the fate of drug during *in vitro* testing. The present study was therefore divided in two parts. In Part 1, a thorough assessment of the solubilization capacity of eight LBF, and their respective solubilization capacities following dispersion and digestion was performed. Subsequently, in Part 2, the performance of drug-containing LBFs during dynamic dispersion and digestion tests was evaluated. The results of the solubility studies in Part 1 therefore allowed the performance of drug-containing LBF in Part 2 to be discussed in terms of supersaturation. Fenofibrate (Fig. 1) was used as a model lipophilic drug. Four different lipid mixtures consisting of long-chain (LC) and medium-chain (MC) lipids were investigated, and were mixed with either Tween® 85 surfactant to generate Type II LBFs or the more hydrophilic Tween® 80 surfactant to form Type IIIA LBFs, providing for a total of eight LBFs (Table 1). It is stressed here that the experiments described in Part 1 were performed with drug-free formulations whereas in Part 2, LBFs were incorporated with fenofibrate at a level equivalent to 80% of the equilibrium solubility in the anhydrous formulation. As the fenofibrate solubility in Type II and IIIA LBFs of the same lipid component were highly comparable, the absolute fenofibrate loading in Type II/IIIA formulation pairs was similar in all cases. Due to some differences in solubility in MC and LC lipids, the target dose across the four Type II and four IIIA LBFs ranged from 80.3 to 101.3 mg and 85.1 to 102.4 mg, respectively. The results show that the lower solubilization capacity of dispersed Type IIIA LBFs compared with Type II equivalents introduced supersaturation and therefore the risk of drug precipitation. Digestion of the all Type IIIA LBFs and Type II LBFs containing predominantly MC lipid led to extensive drug precipitation, which could be rationalized once again by supersaturation.

4.1. LBF performance on dispersion

A Type II LBF by strict definition should contain no water-miscible components (Pouton, 2006), however it remains possible that a small proportion of the excipients, such as the nonionic surfactant Tween® 85 investigated here, will partition into an aqueous medium to in turn lower the solubilization capacity of the LBF towards a hydrophobic drug (Mohsin et al., 2009). In the present study, the solubilization capacity of Type II LBFs decreased on dispersion (Fig. 5B), however the extent of this decrease was small considering that LBFs were diluted 100-fold. As a result, dispersion of fenofibrate-containing Type II LBFs generally did not generate supersaturation (Table 2 and Fig. 8A), and with the exception of one formulation (that did generate supersaturation and show evidence of precipitation), dispersed Type II LBFs were shown to maintain practically all of the drug dose in a solubilized form over 4 days (Fig. 7). This lack of precipitation occurred despite the use of a high drug loading, equivalent to 80% of the equilibrium solubility in the anhydrous LBF. In contrast to Type II LBFs, dispersion of Type IIIA LBFs led to a more marked decrease in solubilization capacity (Fig. 5B) and higher degrees of supersaturation on dispersion of the fenofibrate-containing formulations (Table 2 and Fig. 8B) that led to some evidence of precipitation. This can be rationalized by the fact that Type IIIA LBFs contained the more hydrophilic surfactant Tween® 80. This surfactant has a greater affinity for aqueous media, readily forming a micellar solution, and therefore might be expected to lose a greater proportion of its bulk solubilization properties on dispersion.

More hydrophilic IIIB and IV types of LBF have been reported to undergo a decrease in solubilization on aqueous dispersion that covers two or more orders of magnitude (Mohsin et al., 2009; Pouton, 2006; Williams et al., 2012a). The decrease in solubilization capacity of Type IIIA LBFs in the present study were more modest, as evidenced by the maximum supersaturation ratios (SR^M; Eq. (3a)) (Table 2). The limited drug precipitation during the initial 4 h of dispersion of Type IIIA LBFs therefore suggests that higher supersaturation ratios are required for more rapid precipitation.

Such higher degrees of supersaturation may be produced on dispersion if drug loading in the LBF is increased. However, the Type IIIA LBFs investigated here contained high fenofibrate loadings, equivalent to 80% saturation. The fact that the Type IIIA LBFs generated relatively small degrees of supersaturation on dispersion suggests that the high proportion of lipid in these formulations (50% in this study) may attenuate the loss of solubilization that might be expected when using a water-miscible surfactant, even at high drug loadings. Therefore, unless rendered more hydrophilic through the addition of cosolvent or polar oils (such as MC monoglycerides), limited precipitation following the dispersion of a Type IIIA fenofibrate formulations is expected. Indeed there may be no precipitation in the stomach before the formulation is emptied from the stomach into the intestine. Thus it can be concluded that for fenofibrate formulations, the design of both Type II and Type IIIA LBFs should be focused on the solubilization capacity of the excipients in the digested state (rather than dispersion).

4.2. LBF performance on digestion

As the quantity of lipid in LBFS is sufficient to stimulate gall bladder secretion, and therefore, highly efficient digestion processes (Kossena et al., 2007), digestion of lipid components of a LBF within the small intestine is inevitable. Although the high lipid content in Type II and IIIA formulations may prevent drug precipitation on dispersion, digestion of these lipids will decrease the LBF solubilization capacity, increase supersaturation and, depending on the drug loading, may promote rapid and extensive precipitation of the incorporated drug (Fig. 9). The results presented here also showed that supersaturation and precipitation is intensified by the use of high amounts of MC lipids. The finding that the use of MC lipids is associated with increased risk of precipitation is consistent with several other studies (Anby et al., 2012; Dahan and Hoffman, 2006; Han et al., 2009; Porter et al., 2004a; Williams et al., 2012a). The present work also suggests that substitution of as little as 15% LC lipid for MC lipid is sufficient to significantly increase the rate and extent of drug precipitation.

Triglycerides are readily digested in the small intestine by pancreatic lipase into monoglycerides and fatty acids. Digestion will therefore render a LBF traveling through this region of the GI tract increasingly more hydrophilic and, since the solubilization capacity of this digested LBF towards a hydrophobic drug is inevitably lower than the solubilization capacity of the bulk and/or dispersed LBF, digestion can lead to supersaturation. Supersaturation has two opposing potential roles in drug absorption; either increasing drug absorption through increasing the thermodynamic activity of the absorbable fraction of drug or decreasing drug absorption by driving precipitation (Williams et al., 2013). Critical to the optimal *in vivo* performance of a LBF is the need to avoid factors that promote precipitation, principally a high degree of supersaturation,

since supersaturation lowers the thermodynamic barriers to precipitation. By lowering the solubilization capacity of the LBF, digestion was shown to generate high supersaturation ratios for both Type II and IIIA LBFs (Table 2). Supersaturation ratios were higher when LBFs contained MC lipids. This was because the solubilization difference between the anhydrous/dispersed LBF and the digested LBF is generally more pronounced on using MC lipids, which reflects the relative hydrophilicity of monocaprylin/monocaprin and caprylic/capric acid, and the lower tendency of these digestion products to interact with and supplement the bile salt/phospholipid solubilizing phase (Kossena et al., 2003). In the *in vitro* conditions employed here, the high supersaturation ratios generated by digestion of MC lipids could not be maintained for 30 min, and sometimes, not for even 5 min (Fig. 10B).

The use of MC lipid in Type II and IIIA formulations is effective for the generation of LBFs that result in minimal loss of solubilization on dispersion, and that subsequently generate supersaturation rapidly on entering the intestine. In some instances, this supersaturation may be highly effective in driving drug absorption, though if too high, supersaturation may lead to precipitation before drug absorption occurs. The use of LC lipids reduces the risk of precipitation by slowing the rate and extent of LBF digestion and by increasing solubilization. However, as evidenced in the present study, this approach alone may not be sufficient. Alternative strategies that may also attenuate drug precipitation from Type IIIA LBFs include lowering drug loading (Williams et al., 2012a) (though this may not be practically possible), use of polymer precipitation inhibitors (Anby et al., 2012) or judicious selection of the surfactant (Cuine et al., 2008).

The latter strategy is important since formulation surfactants such as the Tween® surfactants investigated here are substrates pancreatic enzymes including carboxyl ester hydrolase (CEH) and phospholipase (Bakala N'Goma et al., 2012; Christiansen et al., 2010; Cuine et al., 2008; Fernandez et al., 2008). Surfactants are digested to more polar and less amphiphilic molecules, which can lower the solubilization capacity of the LBF (Cuine et al., 2008). This appeared to be the case in the present study where it was shown that the solubilization capacity of Tween® 85 and Tween® 80 surfactant solutions decreased following digestion (Fig. 3). The overall drop in solubilization was more marked in the case of Tween® 85 by virtue of its higher digestibility/number of ester groups (Fig. 4). Thus, the digestion of the formulation surfactant can add to the lower solubilization capacity of LBFs following digestion of triglycerides. The varied solubilization capacity of surfactants, and therefore, capacity to affect supersaturation and precipitation dependent on their digestibility calls for a better understanding of the surfactant effects in LBFs, which is the subject of a separate study (Devraj et al., submitted for publication).

5. Conclusions

The results of the current study show that dispersion and digestion of lipid-based formulations (LBFs) can generate supersaturation. The extent of supersaturation on dispersion was dependent on the hydrophilicity of the formulation components, whereas the extent of supersaturation reflected the digestibility of the LBF and the solubilization capacity of the digestion products. High supersaturation ratios are strongly promoting of precipitation and, in the present study, high supersaturation ratios attained on digestion led to widespread crystallization of the model drug fenofibrate from lipid-rich formulations that did not contain any cosolvent. Therefore, while the use of high lipid concentrations in the LBFs (50% in this case) will minimize risk of precipitation on dispersion, judicious selection of the type of lipid and surfactant is necessary to ensure that the positive effect of supersaturation to drug absorption can be exploited before the drug crystals form in the GI tract. The study emphasises the value of conducting *in vitro* digestion tests on LBFs and the predictive power that can be gained by calculating the maximum supersaturation ratio that occurs during digestion.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2013.04.036.

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Appendix C: Published Version of Paper 3

Choice of Nonionic Surfactant Used to Formulate Type IIIA Self-Emulsifying Drug Delivery Systems and the Physicochemical Properties of the Drug Have a Pronounced Influence on the Degree of Drug Supersaturation that Develops During *In Vitro* Digestion

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ABSTRACT: The performance of self-emulsifying drug delivery systems (SEDDS) is influenced by their tendency to generate supersaturated systems during dispersion and digestion in the gastrointestinal tract. This study investigated the effect of drug loading on supersaturation during digestion of fenofibrate or danazol SEDDS, each formulated using long-chain lipids and a range of nonionic surfactants. Supersaturation was described by the maximum supersaturation ratio (SR^M) produced by *in vitro* digestion. This parameter was calculated as the ratio of the total concentration of drug present in the digestion vessel versus the drug solubility in the colloidal phases formed by digestion of the SEDDS. SR^M proved to be a remarkable indicator of performance across a range of lipid-based formulations. SEDDS containing danazol showed little evidence of precipitation on digestion, even at drug loads approaching saturation in the formulation. In contrast, fenofibrate crystallized extensively on digestion of the corresponding series of SEDDS, depending on the drug loading. The difference was explained by the generation of higher SR^M values by fenofibrate formulations. A threshold SR^M of 2.5–2.6 was identified in six of the seven SEDDS. This is not a definitive threshold for precipitation, but in general when SR^M is greater than 3, fenofibrate supersaturation could not be maintained. J Pharm Sci 103:1050–1063, 2014
Keywords: supersaturation; precipitation; SEDDS; poorly water-soluble drugs; LFCS; lipids; surfactants; in vitro models; SR^M

INTRODUCTION

Examples of lipid-based formulations commonly used in oral drug delivery include simple oil solutions, self-emulsifying drug delivery systems (SEDDS), and cosolvent/surfactant mixtures, each of which have been used to improve the oral absorption of poorly water-soluble drugs (PWSDs).[1–4] SEDDS, consisting of a mixture of drug, oil(s), surfactant(s), and sometimes cosolvent, are perhaps the most widely used type of lipid formulation; Neoral® (the Novartis SEDDS formulation of cyclosporine) is a well-known commercial example. SEDDS are designed to emulsify spontaneously on addition to an aqueous phase, generating colloidal oil-in-water dispersions. The size of the colloidal oil droplets is dependent on the composition of the formulation, particularly the lipid–surfactant ratio and the type of surfactant used.[5–8] Although the average particle size of these systems immediately following dispersion is often determined by formulators,[3,9] the reality is that oil droplet size and the overall structure and composition of the colloids is continually changing during gastrointestinal transit, as the formulation encounters the digestive system, and as individual components are absorbed. In recent years, other, more robust measures of SEDDS performance have been sought. Methods for assessment of the fate of the drug during either *in vitro* dispersion or *in vitro* digestion are increasingly being used to predict the *in vivo* performance of lipid-based systems.[6,10–13] The rationale for such *in vitro* tests stems from the knowledge that SEDDS and other types of lipid formulations may suffer a loss of solubilization capacity following dispersion in the aqueous fluids in the gastrointestinal tract[3,14,15] or following digestion of lipids and/or surfactants in the intestine.[6,13,16,17] Dependent on drug loading, loss of solubilization capacity can lead to drug supersaturation, and the risk of drug precipitation.

The Lipid Formulation Classification System (LFCS), proposed by Pouton,[15,18] provides some initial guidance on SEDDS performance during dispersion and digestion. The LFCS describes four different classes of lipid formulations. Depending on the excipients used, SEDDS fall into either Type II or Type III according to the LFCS. Type II formulations consist of oils and water-insoluble surfactant(s), and form turbid dispersions of oil droplets that typically range from 0.25 to 2 μm in diameter. Because of the lack of water-soluble components, Type II formulations typically result in minimal loss of solubilization capacity on dispersion.[15–17] Type III formulations consist of oils mixed with water-soluble (high HLB) surfactant(s) and sometimes also a water-miscible cosolvent. Type III formulations are therefore more hydrophilic. They may form ultrafine dispersions (<100 nm) but typically lose solvent capacity on dispersion and digestion. Type III A/B subclasses have also been introduced to better differentiate between Type III formulations showing high (IIIA) or low (IIIB) lipid contents. The high

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lipid content (>40%) in Type IIIA formulations is often able to prevent rapid and extensive precipitation on dispersion,[10,16,17] unless the formulation contains high drug loadings and/or cosolvent,[6,13] However, oils present in SEDDS (both Types II and IIIA/B) are likely to be readily digested by pancreatic lipases in the small intestine,[19,20] causing the physicochemical nature of the SEDDS to change dramatically. More specifically, at the molecular level, digestion involves the enzymatic hydrolysis of esters in triglyceride and diglyceride molecules and the formation of less lipophilic monoglyceride and fatty acid molecules. This process at a formulation level causes a progressive depletion of an oil droplet phase and the enrichment of bile salt/phospholipid-mixed micellar phase(s) that include the digestion products. Digestion has the effect of "forcing" drug to partition from the oil reservoir, which is rapidly decreasing in volume, into the micellar phase. As lipophilic drugs typically have lower affinity toward the more hydrated micellar phases, the transfer of drug from an oil-rich phase by digestion is associated with a decrease in drug solubility. This is analogous to other events that are known to create supersaturation by shifting the position of equilibrium, such as solvent-shift phenomena.[21,22]

We and others have shown that digestion of Type IIIA SEDDS can dramatically lower their solubilization capacity for hydrophobic drugs to a point where drug precipitation occurs.[5,11,13,16,17] The effect of precipitation on drug absorption is dependent on the physical form of the drug in the precipitate. The emergence of a crystalline solid with a slow rate of redissolution (often the case for PWSD) is likely to be associated with decreased bioavailability.[1,6,13] Rational lipid formulation design therefore requires an awareness of the factors that may contribute to drug precipitation, the critical factor being the extent of supersaturation generated by a loss of solubilization upon dispersion and digestion.

In our previous study,[17] the performance of a Type IIIA SEDDS consisting of long-chain lipids (soybean oil and Maisine™ 35-1), the surfactant Tween® 80, and a high loading (~85 mg/g) of the PWSD fenofibrate, was examined *in vitro*. Precipitation of fenofibrate during dispersion was moderate (<25% over 24 H). However, during 30 min of digestion, because of exposure to pancreatin and bile, more than 85% of the drug crystallized from solution. The substantial increase in precipitation observed during digestion tests was attributed to a marked increase in the degree of supersaturation caused by digestion of the SEDDS, which decreased the solubilization capacity.[17] The present study was designed to extend our understanding of the performance of Type IIIA SEDDS during *in vitro* digestion testing, by further exploring whether the degree of supersaturation attained during digestion could explain differences in drug precipitation. Model drugs were chosen with high (fenofibrate) or lower (danazol) solubility in anhydrous SEDDS, which allowed a wide range of drug loadings to be evaluated. In this study, we explored the influence of the choice of surfactant. Each SEDDS consisted of long-chain lipids combined with one of seven different nonionic surfactants. The surfactants included various digestible materials[6] [Cremophor® EL, Cremophor® RH40, Tween® 80 and Solutol® HS-15, D-α-tocopherol polyethylene glycol (TPGS) 1000 succinate] and nondigestible materials (Brij® 97 and Brij® 98). The focus on the choice of surfactant is timely, given that recent studies have suggested that the digestibility of the surfactant in SEDDS can dramatically influence the performance *in vitro* and *in vivo*.[6,23,24] Other recent studies have compared various nonionic surfactants and reported their differential capacity to affect the activity of intestinal digestion enzymes,[6,25] the interfacial properties at the oil–water interface,[26–28] and cytochrome-mediated drug metabolism in the gastrointestinal tract.[29–31] These studies all reiterate the need for the judicious selection of formulation surfactant in SEDDS. The studies presented herein aimed to investigate the extent of precipitation of two drugs, fenofibrate and danazol, from a range of formulations that differed only in the identity of the surfactant used to form Type IIIA lipid-based delivery systems. The emphasis of the study was to evaluate precipitation as an unbiased measure of performance, and to ask whether there was any relationship between the extent of precipitation and the degree of supersaturation generated during digestion of the formulations.

MATERIALS AND METHODS

Materials

Details of the nonionic surfactants used in the study are presented in Table 1. Fenofibrate, soybean oil (a long-chain triglyceride), sodium taurodeoxycholate (>95%, NaTDC), porcine pancreatin extract (P7545, 8× USP specifications activity), calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), Tris–maleate, and the lipid digestion inhibitor 4-bromophenylboronic acid (4-BPB) were purchased from Sigma–Aldrich Company (St. Louis, Missouri). Danazol was kindly supplied by Sterling Pharmaceuticals (Sydney, New South Wales, Australia). Maisine™ 35-1 (a blend of long-chain mono-, di-, and some tri-glyceride) was supplied

Table 1. Details of the Nonionic Surfactants Used in the Type IIIA SEDDS

Surfactant	Chemical Name	Quoted HLB Value/Range
Brij® 97[a]	Polyoxyethylene (10) oleyl ether	~12
Brij® 98[a]	Polyoxyethylene (20) oleyl ether	15
Cremophor® EL[b]	Polyethylene glycol (35)-glycerol ricinooleate	12–14
Cremophor® RH40[b]	Polyethylene glycol (40)-glycerol hydroxystearate	14–16
Solutol® HS-15[b]	Polyethylene glycol (15)-hydroxy stearate	14–16
Tween® 80[a]	Polyoxyethylene (20) sorbitan monooleate	15
TPGS[a]	D-α-tocopherol polyethylene glycol (23) succinate	~13

The oxyethylene content of each material is quoted using a common nomenclature, not necessarily used by the manufacturers, where the number in brackets represents the approximate number of $–CH_2CH_2O–$groups per molecule. However, the materials are not synthesized by common methods. The oxyethylene chains are a varied chain length because of their polymeric nature and the materials, particularly the esters, may contain complex mixtures of molecules.

[a] Obtained from Sigma–Aldrich, St. Louis, Missouri.

[b] Obtained from BASF, Washington, New Jersey.

by Gattefosse (Saint-Priest, France). Lecithin [~99.2% egg-phosphatidylcholine (PC, #6573), Lipoid E PCS], was purchased from Lipoid GmBH (Ludwigshafen, Germany). Sodium hydroxide (1.0 M; Univol) was purchased from Ajax Finechem Pty. Ltd. (New South Wales, Australia) was diluted with water (Milli-Q water purification system, Millipore, Bedford, Massachusetts) to produce a 0.6 M titration solution. Methanol and chloroform used in this work were HPLC grade and were obtained from Merck (Victoria, Australia).

Lipid Formulations

The lipid-based formulations investigated in this study were Type IIIA SEDDS as defined by the LFCS.[32,33] SEDDS used in this study contained 50% (w/w) long-chain lipids (soybean oil and Maisine™ 35-1 in a 7:3, w/w, ratio) and 50% (w/w) of one of the nonionic surfactants listed in Table 1. All of the formulations were emulsified rapidly to produce fine submicron dispersions under conditions of gentle agitation. The particle size distributions of dispersions were not evaluated because they change extensively as soon as digestion is initiated.

To measure the danazol and fenofibrate solubility in anhydrous surfactants and SEDDS, crystalline drug was added in excess to 3 g anhydrous surfactant or SEDDS. Mixtures were incubated with continuous mixing at 37°C in an orbital mixer (Ratek Instruments, Melbourne, Victoria, Australia). At 24 h intervals over 6 days, approximately 0.5-g sample was removed and centrifuged (Eppendorf 5408R; Eppendorf AG, Hamburg, Germany) at 1600 *g* for 15 min. Accurately weighed samples of the supernatant were dissolved in 5 mL chloroform–methanol (2:1, v/v), and aliquots (100 μL) were then diluted more than 10-fold in methanol. For fenofibrate in the SEDDS, samples were analyzed for drug content using a UV spectrophotometer (Cecil CE 3021; Cecil Instruments, Cambridge, United Kingdom) measuring absorbance at 286 nm. All other samples were analyzed for drug content by content by HPLC (see section headed 'HPLC Detection of Model Drugs'). Equilibrium solubility in the anhydrous excipients and SEDDS was defined as the value attained when consecutive solubility values differed by less than 5%.

To prepare SEDDS containing one of the two drugs, the required mass of drug was weighed directly into clean screw-top glass vials and drug-free SEDDS was added up to target mass. Vials were sealed, vortex-mixed, and incubated at 37°C for at least 12–24 h prior to testing.

In Vitro Digestion Testing

In vitro digestion experiments were performed as previously described.[34,35] In brief, 0.25 g of SEDDS or 0.125 g of surfactant was dispersed in 9-mL digestion medium (50 mM Tris–maleate, 150 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM NaTDC, 1.25 mM PC, pH 7.5) for 10 min followed by the addition of 1 mL pancreatin containing 10,000 tributyrin units (TBU) of pancreatic lipase, giving a final concentration of 1000 TBU per milliliter of digestion medium. Digestion of the surfactant was continuously monitored using a pH-stat titrator (Radiometer Pacific, Copenhagen, Denmark), which maintained a constant pH within the reaction vessel through the automatic addition of 0.6 M NaOH.

After 30 min, 2 × 4 mL samples were collected from the reaction vessel and digestion was inhibited in these samples using a lipid digestion inhibitor (0.5 M 4-BPB in methanol, 9 μL/mL of digestion sample). Samples were then ultracentrifuged (400,000*g*, 37°C; Optima XL-100K Ultracentrifuge; SW-60 swinging-bucket rotor; Beckman, Palo Alto, California) in soft-walled polyallomer tubes (Beckman) for 30 min to separate the digestion samples into a poorly dispersed oil phase, a colloidal aqueous phase (AP_{DIGEST}), and a pellet phase. We chose to stop digestion at 30 min because differences between 30 and 60 min experiments were limited. Approximately 90% of the digestion occurs within 15 min.

In instances where a drug-containing SEDDS was digested, all three digestion phases were isolated and analyzed for drug content. First, the oil phase (where present) in the digestion samples was carefully aspirated using an adjustable pipette and transferred to a 10-mL volumetric flask, followed by 50 μL of 1 M HCl and chloroform–methanol mixture (2:1, v/v) up to volume. The sample tubes were then pierced near the bottom using a 5-mL syringe-23G needle assembly to extract the AP_{DIGEST}. Finally, to remove the pellet, the polyallomer tube was cut just above the mass of the pellet phase and suspended in 100 μL chloroform–methanol (2:1, v/v). The pellet was then transferred to a 5-mL volumetric flask followed by 50 μL of 1 M HCl and chloroform–methanol (2:1, v/v) up to volume. Each of the recovered phases was further diluted more than 10-fold in methanol prior to HPLC analysis (see section headed 'HPLC Detection of Model Drugs') to determine the fenofibrate content in individual phases. In instances where a drug-free SEDDS was digested, only the AP_{DIGEST} was isolated. Drug solubility in the AP_{DIGEST} was determined according to the method described in section headed 'HPLC Detection of Model Drugs'.

Drug Solubility in AP_{DIGEST}

Drug-free SEDDS (0.25 g) was digested for 30 min according to the method described in section headed 'HPLC Detection of Model Drugs'. Fenofibrate–danazol was subsequently added in excess to 3 mL AP_{DIGEST}, and equilibrated for 48 h at 37°C in an orbital mixer (Ratek Instruments). At intervals (i.e., 4, 8, 24, and 48 h), 1 mL samples were removed, centrifuged (Eppendorf 5408R; Eppendorf AG) at 1600*g* for 15 min. Aliquots (50–100 μL) of each homogenous supernatant were diluted more than 10-fold with methanol before analysis of drug content by HPLC (see section headed 'HPLC Detection of Model Drugs'). Equilibrium solubility in the digested SEDDS was defined as the value attained when consecutive solubility values differed by less than 5%.

Estimated Extent of SEDDS Digestion

Digestion profiles were corrected by subtracting for the fatty acid released upon digestion of the bile salt/phospholipid-mixed micelles (i.e., the fatty acid titrated in blank experiments in the absence of formulations); this concentration was determined in separate experiments undertaken in the absence of surfactant/SEDDS. The total concentration of fatty acid titrated over 30 min (corrected for the background fatty acid) was compared with the theoretical quantity of fatty acid that could be liberated if the surfactants/SEDDS were completely hydrolyzed. This provided an estimation of the extent of digestion using Eq. (1). In line with the previous work,[8,17,36,37] it was assumed that on digestion of lipids *in vitro*, one triglyceride molecule released two fatty acid molecules (plus a nondigestible 2-monoglyceride), and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a single fatty acid molecule. In the case of the surfactants, it is assumed that

all fatty acids are available for hydrolysis, which is consistent with previous work.[6,38] The estimated fatty acid content in Cremophor® EL, Cremophor® RH40, and Tween® 80 were taken from Cuine et al.[6] For TPGS monoesters (molecular weight of 1513 g/mol)), it was assumed that each molecule can liberate one fatty acid molecule. Solutol® HS-15 consists of polyethoxylated 12-hydroxystearic acid and some free polyethylene glycol (30%).[39] As ethoxylation may occur at both the carboxyl moiety and the hydroxyl moiety of 12-hydroxystearic acid, Solutol® is a mixture of monoesters (molecular weight of 961 g/mol) or diesters (molecular weight of 1244 g/mol). Complete digestion of 0.125 g of Solutol® HS-15 (the amount of surfactant present in the digestion studies) would therefore liberate 9.1 and 14.1 mM of fatty acid if consisting entirely of mono- or diesters, respectively. In the interest of simplicity, it is assumed that Solutol® consists of 70% monoesters of 12-hydroxystearic acid and 30% polyethylene glycol.

$$\text{Extent (\%) of digestion} = \frac{\text{Titrated fatty acid(mmol)} \times 100}{\text{Theoretical maximum titratable fatty acid (mmol)}} \quad (1)$$

Supersaturation

To calculate the supersaturation ratio (SR) after 30 min of digestion of lipid-based formulations containing drug, the concentration of drug in $AP_{DIGESTS}$ after 30 min digestion ($AP_{30\ min}$) was divided by the drug solubility in the $AP_{DIGESTS}$ (determined in section headed 'HPLC Detection of Model Drugs'):

$$SR = \frac{\text{Drug concentration in } AP_{30\ min} (\mu g/mL)}{\text{Drug solubility in } AP_{DIGEST} (\mu g/mL)} \quad (2)$$

Equation (3) was used to calculate the maximum SR (SR^M)[16], which is the ratio between the maximum drug concentration in the $AP_{DIGESTS}$ (AP_{MAX}) in the absence of any drug precipitation (i.e., drug dose divided by test volume) and drug solubility in the AP_{DIGEST}:

$$SR^M = \frac{AP_{MAX} (\mu g/mL)}{\text{Drug solubility in } AP_{DIGEST} (\mu g/mL)} \quad (3)$$

HPLC Detection of Model Drugs

All HPLC analyses were performed using a Waters Alliance system comprising a 2695 Separation Module and model 486 tunable absorbance detector (Waters Alliance Instruments, Milford, Massachusetts). The column used for fenofibrate assays was a Phenomenex® Luna C_{18} column (150 × 4.6 mm, 3 μm; Phenomenex, Torrence, California). The column used for danazol assays was a Waters Symmetry® C_{18} column (150 × 15 mm, 5 μm, Waters Symmetry®) with a C_{18} security guard cartridge (4 × 2.0 mm, Phenomenex). For both drugs, the injection volume was 50 μL. UV detection was at 288 nm for fenofibrate and 286 nm for danazol. The mobile phase consisted of methanol and Milli-Q water in a 75:25 (v/v) ratio and was pumped through the column at a flow rate of 1 mL/min.

Polarized Light Microscopy

A Zeiss Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with crossed polarizing filters was used to analyze selected digestion pellets containing danazol or fenofibrate. Each pellet was carefully removed from the sample tube and placed on a microscope slide. Samples were analyzed under cross-polarized light at 20× magnification, and images were recorded using a Canon PowerShot A70 digital camera (Canon, Tokyo, Japan). Pellets were isolated and analyzed in the manner described above (section headed 'HPLC Detection of Model Drugs') on the same day.

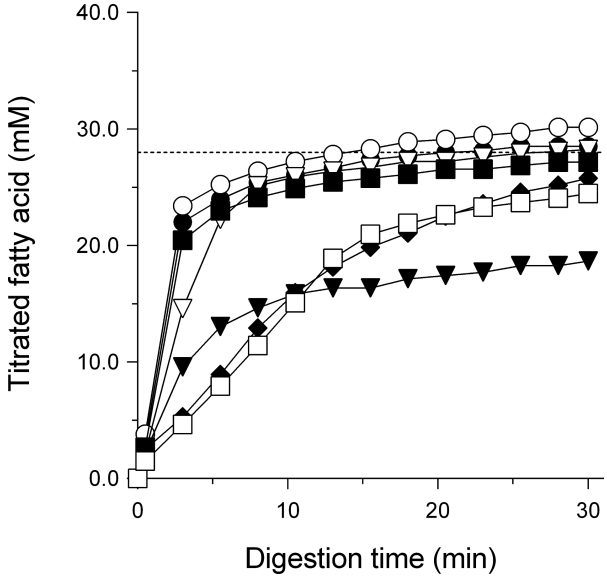

Figure 1. Apparent titration of fatty acids released during *in vitro* digestion of Type IIIA SEDDS, each containing one of seven nonionic surfactants. Formulations were; ▼ $SEDDS_{BR97}$, ▽ $SEDDS_{BR98}$, ■ $SEDDS_{CREL}$, □ $SEDDS_{CRH40}$, ● $SEDDS_{T80}$, ○ $SEDDS_{SOLUTOL}$ ◇ $SEDDS_{TPGS}$. Each SEDDS contained 50% (w/w) lipid (soybean oil–Maisine™ 35-1, 7:3) and 50% surfactant. Digestion was initiated at 0 min on addition of pancreatin, and pH was maintained constant at pH 7.5 during the test. Titrated fatty acid has been corrected for background fatty acid (liberated mainly by digestion of phospholipids) determined in background digestion tests (i.e., digestion medium in the absence of SEDDS). Data for the $SEDDS_{T80}$ have been reproduced from Devraj et al.[17] with permission from Elsevier.

RESULTS

In Vitro Digestion of SEDDS in the Absence of Drug

Profiles of fatty acid titrated during *in vitro* digestion of Type IIIA SEDDS formulated with different nonionic surfactants are shown in Figure 1. Titratable fatty acid corresponds to fatty acid liberated in response to digestion of the formulation components by pancreatic enzymes. $SEDDS_{TPGS}$ and $SEDDS_{CRH40}$ formulations show a near-linear rate of digestion over 25 min, whereas digestion of the other SEDDS was most rapid during the first 5 min of the test. The total concentration of fatty acid titrated varied from 18.7 mM for $SEDDS_{BR97}$ up to 30.1 mM for the $SEDDS_{SOLUTOL}$. Complete digestion of the lipid components in the SEDDS would lead to 28.0 mM of liberated fatty acid (marked by the dashed horizontal line in Fig. 1). Using this value as a measure of complete SEDDS digestion for formulations containing nondigestable Brij surfactants, the calculated extent of digestion of $SEDDS_{BR97}$ and $SEDDS_{BR98}$ (via Eq. (1)) was 66% and 102%, respectively. Similar calculations for the remaining SEDDS are complicated by potential

Table 2. Equilibrium Solubilities of Danazol and Fenofibrate in the Pure Surfactants and the Corresponding SEDDS

	Danazol Solubility[a] (mg/g)		Fenofibrate Solubility[a] (mg/g)	
Surfactant	Pure Surfactant	SEDDS[b]	Pure Surfactant	SEDDS[b]
Brij® 97	34.6 ± 1.1	22.1 ± 0.3	141.7 ± 0.3	100.0 ± 2.5
Brij® 98	34.5 ± 1.4	23.5 ± 0.4	134.3 ± 3.3	105.6 ± 3.0
Cremophor® EL	31.7 ± 0.4	20.7 ± 0.9	113.6 ± 5.8	97.6 ± 4.0
Cremophor® RH40	33.0 ± 1.3	14.2 ± 2.4	117.1 ± 3.3	96.2 ± 3.8
Solutol® HS-15	35.1 ± 2.1	12.2 ± 1.7	124.7 ± 1.5	100.7 ± 4.5
Tween® 80	31.5 ± 2.6[a]	14.2 ± 2.4[c]	102.1 ± 3.3[c]	106.4 ± 8.0[a]
TPGS	30.5 ± 1.4	21.2 ± 0.4	114.8 ± 3.3	101.8 ± 1.2

[a]Solubility was determined at 37°C and values are expressed as means ($n = 3$) ± 1 SD.
[b]SEDDS contained 50% (w/w) lipid (soybean oil–Maisine™ 35-1, 7:3, w/w) and 50% (w/w) of the listed surfactant.
[c]Data for the $SEDDS_{T80}$ have been reproduced from Devraj et al.[17]

surfactant-derived fatty acid, as Cremophor®, Tween®, and Solutol® surfactants all contain ester groups that may be hydrolyzed in *in vitro* digestion tests.[6,8,17,38] The fatty acid titrated in Figure 1 for the equivalent SEDDS is therefore likely to be derived from both lipid and surfactant. By calculating the maximum and minimum mass of available fatty acid (i.e., in the case of either complete digestion or no digestion of the surfactant), the calculated extents of digestion of the remaining SEDDS are (in increasing order of digestion): 58%–87% for $SEDDS_{CRH40}$, 62%–97% for $SEDDS_{CREL}$, 76%–102% for $SEDDS_{T80}$, and 81%–108% for $SEDDS_{SOLUTOL}$. From these values, and from Figure 1, it is evident that the $SEDDS_{CRH40}$ was digested to a lesser extent and more slowly when compared with the equivalent $SEDDS_{CREL}$, which is consistent with the previous work.[6] However, by the end of the test, differences in the extent of digestion among all SEDDS, with the exception of $SEDDS_{BR97}$, were modest. Polyethylene glycol–succinic acid esters in TPGS have been shown to be resistant to hydrolysis by pancreatic enzymes.[38] Similarly to the SEDDS containing the Brij® surfactants, which contain only nondigestible ethers, titrated fatty acids from $SEDDS_{TPGS}$ are likely to be derived only from the mixed glycerides.

Drug Solubility in the Anhydrous Surfactants and SEDDS

Danazol and fenofibrate equilibrium solubilities in the seven surfactants investigated and the corresponding SEDDS are shown in Table 2. Danazol solubility in the pure surfactants was higher (1.4–2.9-fold) than in the respective SEDDS, reflecting its higher solubility in hydrophilic surfactants over long-chain lipids.[40] The highest danazol solubility was determined in the SEDDS containing the Brij® surfactants (22.1 and 23.5 mg/g for $SEDDS_{BR97}$ and $SEDDS_{BR98}$, respectively) and the lowest solubility value was 12.2 mg/g in $SEDDS_{SOLUTOL}$.

Fenofibrate solubility was highest in the $SEDDS_{T80}$ and lowest in the $SEDDS_{CRH40}$. In contrast to the corresponding data for danazol the narrow range of fenofibrate solubility in the SEDDS indicate that its solubility was much less sensitive to the chemistry of the surfactant. The log P values for danazol (log P 4.5[41]) and fenofibrate (log P 5.2[42]) are both high, yet fenofibrate exhibited much higher (5–10-fold) solubility in each lipid formulation.

In Vitro Digestion of SEDDS Containing Drugs at 80% Saturation

Danazol or fenofibrate was incorporated into each of the seven SEDDS at a load equivalent to 80% of the equilibrium solubility values shown in Table 2. These formulations were used to investigate the impact of 30 min digestion on the fate of the drug in each case.

Drug Distribution Following Digestion

Figure 2 shows the effect of *in vitro* digestion of SEDDS on the fate of incorporated danazol (Fig. 2a) or fenofibrate (Fig. 2b). The results are presented as the percentage of the dose recovered from each phase produced by digestion, namely, an oil phase consisting of any undigested triglyceride and lipophilic digestion products; the colloidal AP_{DIGEST} consisting of the majority of the amphiphilic digestion products (but not undigested oil droplets), bile salt and phospholipid; and the pellet phase consisting of insoluble calcium soaps of fatty acid and any precipitated drug.

For both danazol and fenofibrate, digestion samples from $SEDDS_{BR97}$ contained the largest volume of oil phase, which is consistent with the lower extent of digestion of this particular formulation (Fig. 1). Because of better digestibility, the digestion samples from all other SEDDS contained little or no oil phase. Following digestion of the SEDDS, the majority (>90%) of danazol was solubilized in the AP_{DIGEST} (Fig. 2a). This outcome, which represents good, or desirable performance *in vitro* (i.e., a low precipitation tendency), is in agreement with the recent work by Williams et al.[40,43] on formulation of danazol in Type IIIA SEDDS containing long-chain lipids.

The results of identical experiments carried out using fenofibrate (Fig. 2b) however reveal that 55%–88% of the fenofibrate dose was recovered from the pellet phase, indicating that the drug had precipitated extensively during the digestion experiment. Precipitation was lowest in the cases of $SEDDS_{BR97}$ and $SEDDS_{TPGS}$ (though it still amounted to >50%); this was concurrent with the presence of an oil phase after digestion, and was consistent with previous work.[17]

Supersaturation

To determine whether the differences in performance between danazol- and fenofibrate-containing SEDDS shown in Figure 2 could be explained by the degree of supersaturation produced during digestion, the drug solubility in drug-free $AP_{DIGESTS}$ (obtained by digestion of drug-free SEDDS) was determined, and SR and SR^M values were calculated according to Eqs. (2) and (3), respectively. Results are presented in Table 3 for danazol and in Table 4 for fenofibrate.

Danazol solubility values in respective $AP_{DIGESTS}$ (Table 3) are within a 125–202 μg/mL range, lowest in the case of $SEDDS_{T80}$ and highest in the case of the $SEDDS_{TPGS}$. As

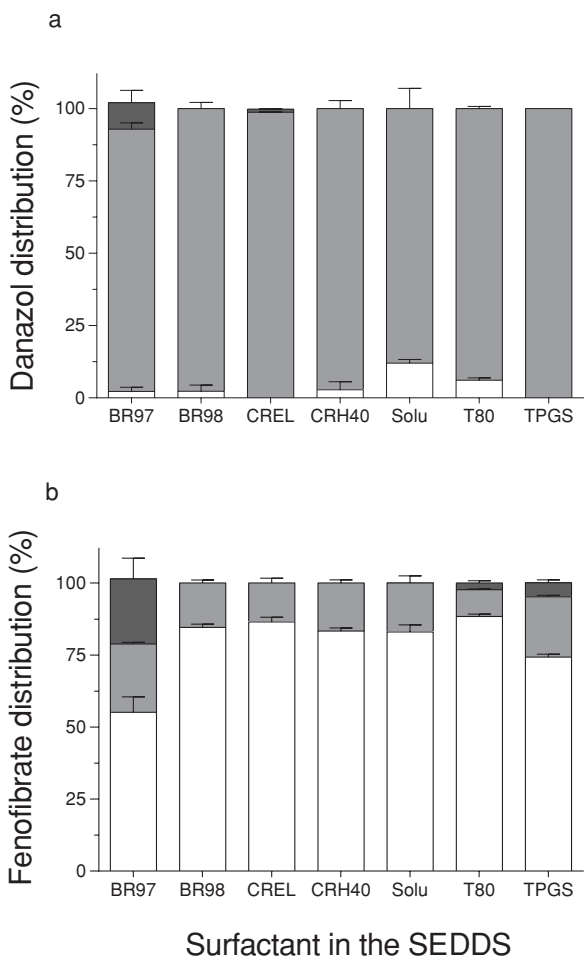

Figure 2. The effect of *in vitro* digestion on the fate of danazol (a) and fenofibrate (b) in SEDDS containing various nonionic surfactants. Surfactants were: Brij® 97 (BR97), Brij® 98 (BR98), Cremophor® EL (CREL), Cremophor® RH40 (CRH40), Solutol® HS-15 (Solu), Tween® 80 (T80), and TPGS. The stacked box-plots show the percentage of total drug distribution within a poorly dispersing oil phase (dark-shaded bars), colloidal aqueous phase, AP_{DIGEST} (light-shaded bars), and pellet phase (white bars). Values are expressed as means ($n = 3$) ± SD. In all cases, each SEDDS contained a drug load that was equivalent to 80% of its equilibrium solubility in the anhydrous formulation. The compositions of the formulations can be found in Table 1. Data for the $SEDDS_{T80}$ containing fenofibrate have been reproduced from Devraj et al.[17] with permission from Elsevier.

danazol concentrations in the $AP_{DIGESTS}$ following digestion of equivalent drug-containing SEDDS ($AP_{30\ min}$) are in excess of these solubility values, it was apparent that digestion led to supersaturation. SR values, however, are modest, ranging from 1.3 ($SEDDS_{SOLUTOL}$) to 2.3 ($SEDDS_{CRRH40}$). The higher SR values in this range are a reflection of a lower solubility in the AP_{DIGEST} (e.g., $SEDDS_{CRH40}$) and/or a slightly higher absolute drug loading because of a higher solubility in the anhydrous formulation (e.g., $SEDDS_{CREL}$). SR^M, the ratio between the maximum danazol concentration in the AP_{DIGEST} in the absence of drug precipitation (AP_{MAX}) and drug solubility in the AP_{DIGEST} captures the maximal driving force of drug precipitation during *in vitro* digestion.[13,16,17] As danazol showed a low propensity to precipitate, values for SR^M are similar to respective SR values (Table 3), slight differences being because of either a small amount of precipitation (e.g., $SEDDS_{SOLUTOL}$), the collection of drug in some cases in a phase-separated oil phase (e.g., $SEDDS_{BR97}$), or some incomplete recovery of drug from the AP_{DIGEST} (although total drug recoveries in the case of danazol was typically >90%).

Incontrast to danazol, SR^M values for fenofibrate were considerably higher (Table 4), ranging from 5.0 ($SEDDS_{SOLUTOL}$) up to 9.3 ($SEDDS_{T80}$). This marked difference in SR^M values between the two drugs can be explained by the higher (between five and10-fold) solubility of fenofibrate in each anhydrous SEDDS, giving an equivalent increase in AP_{MAX} (as all of the SEDDS-contained drug at 80% of its respective solubility in each SEDDS), relative to the much smaller (two-fold) difference in fenofibrate solubility values in the $AP_{DIGESTS}$.

As the majority of the incorporated fenofibrate precipitated during the digestion experiments (Fig. 2b), SR values after 30 min digestion were already well below SR^M. In many cases, SR was below or close to unity, indicating that the extent of this precipitation was such that any supersaturation was effectively removed by precipitation within 30 min. The fact that many SR values are less than 1 may reflect the nonequilibrium conditions generated during dispersion. SR values would be expected to return to unity at equilibrium.

Insummary, the supersaturation values in Tables 3 and 4 together with the results in Figure 2 taken together indicate that digestion of the SEDDS led to supersaturation of both danazol and fenofibrate, but the higher SR^M values for fenofibrate formulations ($SR^M > 5.0$) were sufficiently high to promote drug precipitation.

Table 3. Supersaturation Ratios and SR^M for Danazol (at 80% saturation), Resulting from *In Vitro* Digestion of SEDDS Containing Various Nonionic Surfactants

	danazol concentration (μg/ml)				
Formulation	AP_{DIGEST}[a]	$AP_{30\ min}$[b]	AP_{MAX}[c]	SR[d]	SR^M[e]
$SEDDS_{BR97}$	184 ± 0.3	307.0 ± 33.7	442	1.7	2.4
$SEDDS_{BR98}$	187 ± 0.3	311.4 ± 21.2	470	1.6	2.5
$SEDDS_{CREL}$	131 ± 2.7	281.6 ± 46.5	414	2.1	3.1
$SEDDS_{CRH40}$	130 ± 1.5	298.3 ± 35.5	270	2.3	2.1
$SEDDS_{SOLUTOL}$	135 ± 1.3	182.7 ± 6.5	244	1.3	1.8
$SEDDS_{T80}$	125 ± 7.5[a]	233.8 ± 5.5[a]	284[f]	1.8[f]	2.3[f]
$SEDDS_{TPGS}$	202 ± 2.3	286.8 ± 15.5	424	1.4	2.1

[a] Drug solubility in the AP_{DIGEST} obtained following 30 min *in vitro* digestion of drug-free SEDDS.

[b] Measured drug concentration in the AP_{DIGEST} following 30 min digestion of the drug-containing SEDDS.

[c] Maximum theoretical concentration (i.e., in the absence of drug precipitation) attained in the AP_{DIGEST} during digestion, and is calculated using drug load in the formulation divided by the volume of the test.

[d] Ratio of drug in $AP_{30\ min}$ to the drug solubility in AP_{DIGEST} (see Eq.(2)). Values shown in the table correspond to those obtained using a 80% saturation level in the formulation.

[e] Ratio of AP_{MAX} to drug solubility in AP_{DIGEST} (see Eq. (3)).

[f] This data point is taken from a published study, Devraj et al.[17]

SEDDS contained 50% [w/w, lipid (soybean oil–Maisine™ 35-1, 7:3)] and 50% (w/w) of the listed surfactant.

Danazol solubility in the AP_{DIGEST}, relative to measured ($AP_{30\ min}$) and maximum danazol concentrations (AP_{MAX}) in AP_{DIGEST} allow calculation of SR and SR^M, respectively.

Table 4. Supersaturation Ratios and SR^M for Fenofibrate (at 80% saturation), Resulting from *In Vitro* Digestion of SEDDS Containing Various Nonionic Surfactants

	fenofibrate concentration (μg/ml)				
Formulation	AP_{DIGEST}[a]	$AP_{30\ min}$[b]	AP_{MAX}[c]	SR[d]	SR^M[e]
$SEDDS_{BR97}$	330 ± 3.0	346.6 ± 10.5	2000	1.1	6.1
$SEDDS_{BR98}$	300 ± 8.2	221.7 ± 17.1	2011	0.7	6.7
$SEDDS_{CREL}$	371.1 ± 5.7	212.4 ± 21.7	1952	0.6	5.3
$SEDDS_{CRH40}$	342.8 ± 9.8	224.1 ± 21.4	1924	0.6	5.6
$SEDDS_{SOLUTOL}$	400 ± 9.0	245.2 ± 28.8	2014	0.6	5.0
$SEDDS_{T80}$	230.0 ± 3.4	180.8 ± 11.0	2128	0.8	9.3
$SEDDS_{TPGS}$	370 ± 1.2	321.3 ± 19.4	2036	0.9	5.5

Details of footnotes [a–e] are provided in the footnotes of Table 3.

Fenofibrate solubility in the AP_{DIGEST}, relative to measured ($AP_{30\ min}$) and maximum fenofibrate concentrations (AP_{MAX}) in AP_{DIGEST} allow calculation of SR and SR^M, respectively.

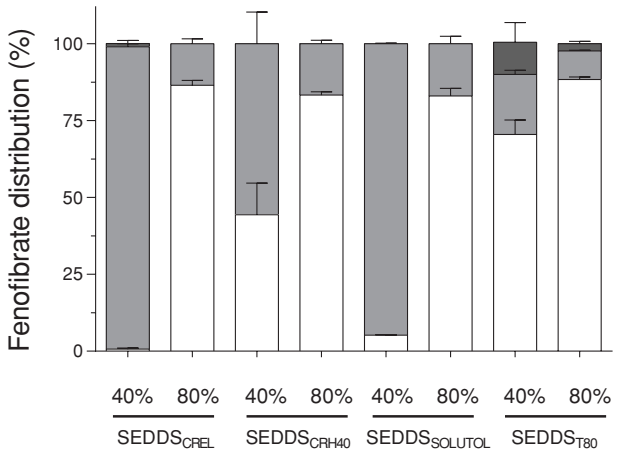

Figure 3. The effect of SR^M produced by each SEDDS on the fate of fenofibrate following 30 min *in vitro* digestion. Fenofibrate loading is expressed as the SR^M values generated using 40% or 80% of the equilibrium solubility in the anhydrous SEDDS, each containing one of four surfactants. The results for 80% loading, now expressed in terms of SR^M are duplicated from Figure 2. SR^M values at 40% and 80% saturation were: 2.6 and 5.3 ($SEDDS_{CREL}$), 2.8 and 5.6 ($SEDDS_{CRH40}$), 2.4 and 4.8 ($SEDDS_{SOLUTOL}$), and 4.7 and 9.3 ($SEDDS_{T80}$), respectively. The legend for Figure 2 contains details of the layout of this figure. Values are expressed as means ($n = 3$) ± SD.

In Vitro Digestion of SEDDS Containing Various Fenofibrate Loadings

To explore the link between SR^M and the fate of drug during *in vitro* digestion of SEDDS, fenofibrate loading in selected SEDDS was reduced by half from 80% to 40% saturation. In accordance with Eq. (3), this also decreases SR^M by half, and therefore, reduces the driving force of precipitation. The results are presented in Figure 3, which shows the data obtained at 80% and 40% saturation to allow comparison of the effect of fenofibrate load (and SR^M) on SEDDS performance. The SEDDS were selected to provide the widest possible range of SR^M values, ranging from 2.5 for $SEDDS_{SOLUTOL}$ up to 4.7 for $SEDDS_{T80}$ when the drug load was equivalent to 40% saturation.

The results show that lowering fenofibrate loading led to a reduction in drug precipitation during digestion (calculated as % of dose). For $SEDDS_{CREL}$ and $SEDDS_{SOLUTOL}$, the reduction in precipitation effectively represented a reversal in the quality of performance of the formulation, such that the majority (>90%) of fenofibrate at 40% saturation was solubilized within the AP_{DIGEST}. Fenofibrate $AP_{30\ min}$ values were 969 ± 56 and 606 ± 40 μg/mL for $SEDDS_{CREL}$ and $SEDDS_{SOLUTOL}$, respectively, more than two-fold higher than the respective $AP_{30\ min}$ values determined at the higher drug loading of 80% saturation (shown in Table 4). By decreasing SR^M, and therefore, the propensity for precipitation, higher solubilized drug concentrations and more sustained supersaturation was attained (at 30 min; SR was 2.6 for $SEDDS_{CREL}$ and 1.5 for $SEDDS_{SOLUTOL}$).

Reducing the fenofibrate loading in $SEDDS_{CRH40}$ and $SEDDS_{T80}$ also reduced the percent drug precipitation, though the change in performance in these cases was more modest, with more than 40% drug precipitation evident in both cases (Fig. 3). Values for SR at 30 min were SR 1.3 for $SEDDS_{CRH40}$ and 0.8 for $SEDDS_{T80}$, indicating that this precipitation practically removed all supersaturation. This observation that the change in performance with $SEDDS_{CRH40}$ and $SEDDS_{T80}$ was less pronounced can be attributed to the fact that a smaller amount of drug precipitation is required to remove supersaturation.

For the SEDDS that showed little evidence of drug precipitation, SR^M values at 40% saturation were 2.4 and 2.6 for $SEDDS_{SOLUTOL}$ and $SEDDS_{CREL}$, respectively. The corresponding values were 2.8 and 4.3 for $SEDDS_{CRH40}$ and $SEDDS_{T80}$, both of which showed evidence of drug precipitation (Fig. 3). These observations suggest that lower SR^M values (≤2.6) were associated with superior SEDDS performance. This provided an estimate of an apparent threshold SR^M, above which precipitation tended to occur, though this estimate was based on only half of the SEDDS used in the study. To probe the validity of the apparent threshold using a wider group of formulations, the fenofibrate loading in all SEDDS was adjusted so that a target SR^M of 2.6 would be attained. This SR^M value was selected for further study based on the results in Figure 3, which shows that the highest SR^M value that could be tolerated before significant drug precipitation occurred was 2.6 (i.e., $SEDDS_{CREL}$). The effect of standardizing SR^M in all SEDDS on fenofibrate fate during digestion is shown in Figure 4 (which includes the $SEDDS_{CREL}$ results reproduced from Fig. 3). The absolute fenofibrate loadings for each SEDDS are shown in the figure legend. Because of differences in fenofibrate solubility in the AP_{DIGEST} (i.e., the values in Table 3), the drug loading in respective SEDDS were varied to achieve the constant SR^M. Absolute drug loadings ranged from 23.9 ($SEDDS_{T80}$) up to 41.6 mg/g ($SEDDS_{SOLUTOL}$). With the exception of $SEDDS_{SOLUTOL}$ and $SEDDS_{CRH40}$, which showed evidence of significant drug precipitation, the different SEDDS maintained the majority of fenofibrate in a solubilized form, with little or no evidence of precipitation. In summary, standardizing the fenofibrate load in SEDDS to achieve a target SR^M of 2.6 led to formulations that were able to maintain drug in a supersaturated state in five of the seven formulations.

Figure 5 compares performance of $SEDDS_{CRH40}$ (Fig. 5a), $SEDDS_{SOLUTOL}$ (Fig. 5b), and $SEDDS_{T80}$ (Fig. 5c) designed to generate a range of SR^M during digestion of the formulations. For each SEDDS, an increase in SR^M results from an increase in fenofibrate load, the details of which are shown in Figure 5 to aid the interpretation of the results. Performance of $SEDDS_{SOLUTOL}$ and $SEDDS_{T80}$ were compared because

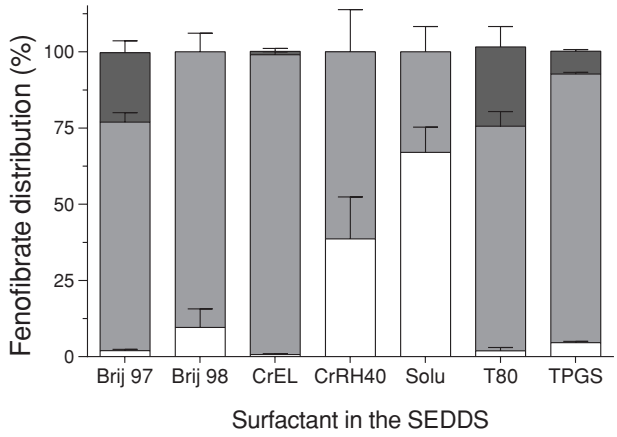

Figure 4. The performance of the seven SEDDS containing a fenofibrate load each of which generated a target SR^M of 2.6. The absolute fenofibrate loadings at this SR^M value were: 30.4 ($SEDDS_{BRIJ97}$), 31.2 ($SEDDS_{BRIJ98}$), 38.6 ($SEDDS_{CREL}$), 35.7 ($SEDDS_{CRH40}$), 41.6 mg/g ($SEDDS_{SOLUTOL}$), 23.9 ($SEDDS_{T80}$), and 38.5 mg/g ($SEDDS_{TPGS}$). The legend for Figure 2 contains details of the layout of this figure. Values are expressed as means ($n = 3$) ± SD.

fenofibrate showed the highest and lowest solubilities in the respective $AP_{DIGESTS}$ of these formulations (400 μg/mL for $SEDDS_{SOLUTOL}$ and 230 μg/mL for $SEDDS_{T80}$), thereby capturing the widest range in absolute loadings required to achieve a particular SR^M. For example, at SR^M 2.6, the fenofibrate load was 41.6 mg/g for of $SEDDS_{SOLUTOL}$ but only 23.9 mg/g for of $SEDDS_{T80}$. Fenofibrate solubility in the AP_{DIGEST} from $SEDDS_{CRH40}$ was intermediate to that of $SEDDS_{SOLUTOL}$ and of $SEDDS_{T80}$.

At 80% saturation, SR^M values for fenofibrate in each of the formulations were 5.0 for $SEDDS_{SOLUTOL}$, 5.6 for $SEDDS_{CRH40}$, and 9.3 for $SEDDS_{T80}$. The increase in SR^M across these formulations reflects the respective decrease in fenofibrate solubility in their $AP_{DIGESTS}$. $SEDDS_{CRH40}$ maintained the majority of fenofibrate in a solubilized but supersaturated state at SR^M 1.7, but there was evidence of increased drug precipitation at $SR^M \geq 2.6$ (Fig. 5a). The SR^M threshold for $SEDDS_{SOLUTOL}$ was comparable at more than 2.5, above which there was significant drug precipitation (Fig. 5b). In contrast, a higher SR^M threshold of more than 3.5 was required for precipitation from $SEDDS_{T80}$ (Fig. 5c).

The highest absolute fenofibrate loading in $SEDDS_{T80}$ that resisted precipitation was 32.2 mg/g (SR^M 3.5), whereas $SEDDS_{SOLUTOL}$ was able to support a slightly higher loading of 40 mg/g (at SR^M 2.5) but not 41.6 mg/g (at SR^M 2.6). Therefore, the higher threshold SR^M value of 3.5 required to promote precipitation from $SEDDS_{T80}$ was not consistent with increased drug load. This is better illustrated in Figure 6, which summarizes the performance of all seven SEDDS with respect to absolute mass of fenofibrate in the formulation. Performance in Figure 6 is compared by plotting the mass of fenofibrate that remains in solution (i.e., drug in the AP_{DIGEST} plus oil phase) following 30 min digestion against the dose. The dashed line denotes a 1:1 relationship between fenofibrate in the formulation and fenofibrate in solution, that is, those points that lie on this dotted line represent the absence of precipitation on digestion. Between approximately 20 and 35 mg/g fenofibrate loadings, all of the SEDDS lie on the dotted line. However, there was

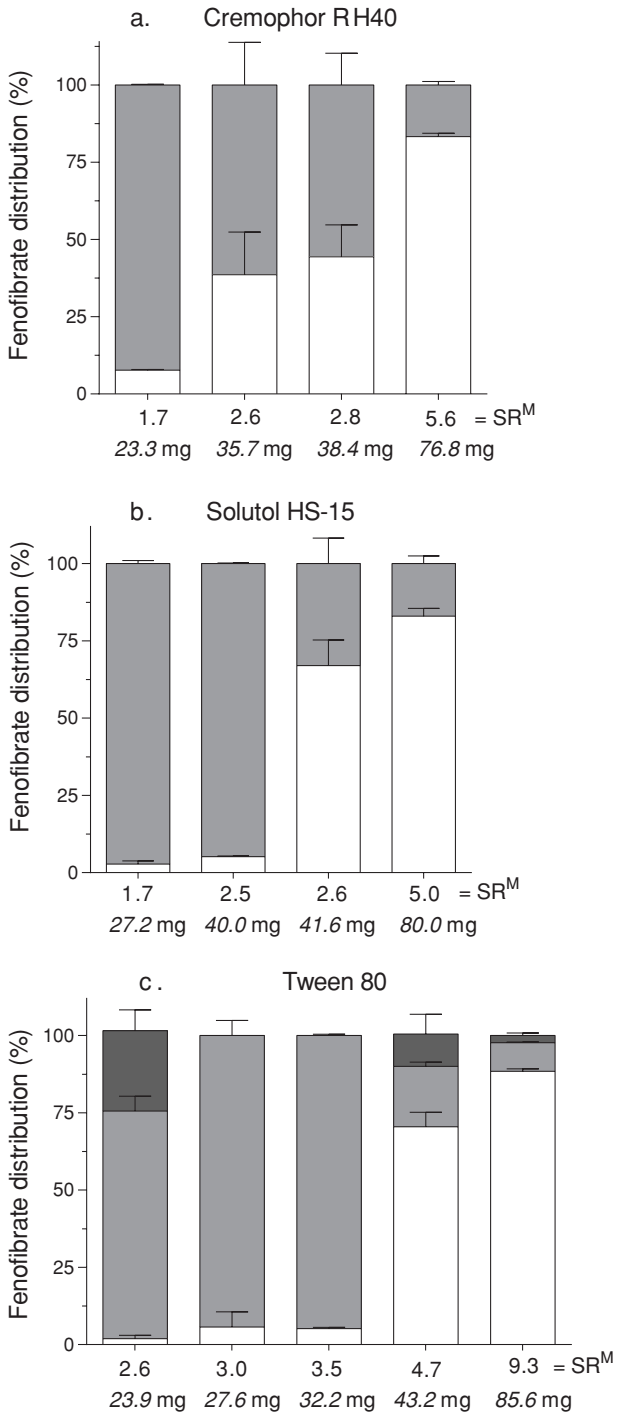

Figure 5. Fenofibrate distribution across the various digestion phases following 30 min *in vitro* digestion of $SEDDS_{CRH40}$ (a), $SEDDS_{SOLUTOL}$ (b), and $SEDDS_{T80}$ (c) with respect to SR^M (and absolute drug load). To allow comparison, certain results are duplicated from Figures 3 and 4. The legend for Figure 2 contains details of the layout of this figure. Values are expressed as means ($n = 3$) ± SD.

considerable variability in performance for SEDDS containing between approximately 35 and 40 mg/g fenofibrate, with some SEDDS exhibiting extensive precipitation (e.g., $SEDDS_{CRH40}$) and others showing no such precipitation (e.g., $SEDDS_{SOLUTOL}$). This variability in SEDDS performance coincided with the

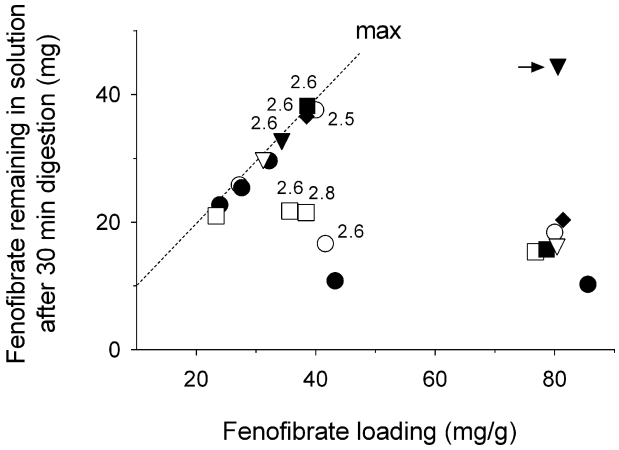

Figure 6. Mass of fenofibrate remaining in solution following 30 min *in vitro* digestion of the SEDDS plotted against absolute fenofibrate loading (mg/g) in the formulation. Symbols represent: ▼, $SEDDS_{BR97}$; ▽, $SEDDS_{BR98}$; ■, $SEDDS_{CREL}$; □, $SEDDS_{CRH40}$; •, $SEDDS_{T80}$; ○, $SEDDS_{SOLUTOL}$; and ◇, $SEDDS_{TPGS}$. Values plotted on the *y*-axis are derived from the dissolved drug concentration at 30 min multiplied by test volume and normalized to 1g of SEDDS. The dashed line represents the maximum amount of drug in solution in the absence of precipitation. Selected SR^M values are also shown. The arrow refers to $SEDDS_{BR97}$, which was partially digested and formed a large oil phase (see text).

generation of SR^M values that were approximately 2.5/2.6, or more than 3.5 in the case of $SEDDS_{T80}$. Further increases in fenofibrate loading produced higher values of SR^M, greater propensity for precipitation, and therefore, reduced variability between formulations. $SEDDS_{BR97}$, which contained fenofibrate at 80% saturation (see arrow in Fig. 6) was the only exception at this high drug loading. The lower amount of precipitation in this case can be attributed to the reduced digestibility of this formulation (and presence of an oil phase). Figure 6 shows how increasing drug loading can be detrimental to the performance of the formulation, and how more sustained supersaturation is possible at lower drug loadings.

Microscopic Analysis of the Digestion Pellet Viewed Under Cross-Polarized Light

Figure 7 shows micrographs of the pellet formed following the digestion of $SEDDS_{T80}$ containing either (a) danazol or (b) fenofibrate. Pellets were viewed under cross-polarized light. The pellets formed from the danazol-containing $SEDDS_{T80}$ showed evidence of birefringence at 5 and 30 min digestion. Birefringent patterns were similar at the two time points, and because there was negligible precipitation of danazol in this case (see Fig. 2a), the birefringence most likely originated from lamellar structures formed by calcium soaps of the fatty acids.[44]

The pellet formed from the fenofibrate-containing $SEDDS_{T80}$ showed a similar birefringent pattern at 5 min; however, the pellet after 30 min digestion was populated by imperfect rod-like crystal particles, indicating that the fenofibrate precipitate was crystalline. Fenofibrate is known to exist in two polymorphic forms.[45] As the starting fenofibrate material consisted primarily of plate-like crystals (not shown), it is possible

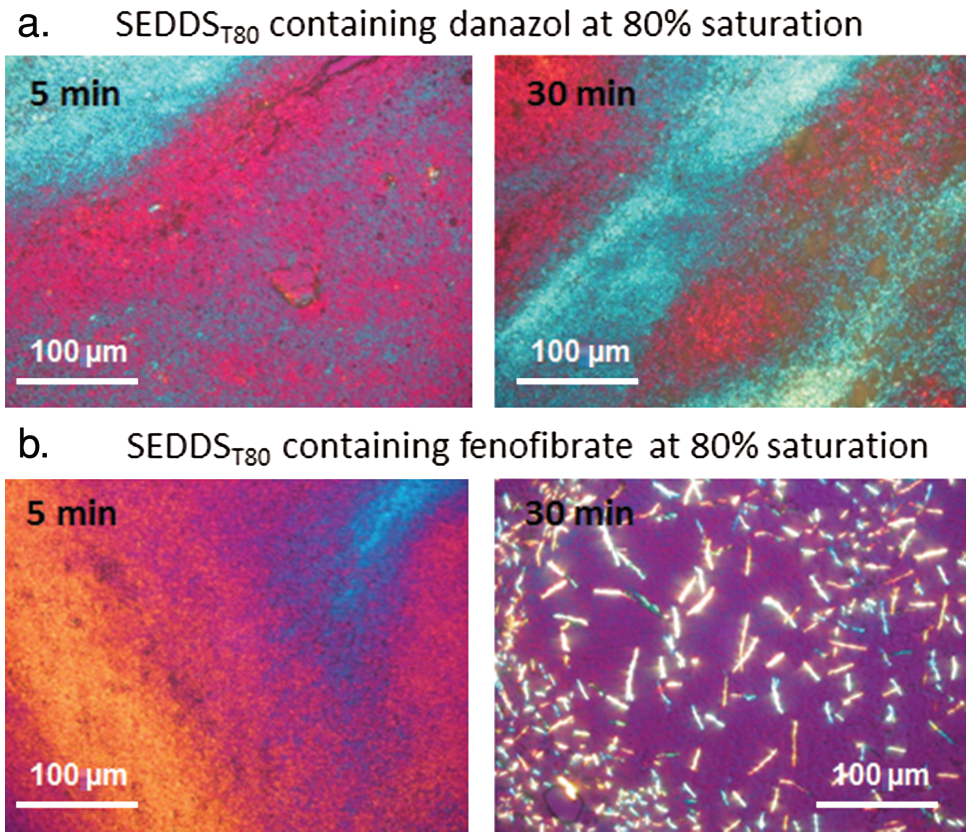

Figure 7. Micrographs of the pellet formed following digestion (5 or 30 min) of the $SEDDS_{T80}$. The formulation contained either (a) danazol or (b) fenofibrate at the 80% saturation level. Images were captured under cross-polarized light.

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that the fenofibrate precipitate in the digestion experiments consisted of the metastable polymorph.

DISCUSSION

Self-emulsifying drug delivery systems consisting of oil(s) and hydrophilic components such as surfactants(s) and cosolvent(s) often exhibit reduced solubilization of drugs following dispersion in aqueous fluids or following digestion of the lipid and/or surfactant.[2,11,13,15,16] *In vitro* dispersion and digestion tests, which determine whether any loss of solubilization is sufficient to promote drug precipitation, are therefore useful to formulators for prediction of SEDDS performance. The emergence of a slowly dissolving precipitate *in vitro* is often predictive of decreased drug absorption *in vivo*.[13,46–49]

In our previous study,[17] a SEDDS of Type IIIA (according to the LFCS[15,18]), containing the drug fenofibrate, was shown to resist precipitation during *in vitro* dispersion testing, but showed a marked decrease in solubilization capacity on digestion, which in turn resulted in extensive crystallization of the drug. The aim of the present study was to better understand the factors that determine the performance of SEDDS during digestion and to inform formulators of SEDDS about the critical parameters that determine the fate of incorporated drug. The investigation involved seven different SEDDS, differing only in the identity of the nonionic surfactant used. Incorporation of danazol or fenofibrate revealed that the fate of the drug during digestion was predominantly governed by the maximum degree of supersaturation generated (SR^M), a parameter that describes the supersaturation pressure that occurs in the system.[16] SR^M and other closely related parameters have previously been used to explain the risk of precipitation following digestion of range of different danazol-containing lipid formulations.[16,17] In the present study, the utility of SR^M to identify the threshold above which the performance of lipid-based formulations becomes more variable is further highlighted.

Self-emulsifying drug delivery systems-containing danazol at loadings equivalent to 80% of the respective equilibrium solubility in the formulations (denoted the "80% saturation level") showed no evidence of drug precipitation during 30 min digestion (Fig. 2). Determination of danazol solubility in digested drug-free SEDDS, revealed that SR^M values generated by digestion of danazol formulations were less than $\leq$3.1. This finding was in general agreement with our previous work.[13,16] In the latter studies when SR^M values were approximately more than 2.5, we observed precipitation during digestion of a range of lipid formulations containing danazol. Further insights into the relationship between SR^M and the performance of SEDDS-containing danazol were limited in the present study by the low solubility of danazol in anhydrous formulations. The addition of a cosolvent such as ethanol to the SEDDS is a common approach to increase drug solubility in the formulation.[1,3] Cosolvents however contribute little to solubilization after dispersion or after digestion of the SEDDS and therefore often result in increased SR^M and promote danazol precipitation.[50]

In contrast, fenofibrate solubility in the anhydrous SEDDS was very high (>95 mg/g), more than fivefold higher than the respective danazol solubility. Fenofibrate solubility in the digested formulations (i.e., the AP_{DIGEST}), was on average only twofold higher; and because of this disproportional change in solubility in the anhydrous and digested formulation, SR^M values produced on digestion of SEDDS incorporated with fenofibrate at 80% saturation were more than 5.0 compared with 3.1 or less for the equivalent danazol-containing SEDDS. These higher SR^M values and the attendant increase in precipitation pressure explain the extensive drug crystallization during digestion of all seven fenofibrate-containing SEDDS (Figs. 2b and 7b).

In an attempt to better discriminate between the SEDDS formulations, the fenofibrate loading was lowered in an effort to decrease the precipitation pressure in the digestion test. The fenofibrate load was initially decreased by half to 40% saturation (thereby also halving SR^M), and this approach led to a clear reduction in precipitation from $SEDDS_{CREL}$ and $SEDDS_{SOLUTOL}$, but not $SEDDS_{CRH40}$ or $SEDDS_{T80}$. It was proposed that, at 40% fenofibrate saturation, the higher SR^M values produced on digestion of $SEDDS_{CRH40}$ and $SEDDS_{T80}$ explained this difference in performance. Therefore, to normalize the effects of digestion on each formulation, the fenofibrate load was adjusted to achieve a SR^M of 2.6 (Fig. 4), which represented the highest SR^M that did not result in precipitation at 40% saturation (Fig. 3). Of the seven SEDDS investigated, five maintained practically the entire mass of drug in a solubilized (and supersaturated) state during the digestion test. Further tests to probe the performance of SEDDS at specific fenofibrate loadings (Fig. 5) revealed that, with exception of $SEDDS_{T80}$, all SEDDS showed evidence of significant drug precipitation above a threshold SR^M of 2.5–2.6. The performance of SEDDS-containing fenofibrate and a diverse range of surfactants could be explained by a single parameter, namely, SR^M. The possibility that there exists a threshold SR, above which supersaturation cannot be maintained during the digestion tests, correlates with previous studies that evaluated danazol supersaturation resulting from digestion of SEDDS formulations containing medium-chain lipids[13,16] and lipid-free cosolvent/surfactant formulations.[16] The latter studies identified a threshold SR^M value for danazol of approximately 2.5, which is very close to the threshold SR^M value for fenofibrate identified in the present study. Thus, the concept of a threshold SR^M value, which predicts the point at which drug precipitation becomes more prevalent *in vitro*, has now been shown to be applicable to a range of lipid formulations and two quite different drugs, fenofibrate being much more lipophilic than danazol. In practice, the precise threshold value will be formulation specific, and we do not wish to suggest that there is a threshold SR^M that is common to all formulations of all drugs. Nevertheless, this and recent studies[13,16] suggest that formulators should be wary if their formulations generate $SR^M > 3$. For fenofibrate, in this study only, the Tween 80 formulation tolerated $SR^M >$ 3, and we attribute that to the lower solvent capacity of this formulation for fenofibrate.

The observation that the threshold SR^M value may be largely formulation and drug independent suggests that the capacity of lipid formulations to support drug for the duration of the digestion test (30 min in this study) may be explained by classical nucleation theory. The rate of nucleation (J) is defined in terms of free energy change (ΔG^*) associated with the formation of a spherical nucleus of critical size:

$$J = K_0 \exp\left(\frac{-\Delta G^*}{k_B T}\right) \quad (4)$$

where T is temperature, k_B is the Boltzmann constant, and K_0 is a kinetic coefficient. ΔG^* is the thermodynamic barrier to nucleation, below which small nuclei form but rapidly decompose. ΔG^* is commonly defined as:

$$\Delta G^* = \frac{16\pi\gamma^3(V_M/N_A)}{3(k_B T \ln S)^2} \quad (5)$$

where V_M is the molar volume of the solute, N_A is Avogadro's number, S is the degree of supersaturation, and γ is the interfacial energy between the emerging new surface (i.e., the nuclei) and the bulk solution.[51,52] This equation implies that increasing supersaturation leads to an exponential decrease in ΔG^*, and in accordance with Eq. (4), an exponential increase in nucleation rate (J).[53]

The degree of supersaturation at which $\Delta G^*$is practically zero and nucleation occurs spontaneously has been termed the "critical supersaturation."[54] When a system is below the critical supersaturation, the higher ΔG^* results in a slower rate of nucleation and enhanced metastability, such that periods of supersaturation are prolonged.[54,55] It is possible that the threshold supersaturation levels identified in the present studies and in previous work,[13,16] which appear to predict the fate of supersaturated drug during the digestion of a lipid formulation, may mark the critical point at which the barrier to nucleation becomes negligible, allowing a drug to crystallize.

The performance of a lipid formulation is expected to be most variable at or near the critical supersaturation, as only minor changes in drug loading and/or drug solubility in the digested formulation will determine whether a system is above (i.e., showing precipitation) or below (i.e., showing no precipitation) the critical point. Such variability was evident in the present study and is clearly illustrated in Figure 6 in approximately 35–40 mg/g fenofibrate loading range. SR^M values within this range of fenofibrate load vary from 2.5 to just 2.8, yet performance varied from no precipitation (e.g., $SEDDS_{CREL}$) to considerable precipitation (e.g., $SEDDS_{CRH40}$). The impact of using different surfactants on the performance of SEDDS was therefore most pronounced close to this threshold. Polymer precipitation inhibitors that slow the rate of nucleation or crystal growth may also be most effective in these instances, and may increase maximum degree of supersaturation that may be maintained.[13,56,57]

Of the seven SEDDS investigated, $SEDDS_{T80}$ was the only formulation that was able to support supersaturation above SR^M 2.6 (i.e., the threshold SR^M value for all other formulations). Although this may suggest that Tween® 80 possesses a greater capacity to support supersaturation than the other surfactants investigated, this scenario was considered unlikely as sorbitan fatty acid monoesters in Tween® 80 are readily hydrolyzed by pancreatic enzymes (such as carboxyl ester hydrolase) when investigated using *in vitro* digestion models.[6,17,19,38] Alternatively, the capacity for $SEDDS_{T80}$ to support higher SR^M may be explained by the lower solvent capacity of this formulation. The concentration of fenofibrate required to generate $SR^M = 2.6$ in $SEDDS_{T80}$ was only 23.9 mg/g, whereas the other formulations contained between 30.4 and 38.5 mg/g. The fenofibrate concentrations at each particular SR^M were lower, which would be expected to reduce the collision frequency and nucleation rate. Figure 8 illustrates how differences in solubilization capacity can lead to marked differences in concentration at equal degrees of supersaturation; SEDDS that exhibit the lowest and highest solubilization capacity after digestion,

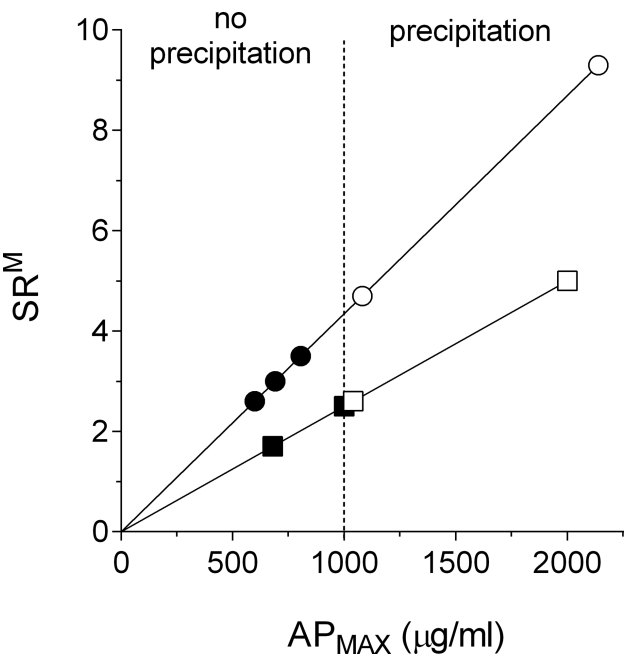

Figure 8. Linear plots of SR^M versus theoretical AP_{MAX} of fenofibrate after digestion of $SEDDS_{SOLUTOL}$ (squares) and $SEDDS_{T80}$ (circles). The theoretical AP_{MAX} is the concentration in the aqueous phase that would occur if no precipitation occurred. The vertical dotted line crosses the x-axis at a fenofibrate concentration of 1000 μg/mL. Open symbols to the right of the dotted line are indicative of incidences of extensive drug precipitation (see Figs. 5b and 5c).

namely, $SEDDS_{T80}$ and $SEDDS_{SOLTUOL}$, are shown. The gradient of each slope represents the inverse of the solubilization capacity (230 and 400 μg/mL for $SEDDS_{T80}$ and $SEDDS_{SOLTUOL}$, respectively) and the symbols capture instances where performance is characterized by negligible (closed symbols) or extensive drug precipitation (open symbols). For $SEDDS_{SOLUTOL}$, the change from no precipitation to extensive precipitation occurs between SR^M 2.5 and 2.6, and at an AP_{MAX} of approximately 1000 μg/mL. However, because of the lower solubilization capacity of $SEDDS_{T80}$ after digestion, equivalent SR^M values of 2.5–2.6 are associated with a much lower AP_{MAX} of approximately 600 μg/mL.

The importance of absolute concentration to nucleation rate (J) is captured by the pre-exponential term in Eq. (4), K_0, which is defined in Eq. (6) as the number of molecules per unit volume, N_0, multiplied by the frequency at which the critical nuclei transform into crystals, V_0[58]:

$$K_0 = N_0 V_0 \quad (6)$$

According to Eq. (6), K_0 will increase as a system becomes increasingly concentrated and the number of collisions between supersaturated molecules and forming nuclei increases. Therefore, at a particular supersaturation, the lower fenofibrate concentration present in $SEDDS_{T80}$ implies that K_0 was lower, which will have resulted in a lower rate of nucleation (relative to other SEDDS at the same degree of supersaturation). This provides a possible explanation for the slightly higher capacity for $SEDDS_{T80}$ to support supersaturation. Bearing in mind the potential differences in collision frequency, deviation from an

apparent common SR^M threshold may be anticipated if formulations in a screening exercise show a wide range in solvent capacities after digestion. Also, although a SR^M threshold of approximately 2.5–2.6 appears to be valid for most danazol[13,16] and fenofibrate formulations, further work will be necessary to determine whether the threshold SR^M values discussed here are representative of common values that can be applied to a more diverse range of drug molecules.

As supersaturation can provide a driver for enhanced absorption (via increases in thermodynamic activity),[49] the design of SEDDS showing a capacity to maintain high drug concentrations in the supersaturated state during dispersion and digestion remains a goal for lipid formulation development. The use of measures of *in vitro* performance such as SR^M discussed in this study provide a mechanistic understanding of the performance of lipid-based formulations that are likely to be of great value to scientists engaged in their development.

CONCLUSIONS

The loss of solubilization capacity resulting from dispersion and digestion of lipid-based formulations, such as the Type IIIA SEDDS investigated in this study, and other lipid formulations, can lead to metastable supersaturated systems that may promote drug absorption. However, as supersaturation may also promote drug precipitation, identification of the threshold level of supersaturation, beyond which extensive precipitation occurs, is necessary to ensure that the positive effects of supersaturation on drug absorption can be fully exploited. Here, the influence of maximum SR generated on digestion (SR^M) on the performance of seven different SEDDS, each containing either danazol or fenofibrate was evaluated. Despite differences in digestibility and solubilization capacity, the comparatively low SR^M values generated on digestion of SEDDS-containing danazol resulted in negligible precipitation, and therefore no significant differences in performance. In contrast, digestion of SEDDS-containing fenofibrate generally led to higher SR^M values and above a threshold SR^M between 2 and 3 led to extensive drug crystallization. The performance of each SEDDS was variable close to a threshold SR^M value of 2.6. Below this value, SEDDS formulations maintained the bulk of the mass of drug in a solubilized form during dispersion and digestion. This study indicates that determination of the solubility of drug in each digested formulation, and the use of this data to calculate the value of SR^M for each formulation, is a simple and powerful tool for formulation scientists. Values of SR^M determined *in vitro* are unlikely to be a true reflection of the maximum SR^M encountered *in vivo*, nor can they predict the influence of the dynamic process of drug absorption on maintenance of supersaturation in the gut lumen. In our view, the *in vitro* digestion test probably overestimates the likelihood of precipitation occurring *in vivo*. The "rules of thumb" emerging from this and other recent *in vitro* studies can be used to take a conservative approach to formulation if desired. The paper suggests that a simple *in vitro* test of solubility in a "digested" formulation may be sufficient to identify the possibility of variable bioavailability caused by possible precipitation in the intestine. We suggest that formulators should adopt a strategy that includes an assessment of possible precipitation during digestion, to allow an informed assessment of risk to be taken into account when a final choice is made on which product to take forward into clinical development.

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