New Insights and Methods for Studying the Impact of Lipid Excipients on the Precipitation Behaviour of Poorly Water-Soluble Drugs

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For Mum and Dad

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Table of Contents

Abstract

The majority of emerging drug candidates are not suited to conventional oral dose forms, as they typically do not dissolve to a high degree in the aqueous environment of the gastrointestinal tract, such drugs are termed poorly water-soluble. The use of lipid based formulations to enable the oral delivery of poorly-water soluble, lipophilic, drugs has received much attention for a number of years. Their premise is to pre-dissolve drug in the formulation and maintain drug in a dissolved state throughout gastrointestinal transit, by benefiting from the lipid digestion pathway, thereby bypassing the rate-limiting dissolution step. An issue with lipid formulations, however, is the loss in solubilisation capacity for drug experienced upon dilution and digestion. This ultimately leads to drug precipitation, which has traditionally been equated with poor therapeutic outcomes, due to an expected decrease in the amount of drug absorbed. It was discovered recently, however, that some drugs do not precipitate as poorly water-soluble crystalline forms during *in vitro* digestion experiments, but rather as high-energy amorphous forms that could potentially re-dissolve under the dynamic digestive conditions encountered *in vivo*. The purpose of this work was to examine why this change in solid-state form upon precipitation occurs, and if this phenomenon can be controlled.

This work involved the development of an *in situ* digestion and synchrotron scattering technique used to examine the precipitation kinetics and solid-state form of the neutral, lipophilic, drug fenofibrate from a model lipid formulation. The trend of weakly-basic drugs precipitating as amorphous forms during *in vitro* digestion of lipid formulations was also examined, with a focus on elucidating ionic interactions between cinnarizine and oppositely charged fatty acid molecules during lipid digestion, as a driver for amorphous precipitation. In addition, the precipitation behaviour of a model weakly-acidic drug, tolfenamic

acid, was assessed in the presence and absence of an oppositely charge excipient, didodecyldimethylammonium bromide, in the starting lipid formulation, in an attempt to gain a level of control over the solid-state form of the precipitated drug upon digestion.

The findings from this thesis provide new methods and insight into the precipitation behaviour of drugs during lipid digestion, which could assist in the development of lipid formulations that are optimised for dealing with the issue of drug precipitation.

Declaration of Authorship

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

This thesis includes (3) original papers published in peer reviewed journals and (1) manuscript in preparation. The core theme of the thesis is the precipitation behaviour of poorly water-soluble drugs upon the digestion of lipid formulations. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Drug Delivery, Disposition and Dynamics unit under the supervision of Professor Ben Boyd.

Signature: 

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Date: 20-04-2017

Publications

1. Khan J., Rades T., Boyd B. *The Precipitation Behavior of Poorly Water-Soluble Drugs with an Emphasis on the Digestion of Lipid Based Formulations.* Pharmaceutical research. 2016;**33**(3):548-62.

2. Khan J, Hawley A, Rades T, Boyd B. *In situ lipolysis and synchrotron small-angle X-ray scattering for the direct determination of the precipitation and solid-state form of the model poorly-water soluble drug during digestion of a lipid-based formulation*. J. Pharm. Sci. 2016, **105**, 2631-39.

3. Khan J, Rades T, Boyd B. *Lipid-based formulations can enable the model poorly water-soluble weakly basic drug cinnarizine to precipitate in an amorphous-salt form during in vitro digestion*. Mol. Pharm. 2016, **13** (11): 3783-3793.

4. Khan J, Rades T, Boyd B. *Proof of Concept for the Use of Cationic Surfactants to Alter the Solubility Profile of Poorly Water Soluble Acidic Drugs and the Solid-State Form of the Precipitate upon In Vitro Lipolysis*. Manuscript in preparation.

In the case of Chapters 1, 3, 4, and 5 my contribution to the work involved was the following:

Thesis chapter	**Publication title**	**Publication status**	**Nature and extent of candidate's contribution**
1	The Precipitation Behaviour of Poorly Water-Soluble Drugs with an Emphasis on the Digestion of Lipid Based Formulations	Published	Manuscript preparation
3	In Situ Lipolysis and Synchrotron Small-Angle X-ray Scattering for the Direct Determination of the Precipitation and Solid-State Form of the Model Poorly-Water Soluble Drug During Digestion of a Lipid-Based Formulation	Published	Research design, performance of data collection and analysis, manuscript preparation
4	Lipid-based Formulations can Enable the Model Poorly Water-Soluble Weakly Basic Drug Cinnarizine to Precipitate in an Amorphous-Salt Form During *In Vitro* Digestion	Published	Research design, performance of data collection and analysis, manuscript preparation
5	Proof of Concept for the Use of Cationic Surfactants to Alter the Solubility Profile of Poorly Water Soluble Acidic Drugs and the Solid-State Form of the Precipitate upon In Vitro Lipolysis	In Preparation	Research design, performance of data collection and analysis, manuscript preparation

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

Student signature:  Date: 20-04-2017

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Chapter 1:
Introduction

Declaration

This chapter consists of a published review article that discusses the current state of knowledge around the solid-state aspects of oral lipid-based formulations. It briefly covers drug precipitation during digestion, conventional and newly emerging techniques used to characterise precipitated drug, and discusses case studies where differences in the solid-state form of the precipitated drug have been observed. The chapter culminates with the hypotheses and aims of this project. The review article was published as: Khan J., Rades T., Boyd B. *The Precipitation Behavior of Poorly Water-Soluble Drugs with an Emphasis on the Digestion of Lipid Based Formulations.* Pharmaceutical research. 2016;33(3):548-62.

Declaration by candidate:

For chapter 1, the nature and extent of my contribution to the work was as follows:

Nature of contribution	**Extent of contribution**
Preparation of manuscript	90%

The following co-authors contributed to the work:

Name	**Nature of contribution**
Thomas Rades	Co-supervisor, preparation of manuscript
Ben Boyd	Main supervisor, preparation of manuscript

The undersigned hereby declare that the above declaration correctly reflects the nature and extent of candidate and co-author contributions:

Candidate's signature: [redacted] Date: 20-04-2017

Main supervisor's signature: [redacted] Date: 20-04-2017

The Precipitation Behaviour of Poorly Water-Soluble Drugs with an Emphasis on the Digestion of Lipid-Based Formulations

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Abstract

An increasing number of newly discovered drugs are poorly water-soluble and the use of natural and synthetic lipids to improve the oral bioavailability of these drugs by utilizing the digestion pathway *in vivo* has proved an effective formulation strategy. The mechanisms responsible for lipid digestion and drug solubilisation during gastrointestinal transit have been explored in detail, but the implications of drug precipitation beyond the potential adverse effect on bioavailability have received attention only in recent years. Specifically, these implications are that different solid forms of drug on precipitation may affect the total amount of drug absorbed *in vivo* through their different physico-chemical properties, and the possibility that the dynamic environment of the small intestine may afford re-dissolution of precipitated drug if present in a high-energy form. This review describes the events that lead to drug precipitation during the dispersion and digestion of lipid based formulations, common methods used to inhibit precipitation, as

well as conventional and newly emerging characterization techniques for studying the solid-state form of the precipitated drug. Moreover, selected case studies are discussed where drug precipitation has ensued from the digestion of lipid based formulations, as well as the apparent link between drug ionisability and altered solid forms on precipitation, culminating in a discussion about the importance of the solid form on precipitation with relevance to the total drug absorbed.

Overview

The aim of using lipid based drug delivery systems (LBDDS) is to enable the effective oral delivery of lipophilic drugs. Issues of formulation dispersion, digestion and post-absorptive fate of both drug and lipid have all been the subject of increasingly intense research in the field. A limited number of products, including Sandimmune®, Neoral® (1) and Aptivus® have achieved some success on the market as LBDDS (2), however their widespread commercialization has been hampered by a lack of understanding on several important fronts. Perhaps one of the most critical of these is the need for a thorough understanding of drug precipitation during the digestion of LBDDS with regard to the solid-state form of the drug, and subsequent effects on re-dissolution in the presence of an absorptive sink (e.g., *in vivo*). In this review the current level of understanding around factors that govern drug precipitation during the dispersion and digestion of LBDDS are examined, in addition to describing the common methods used to inhibit drug precipitation, and perspectives on the emerging realization in the field that the paradigm of preventing drug precipitation during lipolysis may not be necessary are offered that may provide a way forward in understanding how to optimally design LBDDS.

Poorly Water-Soluble Drugs and Lipid Based Drug Delivery

The discovery of drugs with poor aqueous solubility is becoming increasingly common because of bias towards such molecules in combinatorial chemistry and high throughput screening. Both of these drug discovery strategies aim to maximize drug–receptor complementarity, stressing hydrophobic drug moieties that improve ligand–receptor affinity through key interactions (3, 4). Improved ligand–receptor affinity, however, generally remains juxtaposed with decreasing aqueous solubility. Current predictions suggest that over 70% of newly-discovered drugs are poorly water-soluble, and lipophilic (5), or class 2 compounds as termed by the Biopharmaceutics Classification System (BCS) (6). These class 2 compounds are highly permeable across the intestinal membrane, but have limited solubility by gastrointestinal fluids and hence may be slow to dissolve in the gastrointestinal tract, thereby limiting their absorption (7–9).

Advances in oral drug delivery may present formulation options that overcome the solubility limitations of such compounds, thus enabling administration of drug in a form that addresses the issues with poor aqueous solubility through formulation, rather than at the drug molecular structure level.

Poorly water-soluble drugs often exhibit slow dissolution in the gastrointestinal tract, ultimately limiting their absorption (10). Slow dissolution can lead, potentially, to incomplete dissolution of drug on passage through the gastrointestinal tract and excretion of undissolved material. Formulation approaches to improve the bioavailability of such drugs are therefore focused on modifying drug dissolution and solubility characteristics, or avoiding the need for dissolution through the use of a solution dosage form. The Noyes-Whitney dissolution equation dictates that the solubility in the surrounding medium relative

to the concentration of dissolved drug, the drug diffusion coefficient and surface area of the solute determine the dissolution rate of a solid material (11). As a consequence, dissolution is affected by the disposition of gastrointestinal fluids - their pH and solubilizing components, agitation, degree of saturation, and the presence of molecular species that may modify drug dissolution characteristics by adsorption to the solute particles (12). There have been many different formulation approaches to address the slow dissolution of drug by impacting on these variables at both the solid state and solution levels (13).

For BCS Class 2 compounds in particular, the lipophilicity of the drug leads intuitively to the use of lipids to provide a dosage form in which the drug is dissolved prior to administration, and during the gastrointestinal processing of the formulation (dispersion and digestion), to make the drug available in a highly solubilized state for absorption. The use of lipids to improve bioavailability could range from simple fatty meals to highly engineered LBDDS as unit dosage forms. With the exception of LBDDS containing a high fraction of hydrophilic components, such as Type 3B and Type 4 formulations, which will be discussed in a later section, a common aspect is that LBDDS generally require digestion to provide the optimal pre-absorptive environment to maximize bioavailability (9, 14–20).

The digestion of lipids is a complex combination of biochemical and physicochemical processes. Many reviews exist that describe these processes in detail (9, 14–20). Figure 1 provides an overview of the digestion process and the potential fate of both lipids and lipophilic drugs.

Briefly, with relevance to LBDDS administered in a capsule, the digestion of lipids starts in the stomach, where enzyme-driven hydrolysis renders triglycerides to 1,2(2,3)-di-glycerides and free fatty acid molecules. Up to 30% of total triglyceride is

initially broken down by gastric lipase, aided by agitation from the stomach to form a crude emulsion (21). Transit to the small intestine then triggers the major aspect of lipid digestion, where the pancreatic lipase/colipase complex breaks down any diglycerides and remaining triglycerides at the oil–water interface, producing an sn2-monoglyceride and two free fatty acid molecules per molecule of triglyceride (10). Biliary secretions released from the gallbladder, containing other amphiphilic endogenous molecules, such as bile salts, phospholipids and cholesterol, then aid in emulsifying and solubilizing the lipid digestion products by forming different colloidal structures, principally vesicles and mixed micelles (22–26). The commonly proposed mechanism of transport of lipids towards the epithelial surface occurs *via* mass transport, in which the final colloidal phases (typically mixed bile salt micelles) carry the digestion products across the unstirred water layer separating the absorptive cells from bulk intestinal fluid (18).

The premise of digestion-enabled drug delivery is therefore to provide a solubilizing medium (*e.g.,* the mixed micelles) for poorly water-soluble, lipophilic, compounds. This is most simply achieved by administering drug immediately after consuming a high fat meal (10). However, inter-patient differences in the fat content and temporal aspects of taking the medicine often lead to variable pharmacokinetics and consequently variable therapeutic outcomes (27). LBDDS often achieve more consistent absorption profiles, at least in part by providing drug in a pre-dissolved state, thus avoiding the rate-limiting dissolution step (9, 17, 19). There is clear evidence in the literature that when compared to an aqueous suspension, drug administered in solution in a lipid vehicle almost always provides improved absorption (28–31). It is less clear whether lipid solutions are beneficial over lipid suspensions from a bioavailability perspective (32). Other drawbacks for lipid suspensions, such as Ostwald ripening and dose uniformity, mean that they have

not been as extensively studied as lipid solutions.

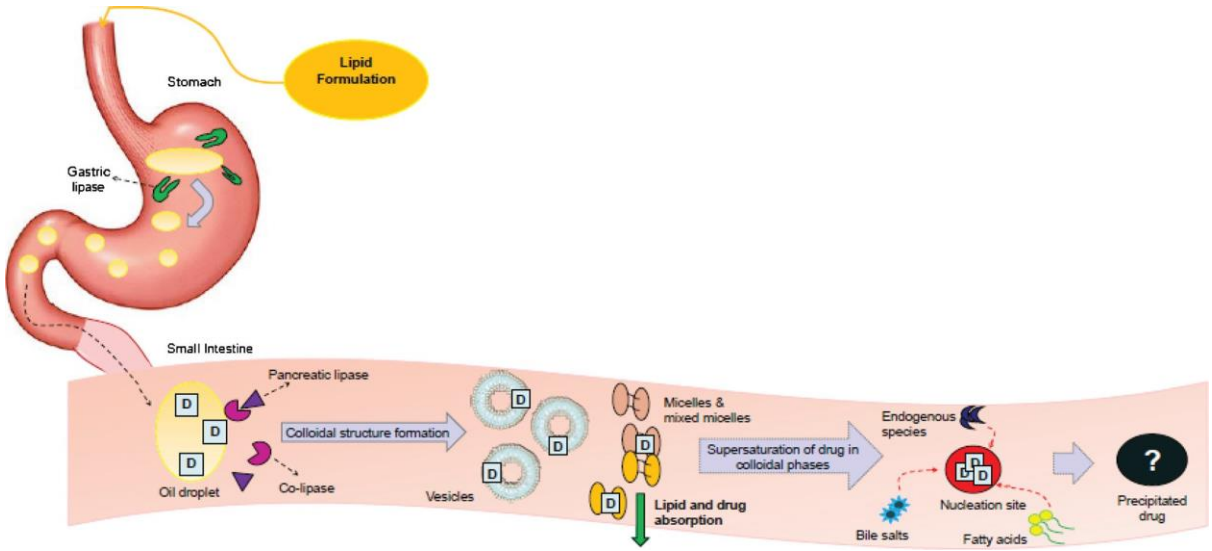

Figure 1: An overview of the digestion of lipids in the stomach and small intestine, and the potential fate of lipids and drug. Adapted from Porter *et al.* (18), with permission from Nature Publishing Group.

There are different types of LBDDS composed of varying amounts of lipids, surfactants (either hydrophobic or hydrophilic) and co-solvents. The lipid formulation classification system (LFCS) groups LBDDS based on their composition and physical properties (9). Briefly, Type 1 formulations comprise oil excipients only, such as triglycerides and other glycerides, requiring digestion for dispersion to occur. Type 2 formulations are self-emulsifying, meaning that upon dilution and provided gentle agitation, a dispersed system forms with a droplet size in the range of 0.25–2 µm. Oils and non-ionic surfactants generally make up Type 2 formulations. Type 3 formulations produce very fine dispersions with droplet sizes < 100 nm, due to the inclusion of water-soluble components, they are also optically clear. Subtypes A and B exist for Type 3 formulations, where the discriminating factor is the fraction of total formulation consisting of water-soluble parts and co-solvents. On the extreme end of the LFCS

are Type 4 formulations, which do not contain any lipid excipients, and are purely composed of surfactants to form fine micellar solutions.

There is growing evidence to suggest that LBDDS improve the extent of absorption of lipophilic, poorly water-soluble drugs. Current approaches to the formulation of LBDDS, however, continue to be largely empirical, based on drug solubility in the formulation, and dispersibility of the formulation during *in vitro* dispersion testing. The complexity of the digestive environment and the dynamic compositional changes over time are currently not well understood, but are critical in dictating drug disposition. To date there has been a focus on drug solubilisation, but the solid-state aspects of drug disposition are emerging as an important aspect of overall drug behavior during dispersion, digestion and absorption from LBDDS. Solid-state aspects that could influence drug disposition are those of the formulation in the case of a suspension formulation, and those of solid material resulting from precipitation during dispersion and digestion. The latter is the focus of this review article.

Supersaturation and Drug Precipitation

Precipitation of drug during the dispersion and digestion of LBDDS may reduce the total amount of drug absorbed, and therefore decrease oral bioavailability (7, 14, 33–36) on the basis that re-dissolution of the drug does not take place. Precipitation is the nucleation and phase separation of solid drug particles from a supersaturated liquid system. A supersaturated state is a prerequisite for precipitation (37), and there are different mechanisms by which supersaturation of drug can occur during digestion. The pH shift mechanism is apparent during the gastric emptying of basic drugs, where supersaturation occurs due to the movement of drug from the highly solubilising, low pH environment of the stomach (~pH = 2), to the higher pH of the small intestine. Consequently, the solubility of the basic drug is

reduced resulting in a transiently supersaturated state (7, 36, 38–41). Another mechanism by which a supersaturated state forms, relevant to LBDDS, is where dispersion and digestion in the small intestine leads to a decrease in solubilisation capacity of the formulation for the drug (20, 34, 36, 40, 42).

Under conditions of supersaturation, nucleation and crystal growth are inevitable. Solute molecules initially gather, either on the surface of an impurity or in three dimensional clusters, to form stable nuclei that are of a sufficient size for subsequent growth to take place (36, 37, 43). Nucleation is aided by the presence of impurities, as they decrease the energy barrier towards successful cluster formation. An increased degree of supersaturation and sufficient level of impurities lessen the time taken for precipitation to occur. Moreover, recent findings suggest the existence of precursor complexes that lead to nucleation and potentially influence precipitation, and therefore the solid material produced. A detailed discussion on the function of these precursors can be found elsewhere (44).

Crystal growth begins once stable nuclei are formed, but growth and nucleation continue concurrently from this point. Dominance of one mechanism over the other largely determines crystal size, for example a few stable nuclei would give rise to a few large crystals, whereas continuous nucleation would form many small crystals. Growth of crystals is driven by the attachment of solute molecules to energetically-favoured growth sites, and the macroscale form of a crystal, or habit, is highly variable, depending on factors such as rate of growth, nature of solvent, degree of supersaturation and agitation, as well as the presence of impurities or excipient material (37, 44, 45). In a system reverting to thermodynamic stability, precipitation proceeds only to the point where there is no longer a supersaturated state.

Three theories exist on the progression of crystallisation, namely the surface energy theory, the diffusion theory and the adsorption layer theory (36). The surface energy theory states that a drop of fluid is most stable when its area/surface energy is at a minimum, and in special cases this minimum is achieved through crystal growth. However, this theory is not well supported due to its inability to explain supersaturation and varying growth rates (36). Diffusion theory describes the integration of units to a lattice structure, where controlled diffusion of the components occurs and a stagnant film can be found on the surface of the crystal. Modifications to diffusion theory were necessary to account for the effects of agitation, which would decrease the thickness of the stagnant film and theoretically lead to rapid crystal growth, but this is never observed in real systems. Therefore, a two-step process has been proposed where, first, diffusion carries solute molecules to the crystal surface, and then rearrangement takes place prior to integration (36). Lastly, the adsorption layer theory is based on the thermodynamics of adsorbed layers at a crystal face. Where adsorbed solute molecules do not immediately integrate on crystal faces, but are rather adsorbed and free to diffuse around the surface, thus creating an equilibrium between adsorbed solute molecules constituting the layer and the bulk solution containing solute molecules. Attractive areas on the crystal face yield growth sites or kinks for crystal step growth (36, 46).

In vitro Lipolysis and Maximum Supersaturation Ratio (SR^M)

In vitro lipolysis models are often used to study the solubilisation and precipitation of drug during dispersion and digestion of LBDDS (18). Briefly, *in vitro* digestion/lipolysis experiments attempt to simulate the processes occurring on oral administration of lipid formulations by mimicking the *in vivo* environment of the gastrointestinal tract, most often conditions reflecting

the upper small intestine. Hydrolysis of lipids occurs upon the addition of lipases to simulated intestinal fluid containing the dispersed lipid formulation in a thermostatted (37°C) glass vessel (17). The simulated intestinal fluid is composed of bile salt and phospholipids in buffer. An automated titration unit maintains pH, generally at 6.5, which is a compromise between the pH of the upper small intestine (47) and the optimal pH for the lipolytic activity of pancreatin enzyme (48). Sodium hydroxide solution is used as the titrant to neutralize free fatty acids liberated by lipase-mediated degradation of triglycerides (49). Information with regard to the extent of digestion of the formulation and the distribution of drug in different phases is commonly acquired from lipolysis experiments.

After a designated time period the *in vitro* lipolysis can be arrested by addition of a lipase inhibitor to the glass vessel, after which the digested contents are centrifuged to produce the relevant post-digestion phases, namely undigested oil (if present), aqueous colloidal phase and a semi-solid or solid pellet (50). Distribution of lipids and drug between the different phases can then be assessed analytically, where precipitated drug in the pellet and solubilized drug in the colloidal phase indicate the likelihood of drug precipitation *in vivo*.

However, the prediction of drug precipitation *in vivo* based on the results from *in vitro* lipolysis experiments may be overestimated (34, 51–53). The absence of an absorptive sink *in vitro* is considered a major drawback of lipolysis experiments, and the lack of a product removal mechanism implies exaggerated results with regard to drug precipitation. Furthermore, the variables such as type and concentration of bile, pH and type and activity of the enzyme have made it difficult to compare results across groups, or to draw definitive conclusions on the biorelevance of the lipolysis methods, leading to recent efforts to standardise *in vitro* digestion protocols for LBDDS (54–57).

As mentioned earlier, solubilisation of drug in the aqueous colloidal phase may be an indicator of likely *in vivo* performance. A high amount of solubilized drug in the aqueous colloidal phase may increase the thermodynamic driving force for absorption, leading to a recent focus on supersaturation of drug in the colloidal phase during and after digestion as a more appropriate indication of the potential for absorption from a LBDDS. The degree to which digestion produces a supersaturated drug-containing medium has been termed the maximum supersaturation ratio (SRM) (56).

The SRM describes the maximum amount of supersaturation obtained before drug precipitation is observed on digestion of lipid based formulations. The SRM is a useful tool with respect to quantifying the influence of formulation components on the kinetic aspects of supersaturation. The absolute value between drugs, however, does not take account of several issues including drug participation in self-assembly processes (58), impact of drug on kinetic aspects of digestion (59), or importantly, differences in solid-state between drug in solubility measurements and precipitated drug in dynamic lipolysis experiments. One particular area of conjecture remains the nature of the centrifuged pellet phase with regard to its molecular composition, and effect on drug precipitation. It has been proposed that during *in vitro* lipolysis the liberation of fatty acids from the breakdown of glycerides yields calcium-fatty acid complexes, which form insoluble soaps residing in the pellet of a centrifuged digestion sample (51). Insoluble soaps are an undesired non-biorelevant byproduct from sequestering liberated fatty acids with calcium in an effort to maximize lipase action. This fatty acid-calcium matrix could, potentially, affect the drug precipitation process. Therefore, if the pellet itself is influencing the solid state form of precipitated drug in the *in vitro* model, this would not be the case *in vivo*, as the absorption of fatty acids would relieve inhibition of lipase.

Conventional Characterization Techniques for Analyzing Precipitated Drug

There are several commonly used characterization methods for determining the solid-state of precipitated drug during digestion-related experiments. These include X-ray diffraction (XRD), Cross Polarized Light Microscopy (CPLM), dissolution testing, Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FTIR).

XRD is typically performed on the pellet phase of a digested formulation, after isolation of the precipitated material. The basis of how XRD works to obtain the structural information of solids is well documented and a detailed review may be consulted for a basic understanding (60). Briefly, X-rays pass through a sample and diffract at angles unique to the crystal structure, effectively providing a fingerprint for the molecular arrangement characteristic of the solid state of the material. Therefore, polymorphic forms of a drug may be identified due to the unique molecular arrangements of the polymorphic crystals. Amorphous drug forms, lacking long range orientational and positional order are also identifiable by XRD, albeit indirectly, by an absence of diffraction peaks and the presence of a largely undefined halo region in the resultant intensity vs angle diffractograms. XRD thus provides an easy means to differentiate between amorphous and crystalline drug forms; however, analyzing precipitated drug in the pellet phase from digested lipid formulations is difficult, due to sample preparation and analysis time requirements, and the resulting diffractograms need to be interpreted with caution. Typically, isolation of the pellet phase requires a period of centrifugation ranging from 30 to 90 min, which is followed by removal of supernatant and sometimes drying before performing the XRD measurement which requires an additional 30 min per sample. Whether the final and observed solid-state form of the drug after performing XRD truly reflects

the precipitated drug during the digestive process remains unclear (i.e., amorphous to crystalline conversion of precipitated drug), but can potentially be confirmed with the development of *in situ* characterization methods, which will be discussed in a later section.

CPLM is an imaging technique that can identify drug crystals in the pellet phase, with birefringence indicating crystalline material. Calcium-fatty acid soaps, however, may also be present in the isolated pellet and show birefringence on CPLM due to their anisotropic lamellar liquid crystalline structure, which makes amorphous drug difficult to detect. Similar to XRD, invasive sample preparation and the potential for temporal changes in structure during sampling and experiment apply for CPLM. In any case, CPLM remains a sound visualization technique that provides a link between crystal morphology and XRD data. A thorough understanding behind the principles of CPLM may be obtained elsewhere (61).

Dissolution testing of precipitated drug in bio-relevant media can inform the likelihood of re-dissolution of drug during the digestion of lipid-based formulations in the presence of an absorptive sink (e.g., *in vivo*). Dissolution tests can be performed on isolated and extracted pellet phases in a standard USP-2 paddle apparatus (62, 63). The solid state form of the pelleted drug precipitated during the digestion of lipid formulations can be compared to the reference crystalline form by spiking crystalline drug into the isolated pellets of corresponding blank (drug-free) lipid formulations (40, 62, 64). Solid state transformations upon contact with solvent should also be considered, where amorphous drug can convert to a crystalline or polymorphic form. Recently, an *in situ* approach that combines dissolution testing with Raman spectroscopy was able to monitor the transition of amorphous indomethacin to its crystalline forms (65).

DSC can be used identify key thermal events intrinsic to the precipitated drug found in the pellet phase after digestion of lipid formulations. A glass transition temperature (Tg) or re-crystallization event would suggest amorphous drug has precipitated during lipolysis. In contrast, exothermic melting should be observed for crystalline drug. However, as discussed above the pellet is not limited in composition to precipitated drug, with calcium-fatty acid complexes also being present, and thermal events due to these components need to be distinguished from those of the drug. A detailed review on the applications of DSC can be found elsewhere (66, 67).

Delaying the Onset of Drug Precipitation with Polymeric Precipitation Inhibitors (PPIs)

Polymeric materials, such as polyvinylpyrrolidone (PVP), or hydroxypropylmethyl cellulose (HPMC), when administered with poorly water-soluble drugs can act to inhibit precipitation of drug for a given time period. Delayed onset of drug precipitation can be a result of these polymers working to inhibit both the nucleation and crystal growth mechanisms, and have not been shown to operate exclusively on one or other of these processes (68, 69). Kinetic stability of supersaturated states is thought to be achieved through polymer-drug interactions, such as hydrogen bonding, hydrophobic interactions or steric disruption of the crystallization phase (70, 71). By doing so, PPIs effectively maintain a supersaturated environment that allows for rapid drug absorption to occur (34). Typically, PPIs do not produce an effect on equilibrium solubility of the drug. PPIs slow the onset of drug precipitation; however, they do not completely stop precipitation, as precipitation is thermodynamically favored. The ‘spring and parachute’ concept has been used in the literature when describing the action of PPIs (72), where drug is maintained in a supersaturated state and the PPIs slow

down the otherwise rapid decrease in solubilized drug, eventually relaxing to a thermodynamically-favored precipitated state (36, 72). In addition, once the drug starts to precipitate and undissolved particles are present, the PPIs may continue to exert their effect by retarding the crystal growth mechanism and slow down further precipitation.

The reported mechanisms and sites of action for PPIs include changing surface tension at the bulk-solution interface (36, 73), changing the adsorption layer at the crystal-solution interface (36, 74), adsorbing to the crystal-surface interface (36, 70), adsorption to growth terraces and blocking access of solute to these growth sites (36, 75), adsorption to surface crevices creating a smooth surface free of growth areas and altering the surface energy of crystal faces and changing the level of solvation (36). Factors that influence polymer-drug binding have been cited previously and include temperature, molecular weight, viscosity, dielectric constant and hydrogen bonding (36). High temperatures lead to greater drug solubility, which weakens intermolecular bonding. Increasing the molecular weight of the polymer generally strengthens polymer-drug interactions through an increased viscosity and number of functional groups for bonding. The rate of diffusion of drug is inversely proportional to the viscosity of the aqueous medium. For poorly water-soluble drugs, lowering the dielectric constant is expected to increase drug solubility, thus negatively affecting drug-polymer binding. Finally, hydrogen bonding between polymer and drug is greater where there are more hydrogen bonding sites on the polymer (36). These act as merely generalized observations and are not necessarily always the case for a given drug-polymer combination.

Link Between Drug Ionisability and Solid State form on Precipitation

Until recently the pervasive view was that drug precipitation is an undesirable event during the dispersion and digestion of lipid based formulations, as the precipitated drug may not be absorbed and therefore could result in decreased bioavailability. Drug precipitation can lead to changes in the rate of drug absorption regardless of where precipitation takes place along the gastrointestinal tract and this will likely have some effect on therapeutic outcomes. However, this view of drug precipitation inherently leading to negative therapeutic outcomes is more applicable where the gastric precipitation of drug upon dispersion of the formulation in the stomach is expected, which in turn is more likely to occur when relatively hydrophilic formulations are administered, for instance Type 3B and Type 4 formulations. A loss in solubilisation capacity for the drug upon dispersion is most often seen with these hydrophilic formulations and generally is not considered a major hurdle for more lipophilic Type 1, Type 2 and Type 3A formulations. The absence of self-assembled lipid digestion products and the low pH environment of the stomach means the potential for re-dissolution of the precipitated drug would be different to that of the small intestine. Conversely, the likelihood of drug precipitation following the administration of more lipophilic formulations (Type 1, Type 2 and Type 3A) is greatest in the small intestine upon digestion of the lipid components in the formulation.

However, drug precipitation does not necessarily preclude re-dissolution in the dynamic environment of the small intestine, where sink conditions may be re-instated, through transient changes in lipid composition, changes in colloidal structures, or absorption of lipid or drug. The solubility of drug in the changing medium will also depend on the solid-state form of

precipitated drug, hence it is possible that differences in solid-state of the precipitated material during administration may account for varying extents of total drug absorption, and bioavailability. Despite the likely importance of the solid-state of precipitated drug in understanding the performance of LBDDS, the issue has received only recent attention in the lipid-based formulation field. There are currently a handful of studies in the literature where the solid-state form of precipitated drug has been investigated after the digestion of lipid and non-lipid formulations, the findings are summarized in Table I. These studies point to a strong correlation between the ionisability of the drugs tested and an altered solid-state form on precipitation, particularly for lipid formulations tested with *in vitro* lipolysis experiments.

Table I: Summary of Amorphous or Crystalline Precipitates During Dilution or Digestion Experiments

Drug	Drug Type	Formulation	Excipients	Solid-state in pellet	Reference
Cinnarizine	Base	LC SMEDDS	Sesame oil, Cremophor RH40, oleic acid, Brij 97 and ethanol	Amorphous	(62)
Carvedilol	Base	Type 3-4 LBF	Miglyol, Imwitor, Cremophor EL, ethanol, Capryol and Transcutol	Amorphous	(40)
Loratadine	Base	Type 3-4 LBF	Miglyol, Imwitor, Cremophor EL, ethanol, Capryol and Transcutol	Crystalline	(40)
Halofantrine	Base	LC and MC SNEDDS	LC - soybean oil/Maisine, MC – Captex/Capmul300, Cremophor RH40 and ethanol	Amorphous	(64)
Ketoconazole	Base	Drug solutions	Non-carbonated mineral water adjusted to pH 2.4, 2.6 and 2.7 using HCl	Amorphous Crystalline	(76) (78)
Dypiridamole	Base	Drug solutions	Non-carbonated mineral water adjusted to pH 2.4, 2.6 and 2.7 using HCl	Crystalline	(76)
Mebendazole	Base	Drug solutions	N,N-dimethylacetamide and HCl	Crystalline	(77)
AZD0865	Base	Drug solutions	PEG 400, ethanol and HCl	Polymorph	(78)
Fenofibrate	Neutral	Type 1-4 LBF LC SNEDDS	See ref. Soybean oil, Maisine, Koliphor RH40 and ethanol	Crystalline	(53, 56)
Danazol	Neutral	LC SMEDDS	Sesame oil, Cremophor RH40, oleic acid, Brij 97 and ethano	Crystalline	(79)
Simvastatin	Neutral	MC-SNEDDS	Captex/ Capmul300, Cremophor RH40 and ethanol	Amorphous	(80)
Tolfenamic acid	Acid	Type 1-4 LBF	See ref.	Crystalline/polymorph	(56)

Cinnarizine is a selective calcium antagonist that prevents contraction of arterial smooth muscle, is poorly water-soluble, and is often used as a model drug in LBDDS experiments (41, 42, 62, 81–84). A recent study by Sassene *et al*. showed that cinnarizine precipitated in a non-crystalline form during *in vitro* lipolysis of a self-micro-emulsifying drug delivery system (SMEDDS) (62). Crossed polarised light microscopy (CPLM) and X-ray diffraction (XRD) suggested that the precipitated drug was in an amorphous form. Dissolution studies performed on solid material collected post-digestion, the results of which are shown in Figure 2, revealed a ten-fold higher dissolution rate for precipitated cinnarizine in the pellet, compared to neat crystalline drug powder spiked in a blank pellet.

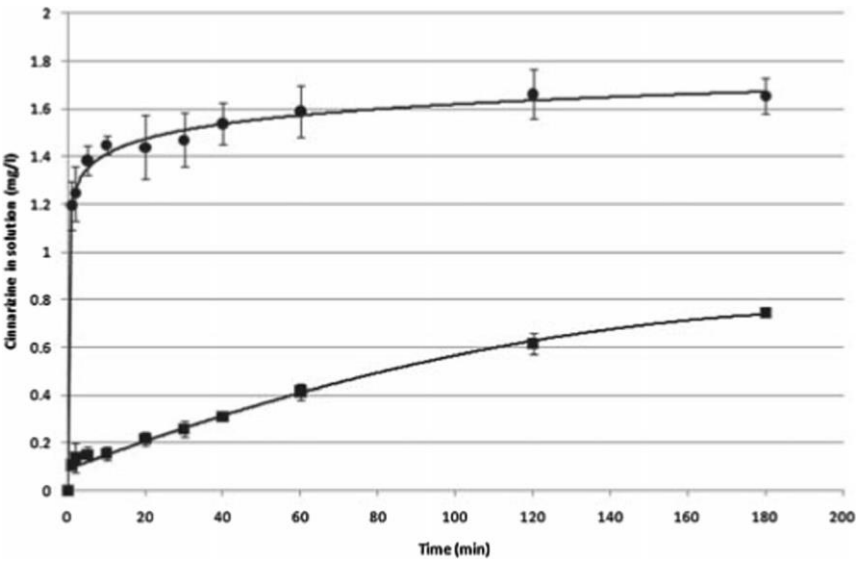

Figure 2: Dissolution rate of pellet with cinnarizine from endpoint *in vitro* lipolysis (circles) and blank pellet spiked with crystalline cinnarizine (squares). Reproduced with permission from Wiley (62)

XRD patterns were compared for raw crystalline cinnarizine, precipitated cinnarizine in the pellet from the digestion sample, a blank pellet from the digestion of drug-free formulation and a blank pellet spiked with crystalline cinnarizine. These diffraction patterns are presented in Figure 3. From Figure 3, raw cinnarizine spiked in a blank pellet from a drug-free formulation (d) showed diffraction peaks that correlated to reference XRD data for cinnarizine (a). Conversely, precipitated drug from the pellet phase post-digestion did not show these peaks (b), suggesting a lack of crystalline cinnarizine in the sample. The conclusion was that cinnarizine, post-digestion, presented itself in a non-crystalline, amorphous form, which may have favourable physico-chemical properties for drug absorption. This paper was the first to acknowledge that the solid-state form of drug upon precipitation during digestion may play a role in determining the total amount of drug absorbed.

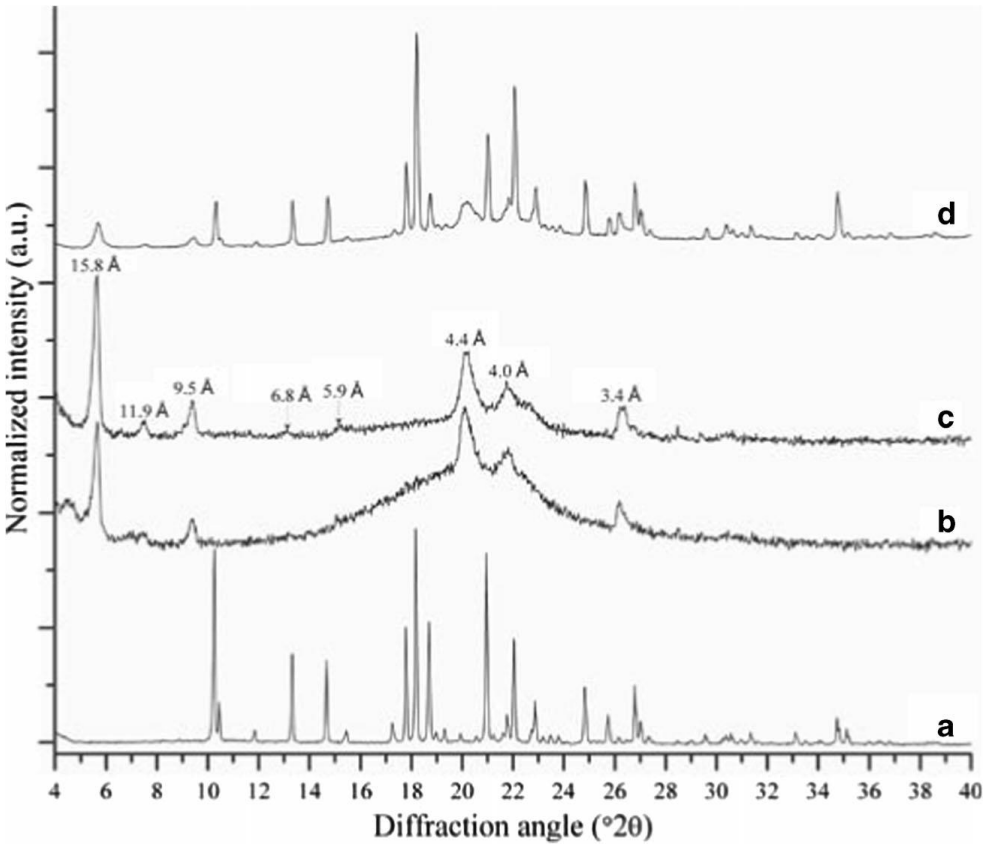

Figure 3: XRD pattern of (a) starting crystalline cinnarizine used in SMEDDS, (b) pellet from the lipolysis of SMEDDS with cinnarizine; CIN pellet, (c) pellet from the lipolysis of SMEDDS without cinnarizine; blank pellet, (d) blank pellet spiked with cinnarizine; blank pellet + CIN. Numbers over the peaks in (c) indicate d-spacings and are also applicable for the peaks of (b) Reproduced with permission from Wiley (62).

The same research group also examined the precipitation of the neutral, poorly water-soluble compound, danazol (79) from the same SMEDDS formulation as for cinnarizine in the aforementioned study. In contrast to the cinnarizine study, danazol precipitated in crystalline form according to XRD data. The authors attributed this finding to the different physicochemical properties of cinnarazine and danazol, and acknowledged that these properties may be important in determining the solid-state formed upon precipitation.

In analogous studies, the *in vitro* precipitation behavior and solid-state characteristics of the poorly water-soluble, weakly-

basic drugs, loratadine and carvedilol, were studied on *in vitro* digestion of lipid-based formulations (40). Formulations containing these drugs were prepared with drug dissolved at up to 80% total drug saturation. Three different formulations were tested with the two compounds, resembling medium-chain Type 3A, medium-chain Type 3B and Type 4 LBDDS. In this study the effects of both formulation dispersion and digestion on precipitated drug were investigated, as opposed to digestion alone. Although the dispersion tests were carried out at a pH of 7.5, the precipitation of drug upon the dispersion of LBDDS can indicate the likelihood of gastric precipitation, as these formulations are first diluted in the stomach before passing to the small intestine, which is the main site for lipid digestion. The solubility of the weakly-basic drugs decreased in dispersed systems compared to undiluted formulations, due to a loss of solubilising capacity upon dispersion of the formulation. As in the case of Sassene *et al*. above, XRD was used to determine the crystallinity of precipitated drug in the pellet. After a dispersion period of 1 h, both drugs precipitated from the Type 3B formulation and the resultant pellet was analysed with XRD, which showed that both drugs had precipitated in their thermodynamically stable crystalline form. After a digestion period of 30 min, however, the drugs precipitated from all three formulations tested, where loratadine was crystalline after digestion in all cases, but the digestion of carvedilol produced an amorphous precipitate, analogous to the cinnarizine study already discussed (62). An explanation for this discrepancy in the solid-state form of precipitated drug on digestion between these two basic compounds is discussed in a later section.

The neutral drug fenofibrate and the weak acid tolfenamic acid, both poorly water-soluble highly lipophilic compounds, were also tested for their solid state form upon precipitation during the *in vitro* digestion of lipid formulations (56). The presence of

fenofibrate crystals after digestion (60 min) was detected by CPLM, and XRD data supported this observation as characteristic peaks corresponding to the thermodynamically stable crystalline form were present. Tolfenamic acid also precipitated during digestion, and crystals were also observed using CPLM, but the crystal form was dependent on the type of lipid formulation. Whilst Type 3B lipid formulations produced crystalline tolfenamic acid in the same form as the reference drug, Type 4 lipid formulations produced a different ('yellow') polymorph upon digestion, as is shown via XRD and CPLM in Figure 4. This latter finding suggests that the composition of the formulation and physico-chemical properties of the precipitated drug products are interrelated.

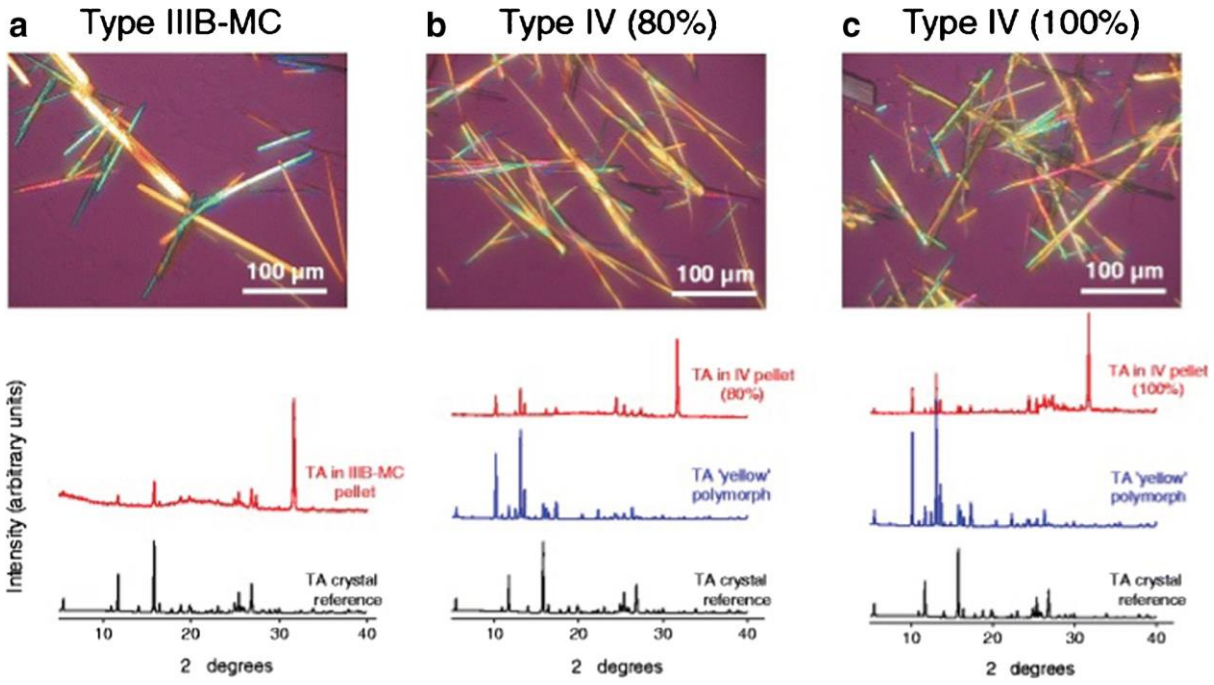

Figure 4: XRD patterns for precipitated tolfenamic acid (TA) after *in vitro* lipolysis of Type 3B and Type 4 lipid based formulations. Digestion of Type 4 formulations at 80 and 100% drug saturation levels produced the 'yellow polymorph' Reproduced with permission from Springer (56).

The link between drug precipitation *in vitro* and formulation performance *in vivo* has also been examined for fenofibrate using a self-nano emulsifying drug delivery systems (SNEDDS), at different drug saturation levels (53). Fenofibrate was shown to precipitate in its thermodynamically stable crystalline form during *in vitro* lipolysis, however, this did not correlate well with *in vivo* data, leading to the idea that the high lipophilicity and permeability of fenofibrate may dictate that a supersaturated state is not maintained for sufficient time *in vivo* for precipitation to occur (7).

A separate study by the same researchers examined the *in vitro* and *in vivo* performance in beagle dogs of SNEDDS and super-SNEDDS formulations containing the poorly water-soluble, weakly basic, drug halofantrine (64). The *in vitro* digestion of these

SNEDDS formulations rapidly induced the precipitation of halofantrine. XRD was performed on the pellet phases after digestion (60 min), where halofantrine was detected in a non-crystalline form. Dissolution of the non-crystalline precipitate was performed in lipolysis media, and the dissolution was shown to be enhanced in comparison to the starting material, similar to the previous observation with cinnarizine discussed above. This enhanced dissolution of the precipitated halofantrine correlated with improved bioavailability *in vivo*, as two SNEDDS capsules were dosed in order to reach a similar AUC and Cmax as a single super-SNEDDS capsule. Findings from this paper are therefore also in direct contrast to the logic that drug precipitation during *in vitro* digestion is inherently linked with a decreased amount of total absorbed drug.

The general trend with the studies discussed thus far, and summarized in Table I, is that poorly water-soluble, weakly-basic drugs tend to precipitate in a non-crystalline form during *in vitro* digestion (cinnarizine, halofantrine and carvedilol), whilst neutral and acidic drugs precipitate in a crystalline form (fenofibrate, danazol and tolfenamic acid, although polymorphism was observed with the latter). Simvastatin, however, which is a neutral compound, went against this trend in a study that compared the performance of super-SNEDDS formulations, in that case at 200% drug loading (80). No sign of crystalline simvastatin within the digested pellet of the 200% super-SNEDDS formulation was seen in the XRD data, which suggests that it is not an absolute rule that only basic drugs precipitate during *in vitro* digestion in a non-crystalline or amorphous form.

Solid-State Precipitation Behaviour in the Absence of Lipid Formulation

The general trend of basic drugs forming non-crystalline precipitates upon digestion is not as apparent in the absence of

lipids. For example, precipitation of mebendazole from dimethylacetamide solutions on dilution with aqueous media resulted in crystalline drug *in vitro* and *in vivo* (77). *In vivo* experiments were performed on dogs with intestinal stomas for sampling, and precipitation of drug in a crystalline form did not appear to negatively impact bioavailability. The results from this study suggest that the components present during and after lipid digestion may play an integral role in dictating the solid-state form of precipitated drug during the digestion of lipid formulations.

Studies on aspirated intestinal samples from humans have shown that the poorly water-soluble, weak base, ketoconazole appears to precipitate in an amorphous form *in vivo* (76), but the drug precipitates in a crystalline form during *in vitro* experiments that were designed to simulate similar conditions (78). This discrepancy in the solid-state form of the precipitated drug between these two studies could be related to the more complex and dynamic environment of humans *in vivo*. As lipids were not used as part of the formulation in either study, however, the amorphous precipitate observed from *in vivo* samples could not be attributed to the presence of lipid digestion products, but could be formed via another unknown mechanism. This observed amorphous precipitate from human intestinal samples implies that the precipitation of weakly-basic compounds in the small intestine, in some cases even in the absence of lipids, may not lead to a decrease in the total amount of drug absorbed; as precipitation of a poorly water-soluble drug in an amorphous form may promote re-dissolution and subsequent absorption. *In vitro* experiments were also performed on the weakly basic compound, AZD0865, which was shown to precipitate in a different polymorphic form to the reference drug material (78).

The inherent supersaturation behavior of certain drugs has also been considered a possible determinant of amorphous or crystalline precipitation. Recently, the solid state properties of 10 different weakly-basic compounds were characterized upon precipitation induced via a pH shift in the dissolution medium (85). Drugs were dissolved at low pH before a base was added as titrant to promote precipitation, subsequently small amounts of base or acid were added to form sub- and supersaturated states in solution, and the extent and duration of supersaturation was determined. Two trends were observed with regard to supersaturation behavior, which were reflected in the solid-state properties of the precipitated drug. Type 1 basic compounds, as described by the authors, exhibited a short-lived, but relatively much longer supersaturation period prior to precipitation compared to their Type 2 counterparts, which appeared to phase separate immediately above the equilibrium solubility of the crystalline form. Consequently, crystalline drug precipitated in experiments containing Type 1 compounds, where molecules supposedly had sufficient opportunity to rearrange themselves during supersaturation to later form a solid crystal, whereas the rapid onset of precipitation of Type 2 compounds gave rise to amorphous, randomly-oriented solid forms. This study provides a mechanistic explanation for the solid-state properties observed with precipitated weak bases by linking the amorphous or crystalline nature of precipitated drug to the intrinsic supersaturation phenomena of the compounds, reflected by one of the two observed trends (i.e., Type 1 or 2 compounds). However, whether this supersaturation behavior is affected by the complex and dynamic environment of the digestive tract remains unclear.

A follow up study by the above researchers examined the pH-induced precipitation of ionisable drugs, this time in the presence of polymers (86). These polymers were proposed to alter the supersaturation behavior intrinsic to the drugs tested, and this change in supersaturation behavior was achieved primarily via

the polymer-driven inhibition of nucleation, as discussed above. Drugs were selected based on their previously determined tendency to rapidly crystallize with the pH shift method. It was shown that whilst some drug-polymer combinations led to stable amorphous precipitates, others did not. Molecular complexity of the drug and the ability to form tautomers were cited as reasons why different polymers were able to maintain the amorphous forms of drugs such as glyburide and warfarin, whereas dipyridamole predominantly precipitated in crystalline form.

The case studies above indicate that the solid-state of some poorly water-soluble compounds is affected by the presence of digested lipids, however, there is a lack of understanding as to why these different solid forms are generated, and exactly what their implication is on drug absorption. A shortcoming of the solid-state analysis methods used thus far to characterize digested drug pellets is that no real information can be deduced with regard to the mechanistic aspects of the solid structures formed, instead CPLM and XRD data alone has pushed forward the notion that amorphous precipitates or polymorphs are formed exclusively. However, solid-state properties are complex and the dynamic compositional changes occurring during digestion provide for a number of other scenarios that could take place upon precipitation, which ultimately affects the solid-state and redissolution potential of these drugs, as well as total amount absorbed.

Amorphous Drug Precipitation or Other Solid-State Forms?

The amorphous and crystalline forms of a drug exhibit different dissolution properties, and these differences may have an impact on bioavailability. An amorphous solid consists of randomly oriented molecules and lacks the long-range order of thermodynamically favoured crystal forms (65, 87). As a result, amorphous forms have high free energy, display faster dissolution rates and have lower melting temperatures than crystalline forms (88),

which are generally all beneficial properties for oral drug delivery. A significant focus of industry has been to manufacture amorphous forms of drugs with an enhanced aqueous solubility and dissolution rate for solid oral dose forms; hence it makes sense that *in situ* generation of similar material should be seen as beneficial over precipitation in crystalline form. The studies presented in Table I that indicate precipitation of amorphous weak bases during the digestion of lipid formulations, in some cases suggest an implication of other components in the digested matrix. The precipitated material may or may not be amorphous drug per se, but could be in alternative amorphous solid-state forms, such as amorphous-salts or co-amorphous systems (87, 89). Moreover, precipitated drug also has the potential to undergo solution-mediated transformations, as well as taking part in excipient-drug interactions.

Whilst significant effort is geared towards stabilizing the supersaturated state (7, 20, 33, 34, 36, 42, 69, 72), it appears inevitable that precipitation is likely to occur at least to some degree *in vivo* with poorly water-soluble drugs in digestible formulations, especially at high drug loadings often seen with LBDDS. The precipitation of drug is highly dependent on the drug loading relative to the solubility of the drug in the different lipid excipients used in the formulation, and also the Type of formulation. For instance preparing a Type 2 formulation with drug loaded at 80% of its solubility in the formulation may be less likely to produce a precipitate on digestion, whereas a saturation level of 80% in a Type 3B formulation would be more likely to produce a precipitate. Hence, a challenge for formulation science is to control the solid state form of drug on precipitation during digestion and prevent rapid conversion to the crystalline form.

In consideration of the many ionisable species present during lipid digestion, the possibility of ion-pairing between precipitating drug and liberated fatty acids, bile salts or other excipients in the formulation has received no direct attention in the literature. The literature evidence to date indicates that, generally, basic drugs appear to precipitate from digesting lipid-based formulations in an amorphous form, while non-ionisable compounds appear to precipitate in a crystalline form, suggesting a possible link between ionisability and solid state form on precipitation, as has been suggested by Stillhart *et al*. (40) previously with the evidence presented in Table I largely supporting this logic. Therefore, it is reasonable to assume that weakly-basic drugs are precipitating during the digestion of lipid formulations as amorphous-salts with fatty acid as the counterion. Moreover, when the pKa values of these weakly-basic drugs, which appear to be precipitating in an amorphous form, are compared to the pH level of the digestion medium in their respective experiments, the link between ionisability and amorphous precipitation becomes more apparent. For example, cinnarizine has a pKa value of 7.47 and the *in vitro* lipolysis experiment performed by Sassene *et al*. (62), mentioned earlier, was set to pH 6.5. At this pH level cinnarizine would be predominantly ionized and available for pairing with oppositely-charged species that are present during digestion, most likely with fatty acids and bile salts, which will also be at least partly ionized at this pH. In contrast, the basic drug loratadine has a pKa value of 5.0 and showed quite different behavior in the study by Stillhart *et al*. (40), where in this case the pH of the digestion medium was set to 7.5 and the drug precipitated in crystalline form after digestion, presumably due to the un-ionized state of the drug at this pH and therefore an inability to form the amorphous salt on precipitation. Whilst the characterization techniques mentioned thus far in the case studies presented above are able to discriminate between amorphous and

crystalline drug, they are unable to elicit ionic-interactions, hence other characterization techniques are needed for this purpose. If this observation is founded in the chemistry as a general effect, it could potentially be utilized directly as a tool in formulation to induce precipitation of drug in an amorphous form.

Re-dissolution Potential and Dynamic Processing During Digestion

Controlling drug precipitation with regard to the solid state form can only be a driver of enhanced drug absorption if re-dissolution takes place *in vivo*. *In vitro* case studies have shown that drugs can precipitate in non-crystalline form, however, in closed *in vitro* models re-dissolution is not possible. Dynamic changes in composition and drug absorption *in vivo*, however, are anticipated to favor re-dissolution of precipitated drug, especially for high-energy solid forms, such as amorphous-salts of drug-fatty acids or drug-bile salt compositions discussed above. The proposed scenario would involve generation of a supersaturated state by the earlier mentioned mechanisms during dispersion and digestion of the formulation, inducing drug precipitation and simultaneous absorption of drug and digestion products. As this dual mechanism proceeds, the level of saturation will decrease and promote dissolution of the high-energy solid/precipitated drug (e.g., amorphous-salt). Meanwhile, digestion will continue to produce a high concentration of colloidal phases (mixed micelles and vesicles), which act to further facilitate the re-dissolution of drug. The role of colloidal structures formed during digestion and changes to their self-assembly and drug carrying capacity during the digestion process is of vital importance in generating the re-dissolution sink and mechanism of transport across the unstirred water layer. In order to consolidate the behavior in these systems beyond the observations to date, there remain two major challenges, namely

to determine the kinetics of drug precipitation during digestion using real-time methods, and to determine the composition of the precipitated material, for example whether drug is actually ion-pairing with fatty acids and/or bile salts.

Newly Emerging *In Situ* Characterization Techniques for Analyzing Precipitated Drug

The need for *in situ* methods that enable the detection and structural interpretation of precipitated drug during digestion is increasingly recognized. Recently, the kinetics of drug precipitation were studied using an *in situ* digestion model with in-line Raman spectroscopy (90). A SMEDDS formulation was loaded with the neutral drug fenofibrate at different concentrations, and *in vitro* digestion with simultaneous Raman was performed. Conventionally, Raman has been used to detect high concentrations of crystallized material in a constant medium, although in this case, a low drug concentration (precipitated drug) needed to be detected and differentiated from the hydrolysis products in a dynamic environment. The kinetics of fenofibrate precipitation was non-linear with respect to lipolysis of the formulation. This finding suggests a supersaturated state formed for several minutes prior to fenofibrate precipitation, and is in contrast to the linear relationship reported between lipolysis progression and the extent of precipitation by Sassene *et al.* with the weakly basic drug cinnarazine (62).

In addition to kinetics, *in situ* structural elucidation may also provide key insights into the solid state behavior of precipitated drug. The relevance of the solid material obtained from the pellet phase after digestion, subsequent to isolation and extraction, has been questioned in the past, due to sample handling and its potential to accelerate the precipitation of drug. To date, structural characterization using *in situ* lipolysis and SAXS has been limited to that of the colloidal phases produced on lipid digestion in a number of studies (23, 91–93). Recently,

however, *in situ* lipolysis and SAXS have also been used to follow the structure of precipitated fenofibrate during the *in vitro* digestion of a highly saturated medium chain lipid formulation, or super-SNEDDS (94), and will further be used to probe the precipitation behavior of ionisable compounds during the digestion of lipid formulations with regard to solid state. Similar time-resolved scattering approaches may involve performing experiments comparable to those reported by Hsieh *et al*. (86), where lipolysis was carried out and samples were taken at different time-points, rapidly centrifuged and placed in the SAXS beamline for analysis. This type of experiment may reduce the influence of sample preparation on drug precipitation, and may be described as *ex situ*. Overall, there is much scope to improve and develop new *in situ* techniques for following drug precipitation during digestion of lipid formulations, as studies look to such techniques for an understanding of what takes place in real-time (35, 95).

As outlined above, experiments that may lead to a mechanistic understanding as to why amorphous and other solid forms of drugs are precipitating during the digestion of lipid formulations is key to designing optimal lipid based formulations for poorly water-soluble compounds. From the trends observed for weakly-basic drugs in Table I, potential interactions between drugs and formulation excipients, as well as drugs and endogenous species in the digestive environment may affect the final solid-state, especially for ionisable compounds. Spectroscopic techniques, such as FTIR and NMR, can potentially reveal these key interactions. For example, if drug-fatty acid binding upon precipitation during digestion is expected to form an amorphous-salt, the resultant FTIR, or NMR spectra would elucidate this change via changes to the absorption bands of the functional groups involved in the ionic interaction.

Conclusion

Lipid based drug delivery has proven to be an effective means to deliver poorly water soluble compounds via the oral route. A major drawback of the formulation, however, remains the loss of solubilisation capacity upon dilution and dispersion in the gastrointestinal tract, which can lead to supersaturation and precipitation of drug. Traditionally, drug precipitation during gastrointestinal transit was believed to be detrimental to bioavailability, and conventional formulation approaches used to inhibit precipitation have been discussed here, nevertheless, recent studies have demonstrated that precipitation of drug is not always confined to poorly water-soluble crystal forms. Consequently, solid-state variability upon precipitation can lead to different bioavailability in some instances, especially where amorphous precipitation is observed. The potential effect of the digestion environment and formulation excipients on the final solid-state of precipitated drug have been explored, as well as the possibility of re-dissolution for precipitated high energy solid forms during digestion. Commonly used characterization methods for analyzing the solid-state of precipitated drug have been mentioned, moreover, the emergence of highly relevant *in situ* characterization techniques have been touched upon. It is becoming increasingly apparent that the solid-state of precipitated drug from lipid based formulations can act as a driver for the total amount of drug absorbed. A better understanding behind the mechanisms of drug precipitation during digestion, and why the solid-state of drug is often altered, are required. Thereby, controlling the solid-state form of precipitated drug during digestion (e.g., amorphous vs crystalline) may prove a valuable formulation strategy to increase the total amount of absorbed drug.

Additional information

From the small number of studies that have examined the solid-state form of precipitated drug during *in vitro* digestion experiments, the majority have looked at weakly-basic drugs. As discussed above, the apparent amorphous precipitation of some weakly-basic drugs during digestion brings into question the factors that lead to this potentially beneficial outcome. One of the aspects of this project was to examine whether ionic interactions between drug and oppositely charged species present during digestion leads to amorphous drug precipitation.

Moreover, the apparent correlation between the state of ionization of weakly-basic drugs during digestion and the solid-state form of the precipitate alludes to a potentially similar effect with weakly-acidic drugs. *In vitro* digestion experiments are typically conducted at pH 6.5 to be reflective of the intestinal environment, meaning the majority of weakly-acidic drugs would also be ionized to some extent. In the presence of oppositely charged-species, weakly-acidic drugs may also form ionic interactions to alter the solid-state form of precipitated drug. Exploring the precipitation behavior of weakly-acidic drugs was therefore another aspect of this project.

Hypotheses and Aims

Based on the current level of knowledge around the solid-state aspects of lipid based formulations the project hypotheses and aims for each experimental chapter were as follows:

Chapter 3: *In Situ* Lipolysis and Synchrotron Small-Angle X-ray Scattering for the Direct Determination of the Precipitation and Solid-State Form of a Poorly Water-Soluble Drug During Digestion of a Lipid-Based Formulation

Hypotheses

1. That synchrotron X-ray scattering could be used to monitor drug precipitation and identify the solid-state form of precipitated drug in real-time during *in vitro* digestion.
2. That the neutral, poorly water-soluble drug fenofibrate precipitates in a crystalline form in the absence of ionic interactions with oppositely charged species during *in vitro* digestion.

Aims

1. To develop a method capable of detecting drug precipitation and elucidating the solid-state form of precipitated drug in real-time during *in vitro* digestion.
2. To combine synchrotron SAXS and the *in vitro* lipolysis model to determine the precipitation kinetics of fenofibrate from a lipid based formulation.

Chapter 4: Lipid-Based Formulations Can Enable the Model Poorly Water-Soluble Weakly Basic Drug Cinnarizine To Precipitate in an Amorphous-Salt Form During *In vitro* Digestion

Hypotheses

1. That medium chain length fatty acids produced upon lipid digestion drive the precipitation of an amorphous fatty acid

salt for the weakly-basic drug cinnarizine through ionic interactions.

2. That the amorphous-salt precipitation of weakly-basic drugs during digestion is pH dependent and therefore affected by the state of drug ionization.

Aims

1. To characterize precipitated cinnarizine after performing *in vitro* lipolysis at pH levels above and below the pKa of the drug (pKa = 7.47).

2. To show using infra-red spectroscopy that medium chain fatty acids produced on digestion of the lipid formulation interact with the weakly-basic cinnarizine.

Chapter 5: Proof of Concept for the use of Cationic Surfactants to Alter the Solubility Profile of Poorly Water Soluble Acidic Drugs and the Solid-State Form of the Precipitate upon *In Vitro* Lipolysis

Hypotheses

1. That the weakly-acidic drug tolfenamic acid interacts with oppositely charged DDAB included in the starting formulation.

2. That the ionic interaction between drug and cationic surfactant affects the solid-state form of the precipitate.

Aims

1. To characterize precipitated tolfenamic acid after performing *in vitro* lipolysis on a model lipid formulation in the presence and absence of the cationic surfactant DDAB.

2. To examine the effect of DDAB on the solubilisation of tolfenamic acid during digestion via HPLC analysis of the digested contents.

References

1. Gursoy RN, Benita S. *Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs*. Biomed Pharmacother. 2004;**58**(3):173–82.

2. Chen S, Dudhedia MS, Wang Z, Darrington RT, Tamblyn T, Smoliga JA, et al. *Drug-excipient complexation in lipid based delivery systems: an investigation of the Tipranavir-1,3- dioctanolyglycerol complex*. J Pharm Sci. 2009;**98**(5):1732–43.

3. Ku MS. *Use of the biopharmaceutical classification system in early drug development*. AAPS J. 2008;**10**(1):208–12.

4. Lindenberg M, Kopp S, Dressman JB. *Classification of orally administered drugs on the World Health Organization Model list of Essential Medicines according to the biopharmaceutics classification system*. Eur J Pharm Biopharm. 2004;**58**(2):265–78.

5. Gupta S, Kesarla R, Omri A. *Formulation strategies to improve the bioavailability of poorly absorbed drugs with special emphasis on self-emulsifying systems*. ISRN Pharm. 2013;2013:848043.

6. Amidon GL, Lennernas H, Shah VP, Crison JR. *A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability.* Pharm Res. 1995;**12**(3):413–20.

7. Bevernage J, Brouwers J, Annaert P, Augustijns P. *Drug precipitation-permeation interplay: supersaturation in an absorptive environment*. Eur J Pharm Biopharm. 2012;**82**(2):424–8.

8. Lipinski CA. *Drug-like properties and the causes of poor solubility and poor permeability*. J Pharmacol Toxicol Methods. 2000;**44**(1): 235–49.

9. Pouton CW. *Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues*

and the lipid formulation classification system. Eur J Pharm Sci. 2006;**29**(3–4):278–87.

10. MacGregor KJ, Embleton JK, Lacy JE, Perry EA, Solomon LJ, Seager H, et al. *Influence of lipolysis on drug absorption from the gastro-intestinal tract*. Adv Drug Deliv Rev. 1997;**25**(1):33–46.

11. Dokoumetzidis A, Macheras P. *A century of dissolution research: from Noyes and Whitney to the biopharmaceutics classification system*. Int J Pharm. 2006;**321**(1–2):1–11.

12. Kawabata Y, Wada K, Nakatani M, Yamada S, Onoue S. *Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications*. Int J Pharm. 2011;**420**(1):1–10.

13. Grohganz H, Priemel PA, Lobmann K, Nielsen LH, Laitinen R, Mullertz A, et al. *Refining stability and dissolution rate of amorphous drug formulations*. Expert Opin Drug Deliv. 2014;**11**(6):977–89.

14. Mohsin K, Long MA, Pouton CW. *Design of lipid-based formulations for oral administration of poorly water-soluble drugs: precipitation of drug after dispersion of formulations in aqueous solution*. J Pharm Sci. 2009;**98**(10):3582–95.

15. Mu H, Holm R, Mullertz A. *Lipid-based formulations for oral administration of poorly water-soluble drugs*. Int J Pharm. 2013;**453**(1):215–24.

16. O'Driscoll CM, Griffin BT. *Biopharmaceutical challenges associated with drugs with low aqueous solubility–the potential impact of lipid-based formulations*. Adv Drug Deliv Rev. 2008;**60**(6):617–24.

17. Porter CJ, Pouton CW, Cuine JF, Charman WN. *Enhancing intestinal drug solubilisation using lipid-based delivery systems*. Adv Drug Deliv Rev. 2008;**60**(6):673–91.

18. Porter CJ, Trevaskis NL, Charman WN. *Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs.* Nat Rev Drug Discov. 2007;**6**(3):231–48.

19. Pouton CW. *Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems*. Eur J Pharm Sci. 2000;**11** Suppl 2:S93–8.

20. Williams HD, Trevaskis NL, Yeap YY, Anby MU, Pouton CW, Porter CJ. *Lipid-based formulations and drug supersaturation: harnessing the unique benefits of the lipid digestion/absorption pathway.* Pharm Res. 2013;**30**(12):2976–92.

21. Liao TH, Hamosh P, Hamosh M. *Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine.* Pediatr Res. 1984;**18**(5):402–9.

22. Holm R, Mullertz A, Mu H. *Bile salts and their importance for drug absorption.* Int J Pharm. 2013;**453**(1):44–55.

23. Phan S, Hawley A, Mulet X, Waddington L, Prestidge CA, Boyd BJ. *Structural aspects of digestion of medium chain triglycerides studied in real time using sSAXS and Cryo-TEM.* Pharm Res. 2013;**30**(12):3088–100.

24. Trevaskis NL, Porter CJ, Charman WN. *Bile increases intestinal lymphatic drug transport in the fasted rat*. Pharm Res. 2005;**22**(11): 1863–70.

25. Yeap YY, Trevaskis NL, Quach T, Tso P, Charman WN, Porter CJ. *Intestinal bile secretion promotes drug absorption from lipid colloidal phases via induction of supersaturation*. Mol Pharm. 2013;**10**(5):1874–89.

26. Kossena GA, Charman WN, Boyd BJ, Porter CJ. *Influence of the intermediate digestion phases of common formulation lipids on the absorption of a poorly water-soluble drug*. J Pharm Sci. 2005;**94**(3): 481–92.

27. van Mourik ID, Thomson M, Kelly DA. *Comparison of pharmacokinetics of Neoral and Sandimmune in stable pediatric liver transplant recipients*. Liver Transpl Surg. 1999;**5**(2):107–11.

28. Han SF, Yao TT, Zhang XX, Gan L, Zhu C, Yu HZ, et al. *Lipid-based formulations to enhance oral bioavailability of the poorly water-soluble drug anethol trithione: effects of lipid composition and formulation*. Int J Pharm. 2009;**379**(1):18–24.

29. Humberstone AJ, Charman WN. *Lipid-based vehicles for the oral delivery of poorly water soluble drugs*. Adv Drug Deliv Rev. 1997;**25**(1):103–28.

30. Chakrabarti S, Belpaire FM. *Biovailability of phenytoin in lipid containing dosage forms in rats*. J Pharm Pharmacol. 1978;**30**(5): 330–1.

31. Carrigan PJ, Bates TR. *Biopharmaceutics of drugs administered in lipid-containing dosage forms. I. GI absorption of griseofulvin from an oil-in-water emulsion in the rat*. J Pharm Sci. 1973;**62**(9):1476–9.

32. Porter CJ, Kaukonen AM, Taillardat-Bertschinger A, Boyd BJ, O'Connor JM, Edwards GA, et al. *Use of in vitro lipid digestion data to explain the in vivo performance of triglyceride-based oral lipid formulations of poorly water-soluble drugs: studies with halofantrine*. J Pharm Sci. 2004;**93**(5):1110–21.

33. Brouwers J, Brewster ME, Augustijns P. *Supersaturating drug delivery systems: the answer to solubility-limited oral bioavailability?* J Pharm Sci. 2009;**98**(8):2549–72.

34. Anby MU, Williams HD, McIntosh M, Benameur H, Edwards GA, Pouton CW, et al. *Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilization on the in vitro and in vivo performance of self-emulsifying drug delivery systems*. Mol Pharm. 2012;**9**(7):2063–79.

35. Arnold YE, Imanidis G, Kuentz MT. *Advancing in-vitro drug precipitation testing: new process monitoring tools and a kinetic nucleation and growth model*. J Pharm Pharmacol. 2011;**63**(3):333–41.

36. Warren DB, Benameur H, Porter CJ, Pouton CW. *Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: a mechanistic basis for utility*. J Drug Target. 2010;**18**(10):704–31.

37. Lindfors L, Forssen S, Westergren J, Olsson U. *Nucleation and crystal growth in supersaturated solutions of a model drug*. J Colloid Interface Sci. 2008;**325**(2):404–13.

38. Bevernage J, Brouwers J, Brewster ME, Augustijns P. *Evaluation of gastrointestinal drug supersaturation and precipitation: strategies and issues*. Int J Pharm. 2013;**453**(1):25–35.

39. Kostewicz ES, Wunderlich M, Brauns U, Becker R, Bock T, Dressman JB. *Predicting the precipitation of poorly soluble weak bases upon entry in the small intestine*. J Pharm Pharmacol. 2004;**56**(1):43–51.

40. Stillhart C, Durr D, Kuentz M. *Toward an improved understanding of the precipitation behavior of weakly basic drugs from oral lipid-based formulations*. J Pharm Sci. 2014; **103**(4):1194-203

41. Yeap YY, Trevaskis NL, Porter CJ. *The potential for drug supersaturation during intestinal processing of lipid-based formulations may be enhanced for basic drugs*. Mol Pharm. 2013;**10**(7):2601–15.

42. Yeap YY, Trevaskis NL, Porter CJ. *Lipid absorption triggers drug supersaturation at the intestinal unstirred water layer and promotes drug absorption from mixed micelles.* Pharm Res. 2013;**30**(12): 3045–58.

43. Kashchiev D. *Forms and applications of the nucleation theorem*. J Chem Phys. 2006;**125**(1):014502.

44. Horn D, Rieger J. *Organic nanoparticles in the aqueous phase-theory, experiment, and use*. Angew Chem Int Ed Engl. 2001;**40**(23):4330–61.

45. Rodriguez-Hornedo N, Murphy D. *Significance of controlling crystallization mechanisms and kinetics in pharmaceutical systems*. J Pharm Sci. 1999;**88**(7):651–60.

46. JW, M, *Crystallisation*. Oxford: Butterworth-Heinemann; 2001.

47. Dressman JB, Berardi RR, Dermentzoglou LC, Russell TL, Schmaltz SP, Barnett JL, et al. *Upper gastrointestinal (GI) pH in young, healthy men and women*. Pharm Res. 1990;**7**(7):756–61.

48. Gargouri Y, Moreau H, Verger R. *Gastric lipases: biochemical and physiological studies*. Biochim Biophys Acta. 1989;**1006**(3):255–71.

49. Zangenberg NH, Mullertz A, Kristensen HG, Hovgaard L. *A dynamic in vitro lipolysis model. I. Controlling the rate of lipolysis by continuous addition of calcium*. Eur J Pharm Sci. 2001;**14**(2):115–22.

50. Sek L, Porter CJ, Kaukonen AM, Charman WN. *Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products.* J Pharm Pharmacol. 2002;**54**(1):29–41.

51. Devraj R, Williams HD, Warren DB, Mullertz A, Porter CJ, Pouton CW. *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.* Int J Pharm. 2013;**441**(1– 2):323–33.

52. Shono Y, Jantratid E, Dressman JB. *Precipitation in the small intestine may play a more important role in the in vivo performance of poorly soluble weak bases in the fasted state: case example nelfinavir*. Eur J Pharm Biopharm. 2011;**79**(2):349–56.

53. Thomas N, Richter K, Pedersen TB, Holm R, Mullertz A, Rades T. *In vitro lipolysis data does not adequately predict the in vivo performance of lipid-based drug delivery systems containing fenofibrate*. AAPS J. 2014.

54. Williams HD, Sassene P, Kleberg K, Bakala-N'Goma JC, Calderone M, Jannin V, et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 1: method parameterization and comparison of in vitro digestion profiles across a range of representative formulations*. J Pharm Sci. 2012;**101**(9):3360–80.

55. Williams HD, Anby MU, Sassene P, Kleberg K, Bakala-N'Goma JC, Calderone M, et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations. 2. The effect of bile salt concentration and drug loading on the performance of type I, II, IIIA, IIIB, and IV formulations during in vitro digestion.* Mol Pharm. 2012;**9**(11):3286–300.

56. Williams HD, Sassene P, Kleberg K, Calderone M, Igonin A, Jule E, et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 3: understanding supersaturation versus precipitation potential during the in vitro digestion of type I, II, IIIA, IIIB and IV lipid-based formulations*. Pharm Res. 2013;**30**(12):3059–76.

57. Thomas N, Holm R, Rades T, Mullertz A. *Characterising lipid lipolysis and its implication in lipid-based formulation development*. AAPS J. 2012;**14**(4):860–71.

58. Salentinig S, Salentinig S, Tangso KJ, Hawley A, Boyd BJ. *pH-driven colloidal transformations based on the vasoactive drug nicergoline*. Langmuir. 2014;**30**(49):14776–81.

59. Arnold YE, Imanidis G, Kuentz M. *Study of drug concentration effects on in vitro lipolysis kinetics in medium-chain triglycerides by considering oil viscosity and surface tension*. Eur J Pharm Sci. 2011;**44**(3):351–8.

60. Harris KD. *Powder diffraction crystallography of molecular solids*. Top Curr Chem. 2012;**315**:133–77.

61. RA, C. *Chapter 2: polarized light microscopy, in pharmaceutical microscopy*. 2011. p. 321p. 139 illus, 102 illus. in color.

62. Sassene PJ, Knopp MM, Hesselkilde JZ, Koradia V, Larsen A, Rades T, et al. *Precipitation of a poorly soluble model drug during in vitro lipolysis: characterization and dissolution of the precipitate*. J Pharm Sci. 2010;**99**(12):4982–91.

63. Carstensen JT, Lai TY, Prasad VK. *USP dissolution IV: comparison of methods*. J Pharm Sci. 1978;**67**(9):1303–7.

64. Thomas N, Holm R, Mullertz A, Rades T. *In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS)*. J Control Release. 2012;**160**(1):25–32.

65. Savolainen M, Kogermann K, Heinz A, Aaltonen J, Peltonen L, Strachan C, et al. *Better understanding of dissolution behaviour of amorphous drugs by in situ solid-state analysis using Raman spectroscopy*. Eur J Pharm Biopharm. 2009;**71**(1):71–9.

66. Verdonck E, Schaap K, Thomas LC. *A discussion of the principles and applications of Modulated Temperature DSC (MTDSC)*. Int J Pharm. 1999;**192**(1):3–20.

67. Gill P, Moghadam TT, Ranjbar B. *Differential scanning calorimetry techniques: applications in biology and nanoscience*. J Biomol Tech. 2010;**21**(4):167–93.

68. Patel DD, Anderson BD. *Effect of precipitation inhibitors on indomethacin supersaturation maintenance: mechanisms and modeling.* Mol Pharm. 2014;**11**(5):1489–99.

69. Warren DB, Bergstrom CA, Benameur H, Porter CJ, Pouton CW. *Evaluation of the structural determinants of polymeric precipitation inhibitors using solvent shift methods and principle component analysis*. Mol Pharm. 2013;**10**(8):2823–48.

70. DiNunzio JC, Miller DA, Yang W, McGinity JW, Williams 3rd RO. *Amorphous compositions using concentration enhancing polymers for improved bioavailability of itraconazole*. Mol Pharm. 2008;**5**(6):968–80.

71. Rupprecht H, Ziller KH. *Characterization of the crystallization behavior of poorly soluble drugs in suspensions*. Pharmazie. 1981;**36**(4):298.

72. Augustijns P, Brewster ME. *Supersaturating drug delivery systems: fast is not necessarily good enough.* J Pharm Sci. 2012;**101**(1):7–9.

73. Machefer S, Huddar MM, Schnitzlein K. *Effect of polymer admixtures on the growth habit of ionic crystals. Study on crystal growth kinetics of potassium dihydrogen phosphate in water/polyol mixtures*. J Cryst Growth. 2008;**310**(24):5347–56.

74. Raghavan SL, Trividic A, Davis AF, Hadgraft J. *Crystallization of hydrocortisone acetate: influence of polymers*. Int J Pharm. 2001;**212**(2):213–21.

75. Gao P, Akrami A, Alvarez F, Hu J, Li L, Ma C, et al. *Characterization and optimization of AMG 517 Supersaturatable Self-Emulsifying Drug Delivery System (S-SEDDS) for improved oral absorption*. J Pharm Sci. 2009;**98**(2):516–28.

76. Psachoulias D, Vertzoni M, Goumas K, Kalioras V, Beato S, Butler J, et al. *Precipitation in and supersaturation of contents of the upper small intestine after administration of two weak bases to fasted adults*. Pharm Res. 2011;**28**(12):3145–58.

77. Carlert S, Akesson P, Jerndal G, Lindfors L, Lennernas H. Abrahamsson, B, *In vivo dog intestinal precipitation of mebendazole: a basic BCS class II drug*. Mol Pharm. 2012;**9**(10): 2903–11.

78. Psachoulias D, Vertzoni M, Butler J, Busby D, Symillides M, Dressman J, et al. *An in vitro methodology for forecasting luminal concentrations and precipitation of highly permeable lipophilic weak bases in the fasted upper small intestine*. Pharm Res. 2012;**29**(12):3486–98.

79. Larsen AT, Sassene P, Mullertz A. *In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems*. Int J Pharm. 2011;**417**(1–2):245–55.

80. Thomas N, Holm R, Garmer M, Karlsson JJ, Mullertz A, Rades T. *Supersaturated self-nanoemulsifying drug delivery systems (Super-SNEDDS) enhance the bioavailability of the poorly water-soluble drug simvastatin in dogs*. AAPS J. 2013;**15**(1):219–27.

81. Boyd BJ, Khoo SM, Whittaker DV, Davey G, Porter CJ. *A lipid-based liquid crystalline matrix that provides sustained release and enhanced oral bioavailability for a model poorly water soluble drug in rats*. Int J Pharm. 2007;**340**(1–2):52–60.

82. Kaukonen AM, Boyd BJ, Porter CJ, Charman WN. *Drug solubilization behavior during in vitro digestion of simple triglyceride lipid solution formulations*. Pharm Res. 2004;**21**(2):245–53.

83. Kossena GA, Charman WN, Boyd BJ, Porter CJ. *A novel cubic phase of medium chain lipid origin for the delivery of poorly water soluble drugs*. J Control Release. 2004;**99**(2):217–29.

84. Larsen AT, Ohlsson AG, Polentarutti B, Barker RA, Phillips AR, Abu-Rmaileh R, et al. *Oral bioavailability of cinnarizine in dogs: relation to SNEDDS droplet size, drug solubility and in vitro precipitation*. Eur J Pharm Sci. 2013;**48**(1–2):339–50.

85. Hsieh YL, Ilevbare GA, Van Eerdenbrugh B, Box KJ, Sanchez-Felix MV, Taylor LS. *pH-induced precipitation behavior of weakly basic compounds: determination of extent and duration of supersaturation using potentiometric titration and correlation to solid state properties*. Pharm Res. 2012;**29**(10):2738–53.

86. Hsieh YL, Box K, Taylor LS. *Assessing the impact of polymers on the pH-induced precipitation behavior of poorly water soluble compounds using synchrotron wide angle x-ray scattering*. J Pharm Sci. 2014;**103**(9):2724–35.

87. Lobmann K, Laitinen R, Grohganz H, Gordon KC, Strachan C, Rades T. *Coamorphous drug systems: enhanced physical stability and dissolution rate of indomethacin and naproxen*. Mol Pharm. 2011;**8**(5):1919–28.

88. Willart JF, Descamps M. *Solid state amorphization of pharmaceuticals*. Mol Pharm. 2008;**5**(6):905–20.

89. Miroshnyk I, Mirza S, Sandlert N. *Pharmaceutical co-crystals an opportunity for drug product enhancement*. Expert Opin Drug Deliv. 2009;**6**(4):333–41.

90. Stillhart C, Imanidis G, Kuentz M. *Insights into drug precipitation kinetics during in vitro digestion of a lipid-based drug delivery system using in-line raman spectroscopy and mathematical modeling*. Pharm Res. 2013;**30**(12):3114–30.

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

92. Salentinig S, Phan S, Khan J, Hawley A, Boyd BJ. *Formation of highly organized nanostructures during the digestion of milk*. ACS Nano. 2013;**7**(12):10904–11.

93. Phan S, Salentinig S, Prestidge CA, Boyd BJ. *Self-assembled structures formed during lipid digestion: characterization and implications for oral lipid-based drug delivery systems*. Drug Deliv Transl Res. 2014;**4**(3):275–94.

94. Khan J, Hawley A. Rades T, Boyd BJ. *In situ lipolysis and synchrotron small-angle x-ray scattering for the direct determination of the precipitation and solid-state form of a poorly water-soluble drug during digestion of a lipid-based formulation*. J Pharm Sci. 2015; **105**(9):2631–9

95. Ueda H, Ida Y, Kadota K, Tozuka Y. *Raman mapping for kinetic analysis of crystallization of amorphous drug based on distributional images*. Int J Pharm. 2014;**462**(1–2):115–

Chapter 2:
General Methods

The foundation for the work carried out during this project was performing *in vitro* digestion experiments on lipid based formulations containing drugs of interest. All subsequent experimental work looked at different aspects of what was taking place during the digestion experiments. From the digestion experiment itself the extent of formulation digested and digestion kinetics were gauged, but other techniques were later employed to investigate the fate of drug, precipitation behaviour and drug-excipient interactions. For example, to probe the distribution of drug between the aqueous colloidal phase that is formed during digestion and the precipitated fraction, samples were taken throughout the experiment and phase separated by centrifugation (see Figure 1). The drug was then quantified in each individual phase via HPLC assay. To examine the morphology of the precipitated drug, the pellet phase was isolated and visualised using CPLM. The pellet was also used to identify the solid-state form of the drug upon precipitation, using XRD, and to test for ionic interactions between drug and oppositely charged components, using FTIR. In the case of chapter 3, *in vitro* digestion and synchrotron SAXS were performed *in situ* to monitor drug precipitation kinetics and solid-state form in real-time during digestion. The general methods for each aspect are outlined below. For more detailed information about the methods and how they relate to specific formulations, or drugs tested, please refer to the methods section of the experimental chapters.

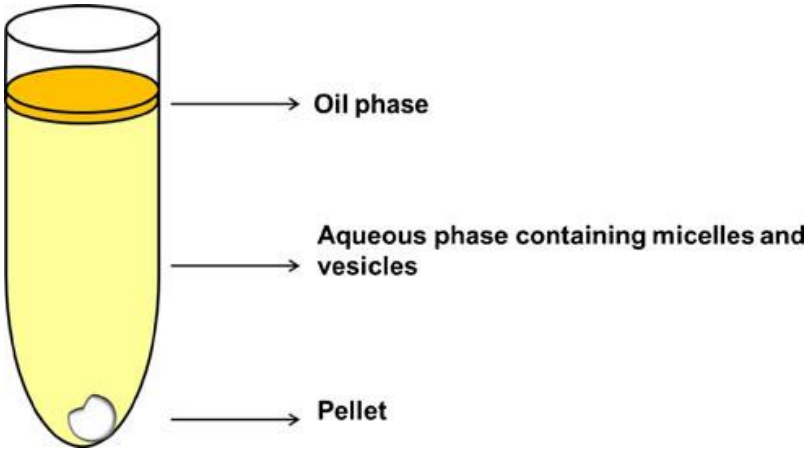

Figure 1: Samples taken during *in vitro* digestion experiments were centrifuged to produce different phases, which could consist of an undigested oil phase, an aqueous colloidal phase and the precipitated pellet phase (1). These phases were then subject to further analysis using different techniques. Image reproduced with permission from Springer.

General Materials

Lipids

Captex 355® [MCT composed of 59% caprylic acid (C_8),40% capric acid (C_{10}), <1% lauric acid (C_{12}) as stated in the product information], and Capmul MCM® [mono/diglycerides composed of caprylic acid (C_8) in glycerol], were obtained from Abitec Corporation (Janesville, Wisconsin). Cremophor EL® was purchased from BASF Corporation (Washington, New Jersey). Soybean oil was purchased from Sigma-Aldrich (St. Louis, Missouri). Maisine 35-1 was obtained from Gattefosse (Lyon, France).

Digestion Materials

Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate, >95%), and 4-bromophenylboronic acid (4-BPBA, >95%) were purchased from Sigma-Aldrich (St. Louis, Missouri). Calcium chloride dihydrate (>99%) was obtained from Ajax Finechem (Seven Hills, New South Wales, Australia). Sodium chloride (>99%) was purchased from Chem Supply (Gillman, South Australia, Australia). Phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC)

was obtained from Trapeze Association Pty. Ltd. (Clayton, Victoria, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia).

Drugs

Fenofibrate was purchased from AK Scientific (Union City, California). Cinnarizine was purchased from Sigma-Aldrich (St. Louis, Missouri). Cinnarizine hydrochloride was synthesised from the free base and hydrochloric acid, the salt was then confirmed by FTIR. Tolfenamic acid was purchased from AK Scientific (Union City, CA). Loratadine was purchased from Haihang Industry Co. Ltd. (Jinuan City, China).

Solvents

HPLC grade methanol and acetonitrile, analysis grade chloroform, and absolute ethanol were purchased from Merck (MA, USA).

In vitro Digestion Experiments

Preparation of digestion buffer, digestion medium and pancreatic lipase

The digestion buffer consisted of 2 mM Tris–maleate, 1.4 mM $CaCl_2 \cdot 2H_20$, and 150 mM NaCl, made up to the appropriate concentration using Milli Q water. The digestion buffer was adjusted to pH 6.5 using NaOH and HCl solutions. The digestion medium was prepared using the digestion buffer, by supplementing the solution with 5 mM NaTDC and 1.25 mM DOPC. This final solution represented fasted state intestinal fluid (2). Briefly, the DOPC was weighed into a round-bottom flask and dissolved in a small amount of chloroform. The chloroform was removed under vacuum at 45 °C to yield a thin layer of DOPC coated on the bottom of the flask. The NaTDC was added to the flask containing the DOPC and digestion buffer was added, before the contents were left

to sonicate until all particles had dissolved (roughly 20 min). The final solution was kept refrigerated at 4°C before use.

To prepare the pancreatic lipase solution, the raw pancreatin extract was weighed (2 – 4g, depending on activity of enzyme) into a small glass beaker, then digestion buffer (5 mL) was added to form a suspension. The contents were stirred for 5 min, transferred to a 12 mL plastic tube and centrifuged at 2205 g for 15 min at 4 °C. The supernatant was collected (1000 TB units/mL of digest) and stored at 4°C prior to use.

Preparation of Lipid Formulations

Excipients that constituted the lipid formulations were weighed and combined into a glass scintillation vial and vortexed for 1 min to mix all components. Relevant drugs were weighed into a separate scintillation vial, before the blank lipid formulation, as described above, was added to the drug. The mixture was further vortexed before it was subject to heating and mixing. More thorough explanation of how each formulation was prepared is provided in the methods section of each experimental chapter.

In vitro Lipolysis

Lipolysis was conducted according to previously established methods (3, 4). A jacketed glass vessel was attached to a waterbath, which maintained the experimental temperature at 37 °C. The vessel sat atop a Metrohm stirring unit, which was attached to a Metrohm titrator, 5 mL dose unit and an iUnitrode pH probe (Metrohm AG, Herisau, Switzerland). Generally, 36 mL of digestion medium was added to the glass vessel and stirred magnetically, followed by the formulation to be digested. The digestion medium and formulation were allowed to mix for 5 min, which was considered the dispersion period. The physical setup of the *in vitro* digestion apparatus is presented in Figure 2.

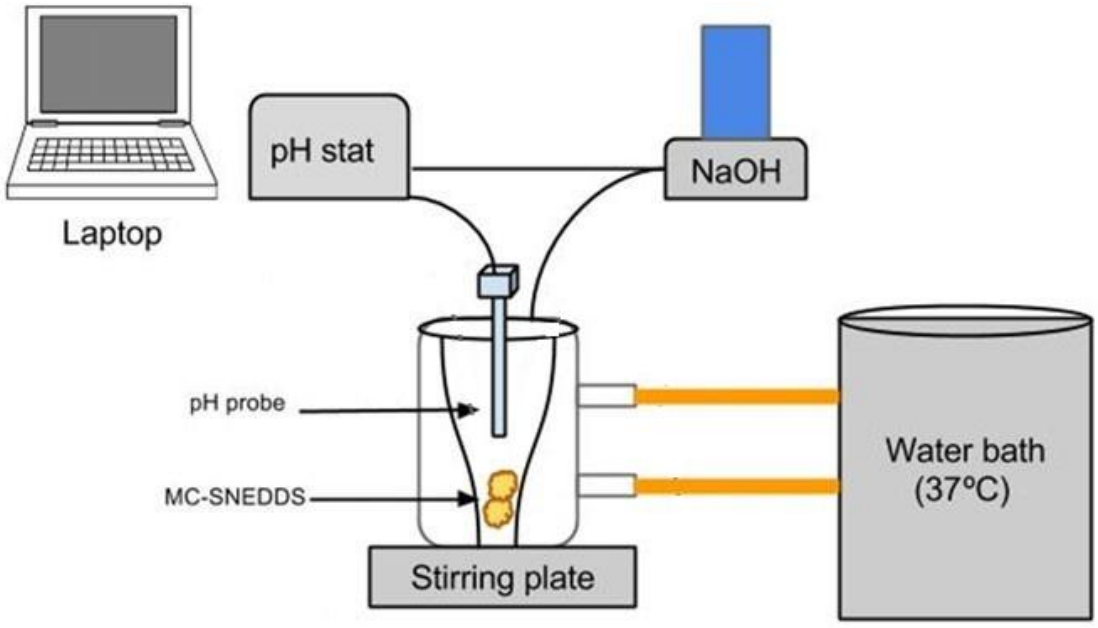

Figure 2: Physical setup of the *in vitro* lipolysis experiment.

Up to 30% of total lipid digestion can occur in the stomach, due to the presence of gastric lipase (5). The majority of lipid digestion, however, takes place in the upper small intestine, where drug absorption and lipid self-assembly processes also occur, and the risk of drug precipitation is at its highest. Therefore, performing *in vitro* intestinal lipolysis was the priority, but the effects of gastric lipolysis on subsequent drug precipitation behaviour would ideally be tested. In the current work, however, it was deemed infeasible to perform a gastric lipolysis step, mainly because gastric lipase is not commercially available (6). In addition, the low pH conditions required to simulate gastric conditions places a limitation on the direct quantification of liberated fatty acid using the pH-stat method, as fatty acids need to be ionised for this purpose (6). Moreover, the focus of the current work was drug precipitation and changes to the solid-state form of the precipitate, due to interactions with digestion products. While a fraction of the formulation would be digested under gastric conditions, drug precipitation is unlikely to occur for lipid-rich formulations,

such as those examined in the following chapters. The solubilisation capacity of lipid formulations can decrease upon dispersion to a degree, but a significant decrease in solubilisation capacity to the point where drug precipitation can occur is expected to take place largely in the upper small-intestine for the formulations examined here (7), with the exception of the Type 3B lipid formulation examined in chapter 5.

During the dispersion period the pH was re-adjusted to experimental pH (usually pH 6.5 except for specific experiments in chapter 4) using NaOH and HCl solutions. Following the 5 min dispersion period, 4 mL of pancreatic lipase solution was added to initiate digestion (to achieve an overall activity of 1000 TB units/mL of digest). The decrease in pH, as a result of fatty acid production during digestion, was titrated with 0.6 M NaOH solution dispensed by the automated dose unit maintaining a pH of 6.5. Lipolysis experiments were set to run for 60 min, after which the digested contents were analysed. For more detailed information about how *in vitro* lipolysis was conducted see the methods section in the subsequent experimental chapters.

Post-Digestion Analysis of Drug

Sample Preparation for HPLC Phase Distribution Studies

To quantify the amount of drug dissolved in the digested contents and the amount of drug precipitated, aliquots (200 µL) were taken at various time points throughout the digestion experiments. These aliquots were accurately pipetted into 1.75 mL Eppendorf tubes prepared with 20 µL of lipase inhibitor (0.05 M 4-BPBA), which were centrifuged for 1 hr at 7708 g. After centrifuging, the supernatant from the samples was transferred to another Eppendorf tube, and this constituted the aqueous colloidal phase of the digestion sample. The remaining pellet phase

consisted of precipitated calcium-fatty acid soaps and precipitated drug.

The aqueous colloidal phase was diluted as necessary with mobile phase and then analysed by HPLC. The pellet phases were first dissolved in a suitable solvent, before they were diluted with mobile phase and analysed by HPLC. The specifications for the HPLC system used and further information on the individual assays for drugs tested can be found in the methods section of the experimental chapters.

Determining the Solid-State Form of the Precipitate using XRD

During XRD measurements a solid sample is subject to X-rays, which scatter according to the unique crystal structure of the sample in question. The X-rays scatter at certain angles due to the nature of the sample, and this information can be used to identify compounds and their different solid-state forms. The application of XRD for determining solid-state structure of precipitated drug after digestion has been demonstrated previously, as was discussed in chapter 1.

Powder XRD was used for some aspects of this project. The three-dimensional scattering information collected from the numerous single crystallites analysed during powder XRD was collated into a one-dimensional intensity vs. diffraction angle (2θ) reading. The diffraction peaks and their position on the 2θ scale were used as a qualitative measurement of drug compounds and precipitated drug for the subsequent chapters.

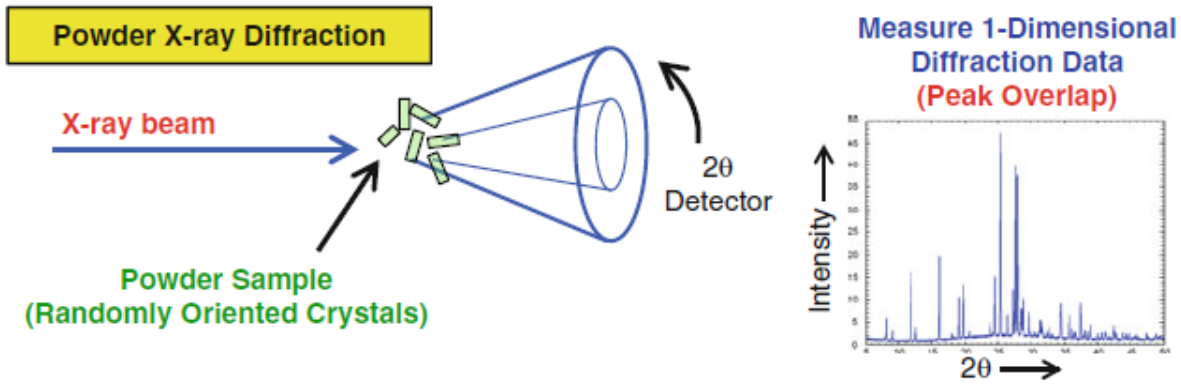

Figure 3: X-rays pass through many single crystallites to produce many 3-dimensional scattering patterns, which are combined to give a 1-dimensional intensity vs. 2θ diffractogram (8).

For all XRD measurements a Bruker D8 Advance X-ray diffractometer with Ni-filtered Cu Kα radiation (1.54 Å) at 40 kV and 40 mA was used to collect XRD data. The samples were analysed in the range of 5–50° in 2θ, with a step size of 0.02° and a scan rate of 0.5 s per step.

Neat crystalline drugs (starting materials) were measured by evenly placing a thin layer of material (~ 20 mg) on the XRD sample holder. For analysis of precipitated drug during *in vitro* digestion experiments the pellet phases were first isolated at the end of lipolysis. The pellet was then spread over a glass microscope with a small spatula and allowed to air dry for approximately one hour prior to the XRD measurement. For all XRD measurements of pellet phases the corresponding *in vitro* digestion experiment was carried out on the same day.

For instances where drug precipitated in an amorphous form there was a clear absence of diffraction peaks corresponding to the neat crystalline drug. A large halo region in the diffractogram was instead observed. This halo region signifies a lack of crystallinity in the sample and is characteristic of amorphous materials that lack long-range order.

In situ Lipolysis and Synchrotron SAXS

All SAXS experiments were conducted at the SAXS/WAXS beamline at the Australian Synchrotron. The physical setup of the flow-through *in vitro* digestion apparatus at the beamline was similar to what has been used previously to determine the evolution of colloidal structures formed from the digestion of lipids (9). Briefly, the setup consisted of a peristaltic pump that was used to move the contents of the digestion vessel through silicone tubing (total volume <1 mL), which led to a 1.5-mm diameter quartz capillary mounted in the X-ray beam, at a flow rate of approximately 10 mL/min. The additional elements that make up the in situ lipolysis and SAXS technique are presented in Figure 4.

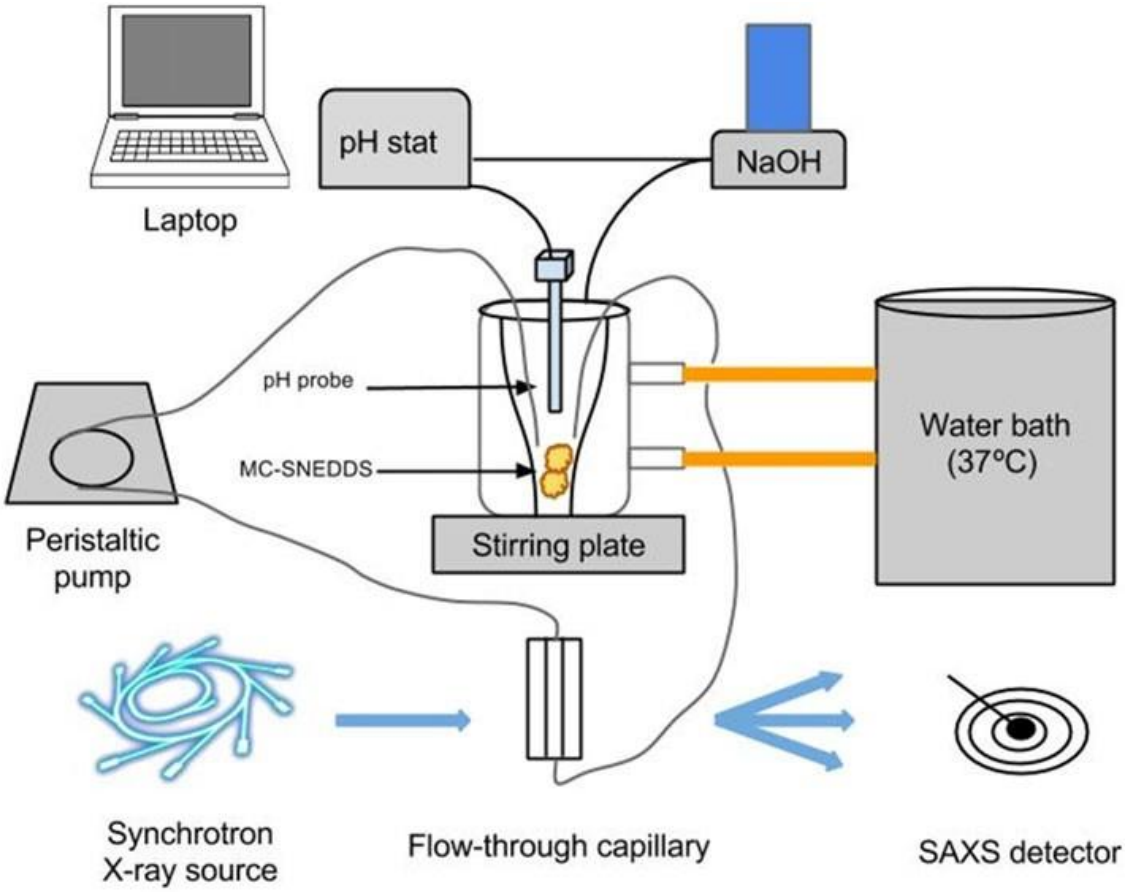

Figure 4: Physical setup of the *in situ* lipolysis and synchrotron SAXS technique.

During SAXS experiments a collimated X-ray beam of a certain wavelength (λ) passes through a sample and is scattered according to the distribution of electron densities in the sample. Typically, SAXS provides structural information for samples in the size range of one to several hundred nanometres. The resultant scattering pattern is converted to a plot of intensity vs. the magnitude of the scattering vector (q), where $q = (4\pi/\lambda)\sin(\theta/2)$. Bragg's Law, $2d\sin\theta = n\lambda$, where d is the interplanar distance between two reflecting planes and λ is the wavelength, describes the arrangement of molecules in a crystal lattice.

The ratio of reciprocal spacings of the Bragg peaks can be used to elucidate the presence of liquid-crystalline structures. In this project, however, SAXS was used as a high-sensitivity method for detecting and determining the solid-state form of precipitated drug during *in vitro* digestion experiments. Conventionally, a separate WAXS detector might be considered for this purpose, as crystalline solids scatter X-rays at wide angles relative to less ordered liquid-crystalline structures. Figure 5 shows how the scattering of the incident X-ray beam at different angles can inform of different types of structures. Relatively large-scale structures, such as colloids and liquid crystals, scatter the X-ray beam at small angles, whereas highly ordered smaller-scale structures, such as crystalline drug, scatter the beam at much wider angles.

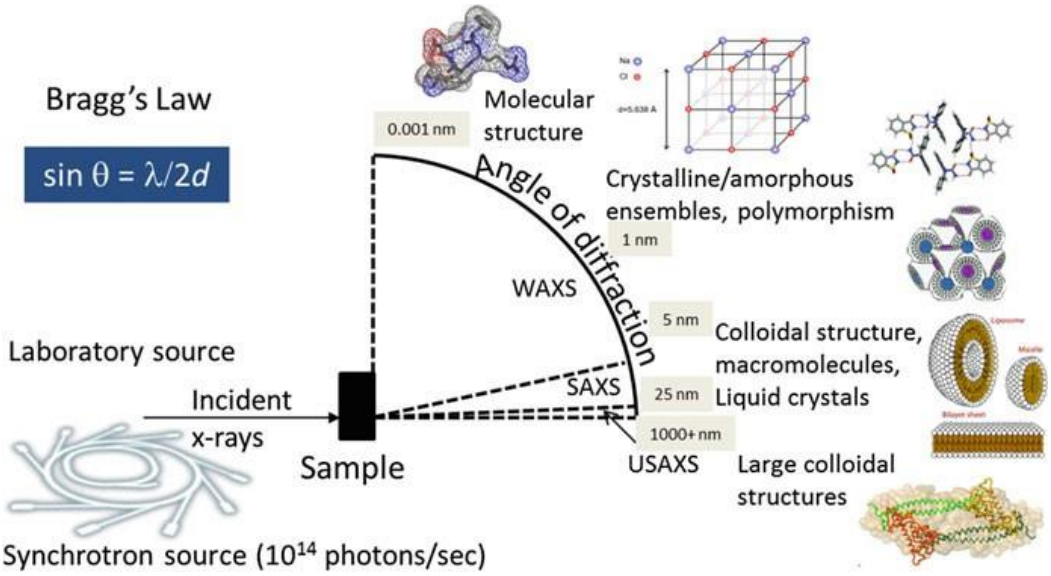

Figure 5: Overview of the relationship between structural dimensions, scattering angle and terminology of techniques. Reproduced with permission (10).

The problem with performing WAXS to examine precipitated drug, however, is the decreased sensitivity to the small amounts of precipitated drug present during the digestion of lipid formulations. The detector for WAXS at the Australian Synchrotron is not fully radial, as opposed to the detector for SAXS, but is rather a vertical strip that captures only a segment of the total sample scattering. Therefore, much more sample is required to achieve similar intensity of peak diffractions, whereas smaller amounts of sample can be assessed with SAXS, as the full radial integration results in much higher sensitivity. For this reason the physical setup of the beamline had to be customised in order to detect scattering at a wide enough angle to pick up crystalline precipitated drug while using the SAXS detector.

To achieve this the sample to detector distance was decreased from a typical SAXS experiment of 1015 mm to 567.5 mm. By shortening the distance of sample to detector the detectable q-range shifted from $0.01 < q < 0.7$ Å^{-1} to $0.03 < q < 1.7$ Å^{-1}. As a result the first few characteristic diffraction peaks for the crystalline drugs could be observed in the scattering profile. An X-ray beam with a wavelength of 1.1271 Å (11 keV) was used. A 5

sec acquisition period was used with a 15 sec delay between frames, providing a diffractogram every 20 sec during digestion. The 2D SAXS patterns were acquired using a Pilatus 1M detector with a pixel size of 172 µm integrated into the one-dimensional scattering function I(q) using the in-house developed software package ScatterBrain. The pancreatic lipase solution was added using a remotely actuated syringe driver during acquisition.

Visualising the Morphology of Precipitated Drug using CPLM

The crystalline morphology of precipitated drug was assessed by visualising the isolated pellet phases from digestion samples under crossed-polarised light. Crystalline materials exhibit birefringence, due to their anisotropic nature, which makes them visible during CPLM. Birefringence refers to a property displayed by doubly-refracting materials, where polarised light from a light source travels through the material and is refracted in more than one direction, before the refracted light is recombined at the second polariser and can be viewed as an illumination of the material through the microscope (11). In contrast, isotropic, or non-birefringent structures, refract light in one direction only. Amorphous materials that lack the long-range order of crystalline materials are non-birefringent. When these amorphous materials are visualised under crossed-polarised light they cannot be distinguished as there is no recombination of doubly-refracted light to produce an illumination of the material.

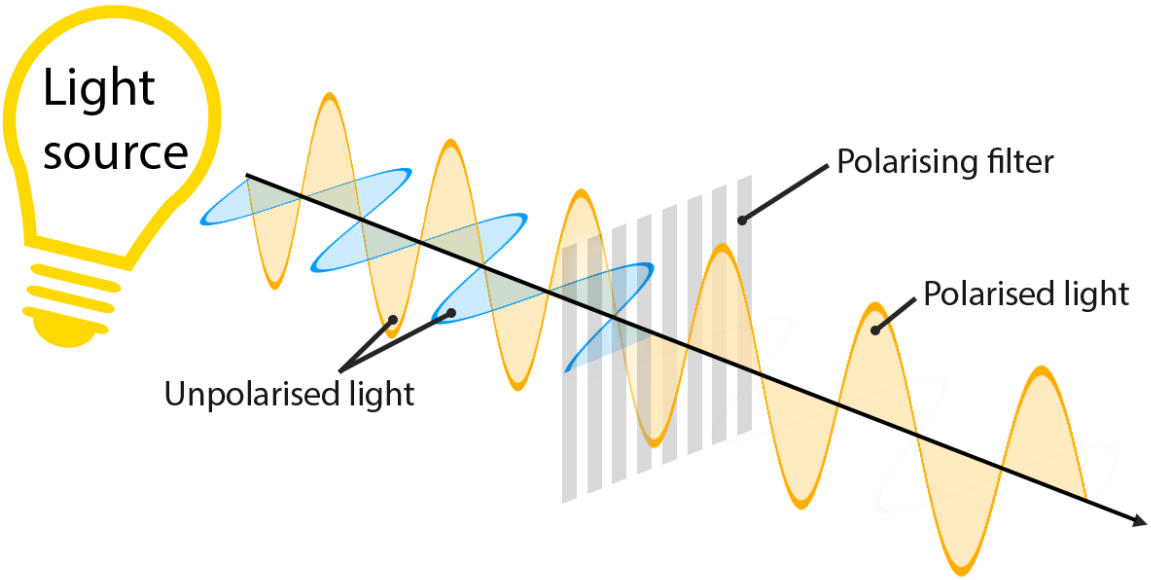

Figure 6: The polarising filter of the microscope turns unpolarised light, propagating in all directions, to polarised light, propagating only vertically, which passes through the sample and is doubly-refracted by anisotropic materials, such as crystalline drug. The ordinary and extra-ordinary light emitted from the sample is then recombined at the second polariser which makes the sample visible under the microscope setup (12).

The isolated pellets were imaged prior to performing XRD measurements. The pellet phases were carefully spread onto a glass microscope slide using a small spatula and then placed under the microscope at room temperature. A Nikon ECLIPSE Ni-U upright microscope fitted with crossed polarizing filters and a DS-U3 digital camera control unit (Nikon, Tokyo, Japan) was used for all imaging conducted.

Probing Ionic Interactions during Digestion using FTIR

During FTIR experiments the molecular vibrations of the sample were examined upon the exposure and absorption of infrared radiation. It is a powerful technique that results in a unique molecular fingerprint for the sample, where the absorption of infrared radiation at certain wavenumbers on the final spectrum informs of specific types of chemical bonds present in the sample

(13). This is especially useful for probing the presence of functional groups and the changes these groups may undergo during *in vitro* digestion experiments. The technique was used in this project to confirm that the tertiary amine in the weakly basic drug cinnarizine underwent an ionic-interaction with fatty acid during digestion to affect the solid-state form of the precipitate.

The pellet phase of a digested formulation was left to air-dry on a microscope slide for a few hours, before it was collected then analysed using a PerkinElmer Frontier FTIR with attenuated total reflectance (ATR) (Waltham, Massachusetts). A small amount of sample was carefully placed onto the ATR crystal at room temperature, and the pressure valve was used to improve the uniformity of contact between the sample and the ATR crystal of the instrument. Samples were analysed using 32 scans at a resolution of 4 cm^{-1}. A background scan was collected initially, and the sample spectrum was collected subsequently with the Spectrum software automatically subtracting the background.

References

1. Thomas N, Holm R, Rades T, Mullertz A. *Characterising lipid lipolysis and its implication in lipid-based formulation development*. The AAPS journal. 2012; **14** (4) : 860-71

2. Fatouros DG, Walrand I, Bergenstahl B, Müllertz A. *Colloidal structures in media simulating intestinal fed state conditions with and without lipolysis products*. Pharmaceutical research. 2009;26(2):361-74.

3. Williams HD, Sassene P, Kleberg K, Bakala-N' Goma JC, Calderone M, Jannin V, et al. *Towards the establishment of standardized in vitro tests for lipid-based formulations, part 1: method parametrization and comparison of in vitro digestion profiles across a range of representative formulations*. Journal of Pharmaceutical Sciences. 2012; **101** (9):3360-80.

4. Porter CJ, Trevaskis NL, Charman WN. Lipids and lipid-based formulations: optimizing the oral delivery of poorly-water soluble drugs. Nature reviews Drug discovery. 2007; **6** (3) : 231-48

5. Liao TH, Hamosh P, Hamosh M. *Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine.* Pediatr Res. 1984;**18**(5):402-9.

6. Bakala-N'Goma JC, Williams HD, Sassene PJ, Kleberg K, Calderone M, Jannin V, et al. Toward the establishment of standardized in vitro tests for lipid-based formulations. 5. Lipolysis of representative formulations by gastric lipase. Pharmaceutical research. 2015;32(4):1279-87.

7. Pouton CW. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. European journal of pharmaceutical sciences : official journal of the

European Federation for Pharmaceutical Sciences. 2006;29(3-4):278-87.

8. Rissanen K. Advanced X-ray Crystallography. Topics in current chemistry. 2012.

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

10. Boyd B and Rades T. *Applications of Small Angle X-ray Scattering in Pharmaceutical Science*. In: Müllertz A, Perrie Y, Rades T, editors & SpringerLink. (2016). *Analytical techniques in the pharmaceutical sciences* (Advances in delivery science and technology). New York: Springer.

11. Carlton R.A. *Chapter 2: Polarized Light Microscopy*. Pharmaceutical Microscopy 2011.

12. *Linear polarized 3D glasses and the physical shape of light waves* 2016, accessed 6-02-2017 http://physics.stackexchange.com/questions/231962/linear-polarized-3d-glasses-and-the-physical-shape-of-light-waves

13. Berthomieu C, Hienerwedal R. Fourier Transform infrared (FTIR) spectroscopy. Photosynthesis Research. 2009; **101** (23)

Chapter 3:
In Situ Lipolysis and Synchrotron Small-Angle X-ray Scattering

Declaration

This chapter consists of a published journal article that showcases the development of a real-time method for detecting the precipitation of drug during *in vitro* digestion. *In situ* lipolysis and synchrotron SAXS were used for this purpose. The precipitation behaviour of the model neutral compound, fenofibrate, was evaluated. The article was published as: Khan J, Hawley A, Rades T, Boyd B. *In situ lipolysis and synchrotron small-angle X-ray scattering for the direct determination of the precipitation and solid-state form of the model poorly-water soluble drug during digestion of a lipid-based formulation*. J. Pharm. Sci. 2016, 105, 2631-39. This chapter then looks at precipitation behaviour with different loadings and a different drug using the same synchrotron setup.

Declaration by candidate:

For chapter 1, the nature and extent of my contribution to the work was as follows:

Nature of contribution	**Extent of contribution**
Perform experiments, preparation of manuscript	80%

The following co-authors contributed to the work:

Name	**Nature of contribution**
Adrian Hawley	Beamline scientist, assistance with experiment
Thomas Rades	Co-supervisor, preparation of manuscript
Ben Boyd	Main supervisor, assistance with experiment, preparation of manuscript

The undersigned hereby declare that the above declaration correctly reflects the nature and extent of candidate and co-author contributions:

Candidate's signature: [redacted] Date: 20-04-2017

Main supervisor's signature: [redacted] Date: 20-04-2017

In Situ Lipolysis and Synchrotron Small Angle X-ray Scattering for the Direct Determination of the Precipitation and Solid-State Form of a Poorly Water-Soluble Drug During Digestion of a Lipid-Based Formulation

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Abstract

In situ lipolysis and synchrotron small-angle X-ray scattering (SAXS) were used to directly detect and elucidate the solid-state form of precipitated fenofibrate from the digestion of a model lipid-based formulation (LBF). This method was developed in light of recent findings that indicate variability in solid-state form upon the precipitation of some drugs during the digestion of LBFs, addressing the need to establish a real-time technique that enables solid-state analysis during *in vitro* digestion. In addition, an *ex situ* method was also used to analyse the pellet phase formed during an *in vitro* lipolysis experiment at various time points for the presence of crystalline drug. Fenofibrate was shown to precipitate in its thermodynamically stable crystalline form upon digestion of the medium-chain LBF, and an increase in scattering intensity over time corresponded

well to an increase in concentration of precipitated fenofibrate quantified from the pellet phase using high-performance liquid chromatography. Crossed polarised light microscopy served as a secondary technique confirming the crystallinity of the precipitated fenofibrate. Future application of *in situ* lipolysis and SAXS may focus on drugs, and experimental conditions, which are anticipated to produce altered solid-state forms upon the precipitation of drug (i.e., polymorphs, amorphous forms, and salts).

Introduction

Lipid-based formulations (LBFs) provide an effective means to improve the oral absorption of poorly water-soluble compounds. In particular, lipophilic drugs, or more generally Class 2 drugs as defined by the Biopharmaceutics Classification System (BCS), are well suited to LBF technology, as in these formulations the drug is presented to the gastrointestinal tract in a solubilised form, avoiding the rate-limiting dissolution step during gastrointestinal transit. In addition, LBFs may also drive the lymphatic uptake of some highly lipophilic drugs from the small intestine, which limits the effect of first-pass metabolism on bioavailability. The enzymatic degradation of lipidic components during digestion leads to the formation of self-assembled structures in the small intestine, such as mixed micelles, which enable the transport of lipophilic drugs to sites of absorption. Endogenous bile salts and phospholipids interact with liberated fatty acids from the digestion of lipids to form these self-assembled structures. There is, however, potential for a loss of solubilisation capacity of the formulation during dispersion and digestion. The drug may then become rapidly supersaturated in the formulation, in which case there is an increased risk of drug precipitation. The supersaturation of drug is in itself complex; some drugs persist in a supersaturated state for an extended time interval while others rapidly precipitate (12).

Supersaturating formulations aim to extend this time interval of drug supersaturation for as long as possible (13–15), as a means to harness the driving force for absorption of drug whilst in the supersaturated state. This inhibition of drug precipitation to enhance absorption is commonly achieved with the use of polymeric materials (16, 17). The use of non-ionic surfactants with polyethylene glycol head groups, or "stealth" components, has also recently been investigated as a means to address the rapid loss of solubilisation capacity seen with the digestion of medium-chain (MC) lipids in particular (18).

In vitro digestion experiments are used to detect drug precipitation during digestion by centrifugation of the digesting medium to sediment precipitated drug. Until recently, drug precipitation during the digestion of LBFs was viewed as inherently detrimental to drug absorption; hence, the strong focus on inhibiting drug precipitation with the use of polymers in supersaturating formulations. This paradigm, however, has come under question with a number of studies showing that the precipitation of some drugs during *in vitro* digestion is not limited to poorly water-soluble crystalline forms. Most notable are the recent findings of Sassene *et al*. (19), Stillhart *et al*. (20) and Thomas et al. (21), which all point to the presence of non-crystalline cinnarazine (19) carvedilol (20) and halofantrine (21) in the endpoint pellet phase of digested LBFs containing these weakly basic drugs. The dissolution performance of these noncrystalline drug precipitates, in bio relevant media, was significantly improved as compared with their thermodynamically stable crystalline forms. Moreover, Williams *et al*. (22) has shown tolfenamic acid to precipitate as a high-energy polymorph during the *in vitro* digestion of an LBF. The findings above indicate that the solid state of the precipitated drug during digestion may play an integral role in influencing the total amount of absorbed drug.

In vitro lipolysis testing and subsequent characterization techniques may be used to examine the solid-state aspects of drug precipitation. The solid-state characterisation of precipitated drug from *in vitro* digestion experiments has, thus far, been largely limited to X-ray diffraction (XRD), crossed polarised light microscopy (CPLM) and dissolution testing, performed on the pellet phase of endpoint digested contents. Although XRD and CPLM can reveal the crystallinity and morphology of precipitated drug at the endpoint of *in vitro* digestion, these techniques provide no real-time information with regard to the solid-state aspects of drug precipitation during digestion. Another drawback of performing XRD and CPLM on pellet phases produced during *in vitro* digestion experiments is the extensive sample handling and time required to produce the pellet phase containing the solid drug material in question, which often involves ultracentrifugation of samples for up to 90 min, as well as analysis time with benchtop XRD instruments requiring at least 10 min per sample. This extensive sample treatment and analysis time needed for the conventional XRD and CPLM methods brings into question the relevance of the solid drug material measured with respect to what actually takes place during digestion, and highlights the need for developing suitable real-time methods capable of monitoring the precipitation of drug and its solid state during digestion.

In-line Raman spectroscopy was recently applied to monitor the precipitation kinetics of the neutral and poorly water soluble drug fenofibrate, in real-time, during the *in vitro* digestion of an LBF (23). Raman spectroscopy proved effective in this instance; however, extensive data analysis was necessary to distinguish precipitated drug from the hydrolysis products of digestion, and the analysis relied on a priori knowledge of the reference crystalline form to correlate with the solid state of precipitated drug. Hence, the need to develop a method that

provides the *in situ* lipolysis of LBFs with real-time determination of the solid-state form of the drug becomes apparent. Boetker *et al.* (24) have recently demonstrated the direct in situ elucidation of transitions between different solid-state forms of drugs in a suspension using synchrotron X-ray scattering. Warren *et al.* (25) have also recently established the use of *in situ* lipolysis and small-angle X-ray scattering (SAXS) for the purpose of following the formation of colloidal structures during the digestion of lipids. In this study, we demonstrate the potential for convergence of these approaches to enable the real-time solid-state structural elucidation of precipitated drug (fenofibrate) during the digestion of a typical LBF (self nano-emulsifying drug delivery systems, SNEDDS). The precipitation kinetics was also independently measured by high performance liquid chromatography (HPLC), and the presence of crystalline material was verified using CPLM.

Experimental

Materials

Fenofibrate was purchased from AK Scientific (Union City, California). Captex 355® [MCT composed of 59% caprylic acid (C_8),40% capric acid (C_{10}), <1% lauric acid (C_{12}) as stated in the product information], and Capmul MCM® [mono/diglycerides composed of caprylic acid (C_8) in glycerol], were obtained from Abitec Corporation (Janesville, Wisconsin). Cremophor ELR® was purchased from BASF Corporation (Washington, New Jersey). Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate, >95%), and 4-bromophenylboronic acid (4-BPBA, >95%) were purchased from Sigma-Aldrich (St. Louis, Missouri). Calcium chloride dihydrate (>99%) was obtained from Ajax Finechem (Seven Hills, New South Wales, Australia). Sodium chloride (>99%) was purchased from Chem Supply (Gillman, South Australia, Australia). HPLC grade methanol and acetonitrile were purchased from Merck (MA, USA).

Phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) was obtained from Trapeze Association Pty. Ltd. (Clayton, Victoria, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia).

Selection and Preparation of Drug and Formulation

Fenofibrate was chosen as the poorly water-soluble model compound for this study, as it has previously been shown to precipitate extensively at the endpoint of *in vitro* digestion in its thermodynamically stable crystalline form (22, 23, 26). Hence, it was deemed suitable for the purpose of validating the proposed *in situ* lipolysis and SAXS method, as characteristic diffraction peaks were anticipated to appear when the drug first precipitates during the experiment and increase in scattering intensity over time, to correspond with the progressive precipitation of drug. Moreover, the use of fenofibrate allowed for a comparison of the *in situ* technique described here, and the *in situ* Raman technique described by Stillhart *et al*. (23).

The formulation was a MC-SNEDDS, and was prepared in 1 g batches with 0.3 g of Captex® 355, 0.3 g of Capmul® MCM, 0.3 g of Cremophor EL® and 0.1 g of ethanol all weighed into the same 20 mL glass scintillation vial. The resultant mixture was vortexed for 1 min before the drug was incorporated. This produced a formulation that was an optically clear solution, which self-emulsified upon agitation in the lipolysis vessel to form a nano-emulsion. MC lipids were selected because of their higher solubilisation capacity for fenofibrate compared with LC lipids (27), and because the rapid enzymatic breakdown of the MC lipids was expected to result in the rapid precipitation of drug.

The loading of fenofibrate in the formulation was selected according to previously established equilibrium solubility values for the drug in a similar MC-SNEDDS formulation, where the equilibrium solubility was shown to be 143.1 mg/g (22). The drug

loading (200% of solubility) was chosen in order to provide a large fraction of precipitated drug and increase the likelihood of detection. The term 'super-SNEDDS' has been used to describe such formulations containing supersaturated levels of drug (28) Hence, 286.2 mg of fenofibrate was weighed and added to the scintillation vial containing the blank MC-SNEDDS formulation. The vial was vortexed for 1 min, then placed in a 70°C oven for approximately 5 h, and finally placed in a 40°C roller oven overnight. The formulation was visually examined for the absence of crystals before use, and placed back into the 70°C oven until the solution was clear. The formulation was vortexed for 1 min prior to performing lipolysis experiments.

Preparation of Digestion Medium and Pancreatic Lipase

The digestion buffer was used to prepare both the digestion medium and pancreatic lipase solutions. Digestion buffer was prepared with 2 mM Tris–maleate, 1.4 mM $CaCl_2 \cdot 2H_20$, 150 mM NaCl and adjusted to pH 6.5 using NaOH and HCl solutions. Digestion medium consisted of the above buffer solution, supplemented with 5 mM of NaTDC and 1.25 mM of DOPC to represent fasted-state simulated conditions. Briefly, the DOPC was dissolved in a suitable amount of chloroform in a round-bottom flask, before the chloroform was removed under vacuum to leave an evenly coated lipid film at the bottom of the flask. The required amount of digestion buffer and NaTDC was added. Sonication was then used to dissolve any remaining solid particles. The digestion medium was kept refrigerated at 4°C before use.

Pancreatic lipase was prepared at the equivalent of approximately 1000 TB units/mL of digest by weighing 2 g of pancreatin extract into a small glass beaker, and adding 5 mL of digestion buffer to form a suspension. The suspension was stirred for 5 min and then transferred to a 12-mL plastic centrifuge tube and centrifuged at 2205g at 4°C for 15 min. This produced roughly 4.5 mL of supernatant, which was stored at 4°C prior to use, and

4 mL of this solution was used to initiate each lipolysis experiment.

In vitro Lipolysis Experiments

Lipolysis experiments were performed according to previously established methods (29, 30). A pH stat apparatus was used and coupled to a 5-mL dosing unit with autoburette, stirring unit and a glass pH electrode (iUnitrode) (Metrohm® AG, Herisau, Switzerland). Tiamo 2.0 software was used to operate the pH stat. A thermostatted glass reaction vessel (37°C) was connected to the stirring unit, with a small magnetic stirrer. Digestion medium (36 mL) was added to the glass vessel, followed by 1 g of the MC SNEDDS containing fenofibrate. An initial dispersion duration of 5 min allowed for thorough mixing of the digestion medium and formulation, and for adjusting the pH to 6.5 ± 0.003. The pH was set to 6.5 as a compromise between the recommended range for pancreatic lipase activity (pH 6–10) (31) and duodenal pH (5.9–6.5) (32). Lipolysis was then initiated by transferring 4 mL of the previously prepared pancreatic lipase solution into the glass vessel, and a 0.6 M NaOH solution was used as titrant to maintain a pH environment of 6.5. The titrant countered the decrease in pH resulting from the liberation of ionised-free fatty acids upon the digestion of lipids in the formulation. The duration of all lipolysis experiments was set to 60 min. At the end of the 60-min digestion period, the pH of the contents in the glass vessel was rapidly increased to pH 9.0, using the NaOH titrant, to ionise all fatty acid molecules present in the digestion mixture. The amount of unionised fatty acid molecules was thereby determined from the amount of titrant required to reach pH 9.0. The extent of LBF digested was calculated according to Equation 1.

$$\textit{Extent of LBF digested}\ (\%) = \frac{\textit{Ionised fatty acid}\ (mol) + \textit{Unionised fatty acid}\ (mol)}{\textit{Theoretical fatty acid in LBF}\ (mol)} \times 100$$

Equation 1

In Situ Lipolysis and SAXS

The SAXS measurements were performed at the SAXS/WAXS beamline at the Australian Synchrotron (33). The physical setup of the experiment was similar to those previously conducted with coupled *in situ* lipolysis and SAXS experiments, which have focused on the evolution of colloidal structures during digestion (25). Figure 1 depicts the *in vitro* lipolysis and SAXS configuration. A peristaltic pump was used to move the contents of the digestion vessel through silicone tubing (total volume <1 mL), which led to a 1.5 mm diameter quartz capillary mounted in the X-ray beam, at a flow rate of approximately 10 mL/min. An X-ray beam with a wavelength of 1.1271 Å (11 keV) was used. A variation in detector configuration compared with Warren *et al*. (25) was introduced in this study to increase sensitivity to the detection of small quantities of precipitated drug, with a short sample to detector distance of 567.5 mm providing the q-range $0.03 < q < 1.7 Å^{-1}$, where q is the length of the scattering vector, defined by $q = 4\pi/\lambda \sin(\theta/2)$, λ being the wavelength and θ the scattering angle. A 5 sec acquisition period was used with a 15 sec delay between frames, providing a diffractogram every 20 sec during digestion. The 2D SAXS patterns were acquired using a Pilatus 1M detector with a pixel size of 172 µm integrated into the one-dimensional scattering function I(q) using the in-house developed software package ScatterBrain. The pancreatic lipase solution was added using a remotely actuated syringe driver during acquisition.

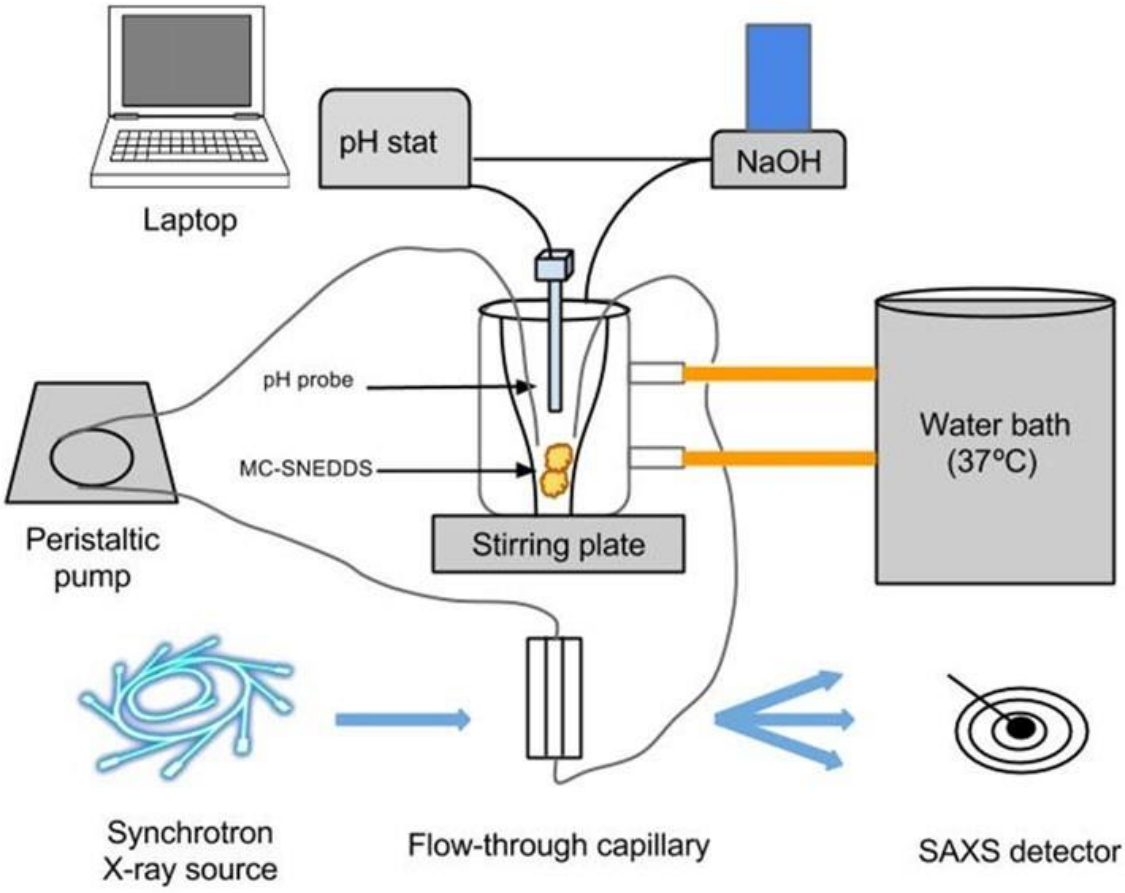

Figure 1: Configuration used for the *in situ* lipolysis and SAXS experiment, where the solid state of precipitated drug was monitored in real-time during the digestion of a MC-SNEDDS containing fenofibrate.

Ex Situ Lipolysis and SAXS

The *ex situ* lipolysis and SAXS method was designed as an alternative technique to the in situ flow-through method for comparative purposes, as it mimics the sample handling of 'offline' approaches (retrieval and centrifugation) while still providing structural information in real-time. The technique involved the analysis of pellet phases in real time, by taking samples at various time points throughout digestion, immediately centrifuging and acquiring the scattering from the pellet within 5 min of taking the sample. Samples (200 µL) were collected from the lipolysis vessel at predetermined time points (0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min after the addition of pancreatic lipase), and placed in HPLC glass insert vials that were treated

with lipase inhibitor (10 µL of 0.1 M 4-BPBA in methanol). After collecting a specific time point sample, the glass vial was immediately placed inside a 1.5 mL Eppendorf tube and centrifuged for 30 sec at 7378g. The vial was then placed inside a custom-built holder in the path of the X-ray beam at the SAXS/WAXS beamline. The beam was adjusted to penetrate the center of the pellet phase for a 5 sec acquisition time. This process was repeated for all time point samples, effectively providing real-time measurements with potentially greater sensitivity to detect precipitation of drug compared with the in situ flow-through approach by concentrating precipitated material in the bottom of the vial.

Crossed Polarised Light Microscopy

A Nikon ECLIPSE Ni-U upright microscope fitted with crossed polarising filters and a DS-U3 digital camera control unit (Nikon, Tokyo, Japan) was used for CPLM imaging of pellet phases. CPLM was performed on the pellet phases of selected time point samples from *ex situ in vitro* lipolysis experiments, as a secondary technique to confirm the presence of precipitated drug (fenofibrate crystals). Crystalline fenofibrate exhibits birefringence under crossed polarised light; hence, CPLM provided an indication of when the drug first crystalized during lipolysis and was correlated to the SAXS data.

HPLC Quantification of Drug and Sample Treatment

High-performance liquid chromatography was performed according to a previously reported method (22) to determine the phase distribution behaviour of fenofibrate upon digestion of the model SNEDDS formulation. The HPLC system included a Shimadzu CBM-20A system controller, LC-20AD solvent delivery module, SIL-20A auto sampler and a CTO-20A column oven set at 40°C, coupled to an SPD-20A UV-detector (Shimadzu Corporation, Kyoto, Japan). A reverse-phase C_{18} column was used (4.6 × 75 mm^2, 3.5 µm; Waters

Symmetry®, MA, USA), and the UV detector was set to 286 nm for detection of fenofibrate. The mobile phase consisted of acetonitrile and water in an 80:20 (v/v) ratio with 0.1% (v/v) formic acid, eluted at a flow rate of 1 mL/min. A standard curve was constructed from fenofibrate in acetonitrile at concentrations of 40, 20, 10, 4, 2, 1 and 0.1 µg/mL, which were diluted from a stock solution of 1 mg/mL.

The lipolysis samples (200 µL) were taken from the reaction vessel at time points identical to those used in the *ex situ* scattering experiment, and transferred to 1.5 mL Eppendorf tubes treated with lipase inhibitor (10 µL of 0.1 M 4-BPBA in methanol). The samples were immediately centrifuged (30 sec at 7378g), before the supernatant was transferred to a separate Eppendorf tube. A second set of HPLC data was obtained by collecting samples at the same time points as above, but centrifuging for 1 hr at 7378g, to force as complete sedimentation of drug as possible. In all cases, the aqueous phase was diluted 10-fold in acetonitrile, and the pellet phase was diluted 100-fold in acetonitrile before injecting (50 µL) into the HPLC.

Results

The MC-SNEDDS formulation immediately dispersed upon addition to the digestion medium in the glass vessel. Rapid hydrolysis of the lipid components in the formulation was evident upon addition of pancreatic lipase at the end of the 5 min dispersion period (lipase added at time = 0 in Figure 2).

Previous studies have shown that SNEDDS formulations, such as the one used in this study, composed mostly of MC lipids, digest to a high degree in *in vitro* lipolysis experiments with similar fasted-state conditions (20, 22). The lipolysis curve shown in Figure 2 resembles the curves of the MC lipid formulations in the studies mentioned above. From Figure 2, 60% of the MC-SNEDDS

was digested within the first 10 min of the experiment, demonstrating the fast kinetics of lipid hydrolysis associated with MC lipids. At the end of the 60 min digestion, approximately 82% of the MC-SNEDDS was digested, taking into account both ionised fatty acid titrated during the 60 min lipolysis and unionised fatty acid titrated during the increase to pH 9.0 after the 60 min lipolysis. The calculated extent of formulation digested (82%) is reflected by the shape of the lipolysis curve, which appeared to be still drifting upwards at the end of the 60 min digestion period. A plateau in the lipolysis curve was not observed, which indicated that the formulation had not yet digested completely.

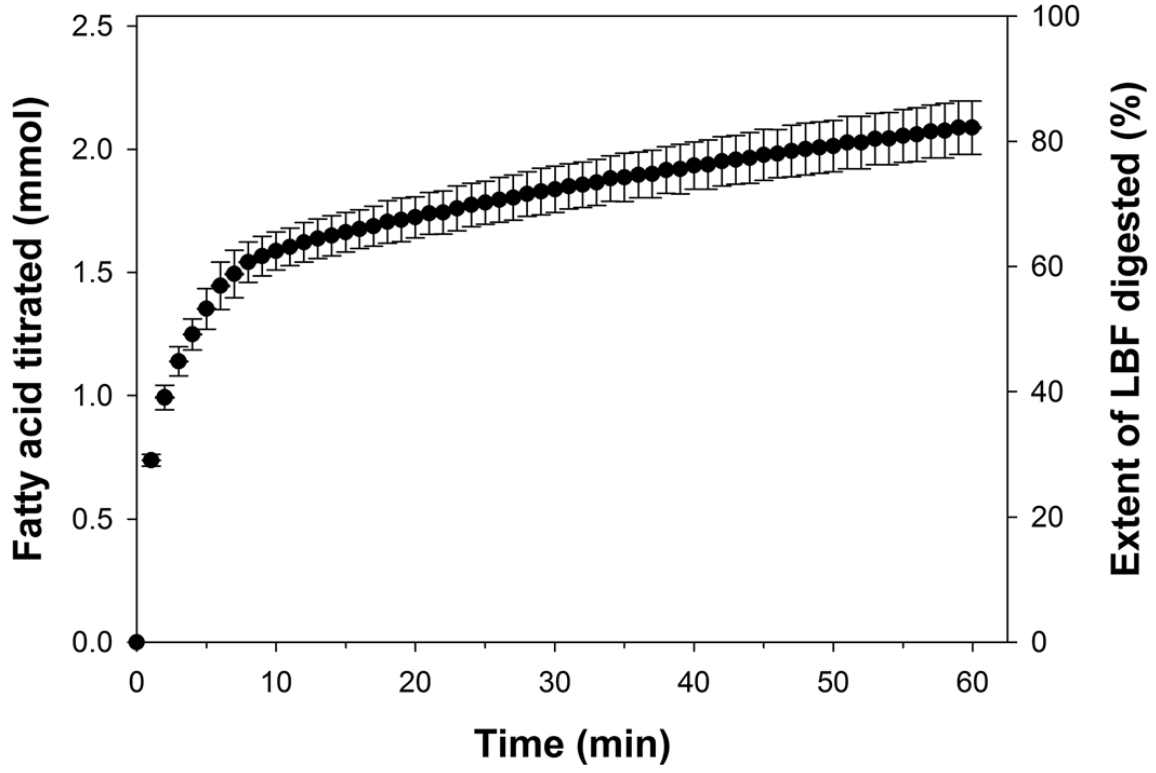

Figure 2: Titration profile of ionised and unionised fatty acid produced during *in vitro* lipolysis of the MC-SNEDDS formulation (mean ± SD, n = 3 different digestions). The profile also shows the extent of LBF digested over the 60-min lipolysis.

Phase Distribution of Fenofibrate during Lipolysis

The concentration of fenofibrate in both the aqueous colloidal phase and pellet phase from samples taken at various time points throughout *in vitro* lipolysis was measured by HPLC. The phase distribution of fenofibrate on lipolysis is presented in Figure 3

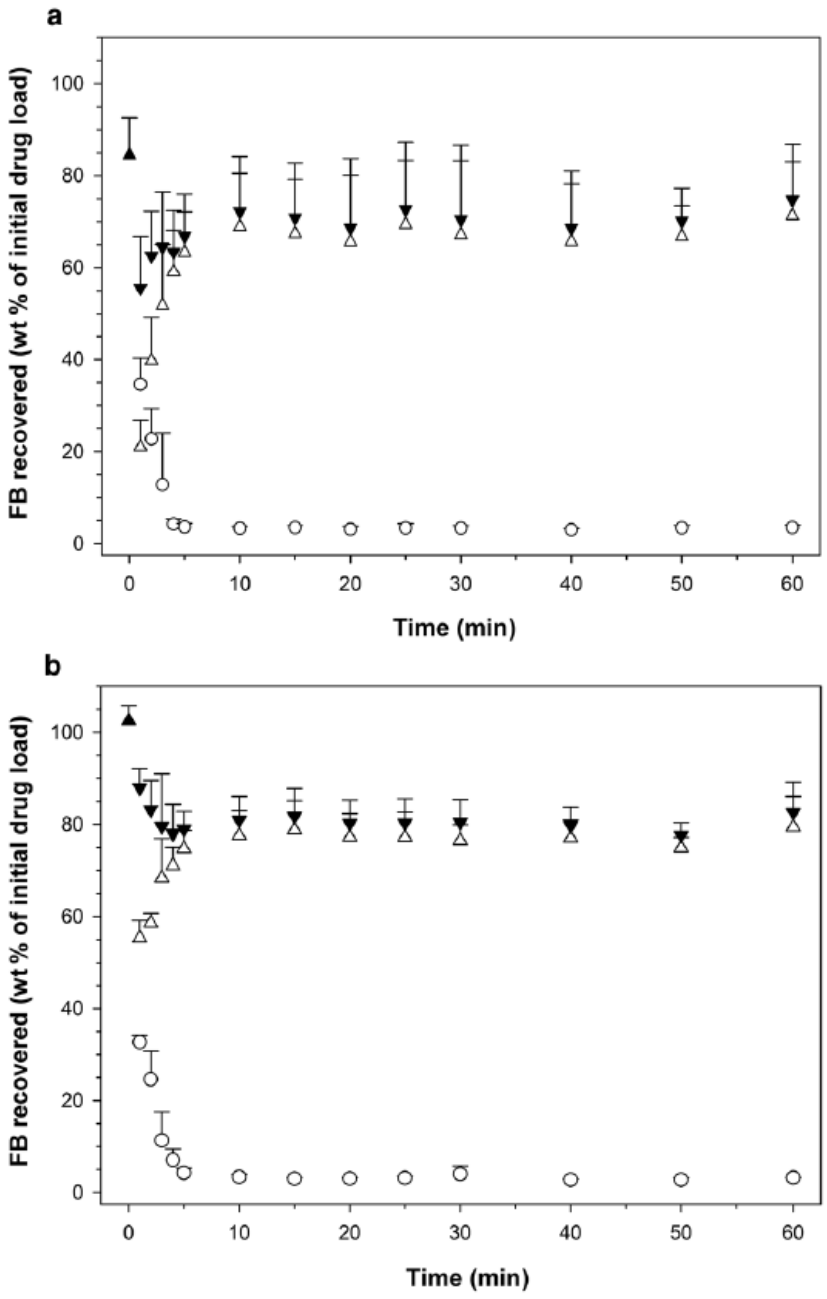

Figure 3: Distribution of fenofibrate in the aqueous colloidal phase and pellet phase during lipolysis with (a) centrifugation of samples for 30 sec at 7378*g* and (b) centrifugation for 1 hr at 7378*g*. Where (O) = wt % fenofibrate in the aqueous colloidal phase; (△) = wt % fenofibrate in the pellet phase; (▼) = wt % fenofibrate recovered in total; and (▲) = wt % fenofibrate after the 5 min dispersion period (before lipolysis). Samples taken at the various time points throughout lipolysis were diluted with acetonitrile and then analysed using HPLC. Experiments were performed in triplicate (*n* = 3 different digestions, mean ± SD).

The HPLC data demonstrates the rapid precipitation of drug, with an increasing amount of drug in the pellet phase, and a parallel decrease in the aqueous colloidal phase. From Figure 3, the concentration of fenofibrate increased in the pellet phase up until 10 min into digestion. Thereafter, for the remaining 50 min of lipolysis, the concentration of fenofibrate in the pellet phase plateaued. Concurrently, the concentration of fenofibrate in the aqueous colloidal phase decreased in the first 10 min after starting lipolysis, and similarly reached a plateau for the remaining 50 min of digestion. This trend of a decreasing amount of drug in the aqueous colloidal phase and an increasing amount in the pellet phase within the first 10 min of lipolysis indicated that precipitation of fenofibrate occurred in this time period, and then levelled off for the remainder of the experiment. The rapid precipitation kinetics can be attributed to the high drug loading in the formulation and the rapid hydrolysis of MC lipids driving a loss of solubilisation capacity and precipitation of fenofibrate. Although it may appear that the recovery for the time zero data point from HPLC in Figure 3a is similarly low compared with the time-resolved samples, which may indicate a systematically low recovery, the inherent variability in the data resulting from the short centrifugation time makes it difficult to test whether there is any statistical significance between the two data sets.

Both centrifugation methods yielded similar concentrations of fenofibrate in the respective phases over time (comparing Figures 3a and 3b), and hence justified the use of the shorter centrifugation time during the *ex situ* scattering experiment for the purpose of obtaining qualitative information on the solid-state of precipitated drug during digestion. However, a higher and less variable concentration of fenofibrate was recovered in the pellet phases with the longer centrifugation time. From Figure 3b, more than 50 wt % of the total fenofibrate was present in the pellet phase after 1 min of lipolysis, which suggested

that drug precipitation ensued very shortly after the addition of pancreatic lipase, an observation which has been reported for fenofibrate in the past using similar centrifugation methods of isolating the pellet phase for analysis (23).

The centrifuged pellet phases collected at selected time points throughout lipolysis were visualised under crossed polarised light, as a means to examine the presence and crystallinity of precipitated drug.

The CPLM images in Figure 4 showed that fenofibrate precipitated in a crystalline form, and followed the trend that was observed with the HPLC data discussed above, where precipitated drug was evident immediately after commencing lipolysis (1 min was the earliest time point for both HPLC and CPLM). An increasing number of crystals could also be observed from the images with a small number present at 1 min, increasing in number up to 10 min. The images also showed that although the number of crystals increased, the size of the crystals appeared to stay the same.

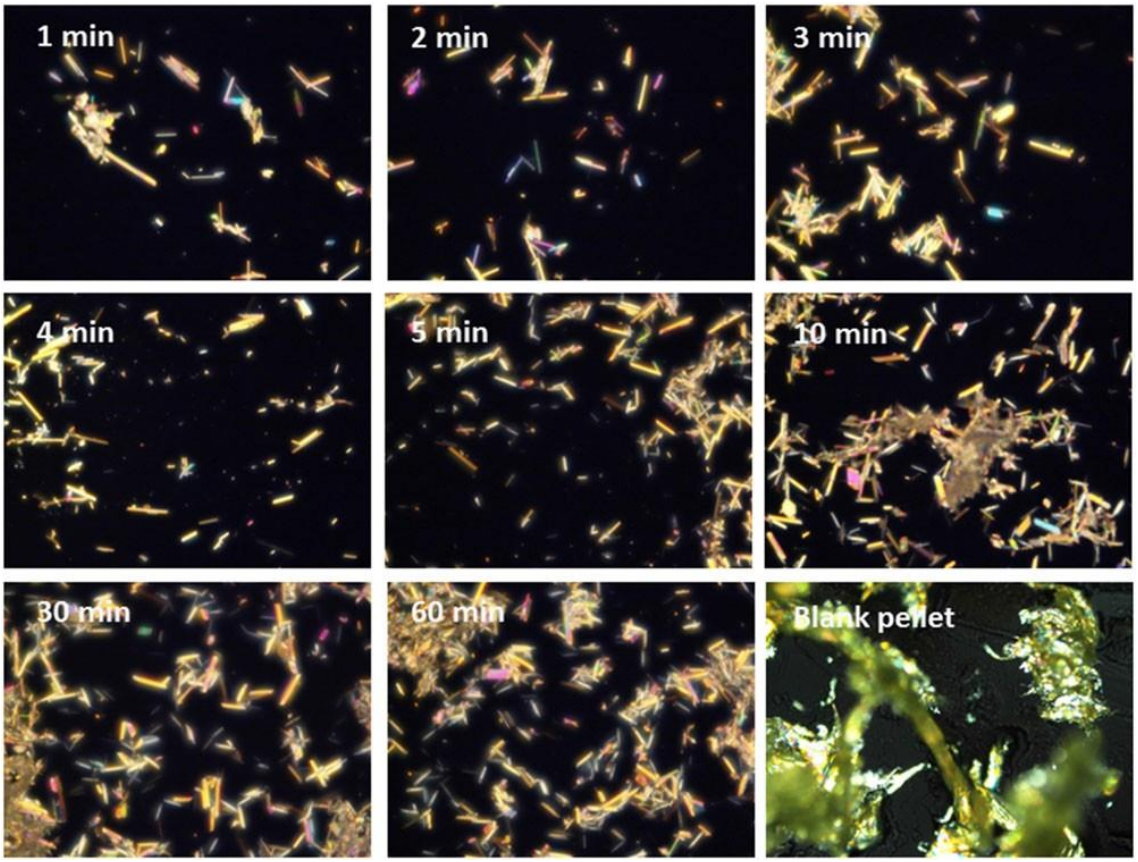

Figure 4: Crossed polarised light microscopy images of the pellet phase obtained at various time points over the 60 min lipolysis. Crystalline fenofibrate was observed 1 min after starting lipolysis, and the number of fenofibrate crystals increased over the duration of the experiment. The blank pellet was shown to consist only of calcium fatty acid soaps in the absence of the drug in the formulation.

Solid-State Structural Elucidation in Real-Time Using SAXS

In Situ Lipolysis and SAXS

Synchrotron SAXS was used to follow the solid-state of precipitated fenofibrate during the lipolysis of the MC-SNEDDS formulation. The scattering of the buffer solution was subtracted from the raw intensity values obtained during the lipolysis of the formulation. The kinetic profile during the lipolysis is presented in Figure 5a. Crystalline material was first detected at 4 min after the addition of pancreatic lipase.

Fenofibrate precipitated in its thermodynamically stable crystalline form, evident by the characteristic diffraction peaks of the drug, in accordance with the scattering from the crystalline reference fenofibrate (Figure 5b). Further interrogation of the data in Figure 6 through integration of the major diffraction peak at q = 1.15 $^{\circ}A^{-1}$ and comparison with titration data reveal that there is a critical percentage digestion at which the bulk of the precipitation occurred at approximately 60%.

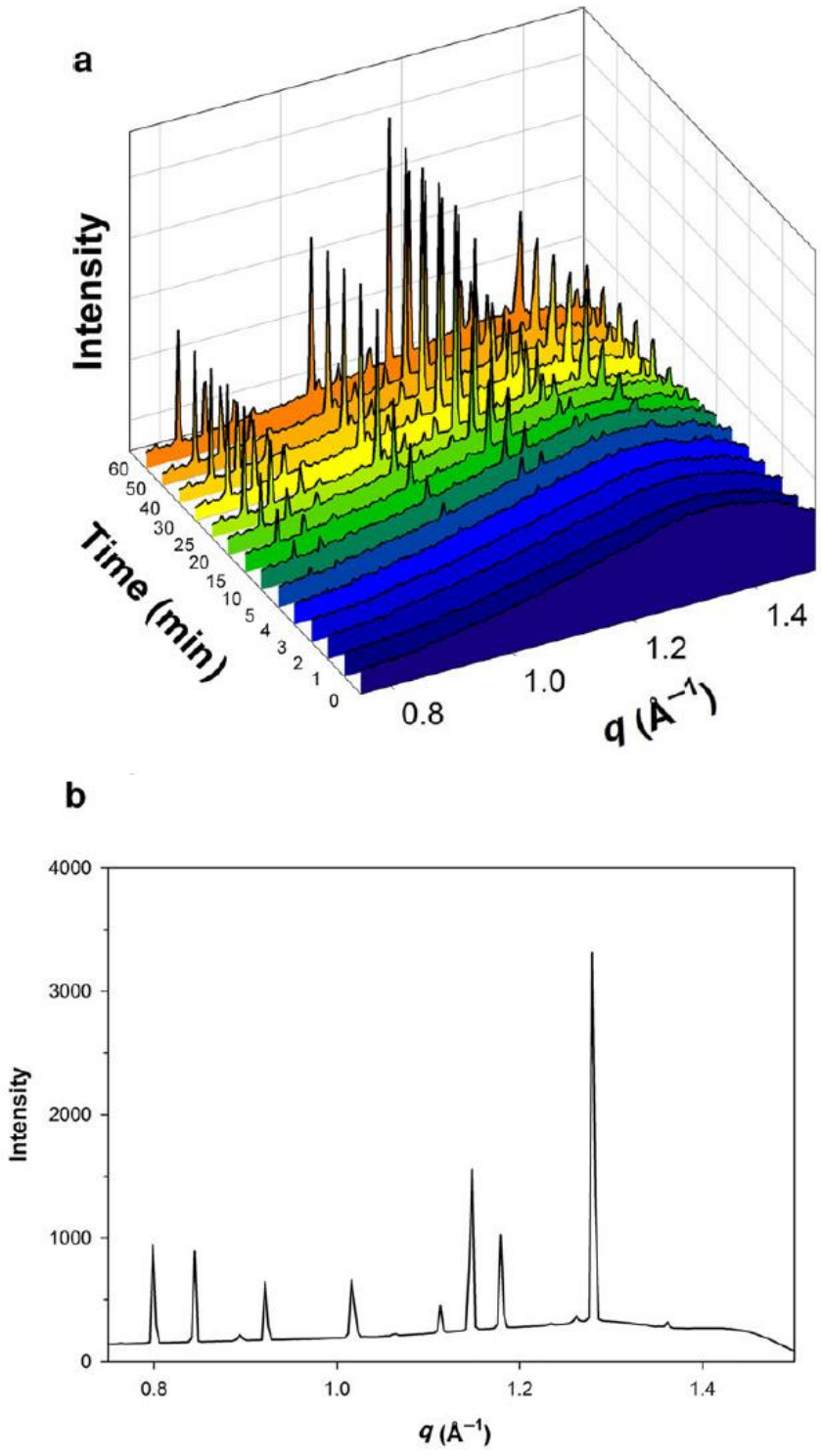

Figure 5: (a) *In situ* SAXS profiles during the lipolysis of the MC-SNEDDS formulation containing fenofibrate. Drug precipitation was evident at 4 min after the addition of pancreatic lipase, with the characteristic diffraction peaks for fenofibrate (b, shows crystalline reference material) visible and steadily increasing in intensity as lipolysis continued and further drug precipitation occurred.

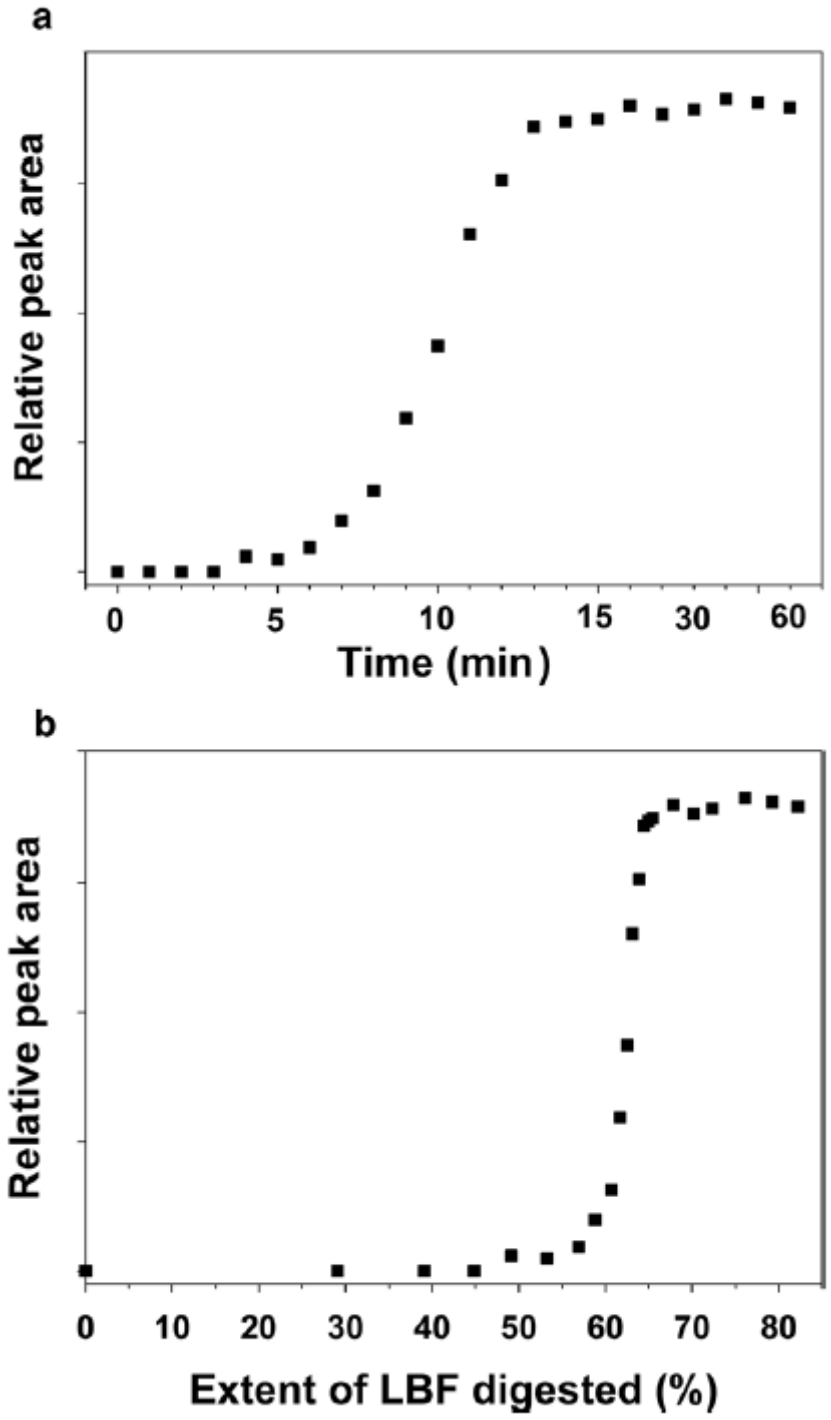

Figure 6: (a) Change in peak area of the most pronounced diffraction peak for precipitated fenofibrate (q = 1.15 Å $^{-1}$) over the 60-min digestion period (the x-axis is presented in 1 min intervals from 0 to 15 min, 5 min intervals from 15 to 30 min and 10 min intervals from 30 to 60 min). (b) Change in peak area of precipitated fenofibrate against the extent of LBF digested during lipolysis.

Ex Situ Lipolysis and SAXS

The SAXS profiles of the pellet phases collected and analysed using SAXS immediately after centrifugation at certain time points during lipolysis are shown in Figure 7.

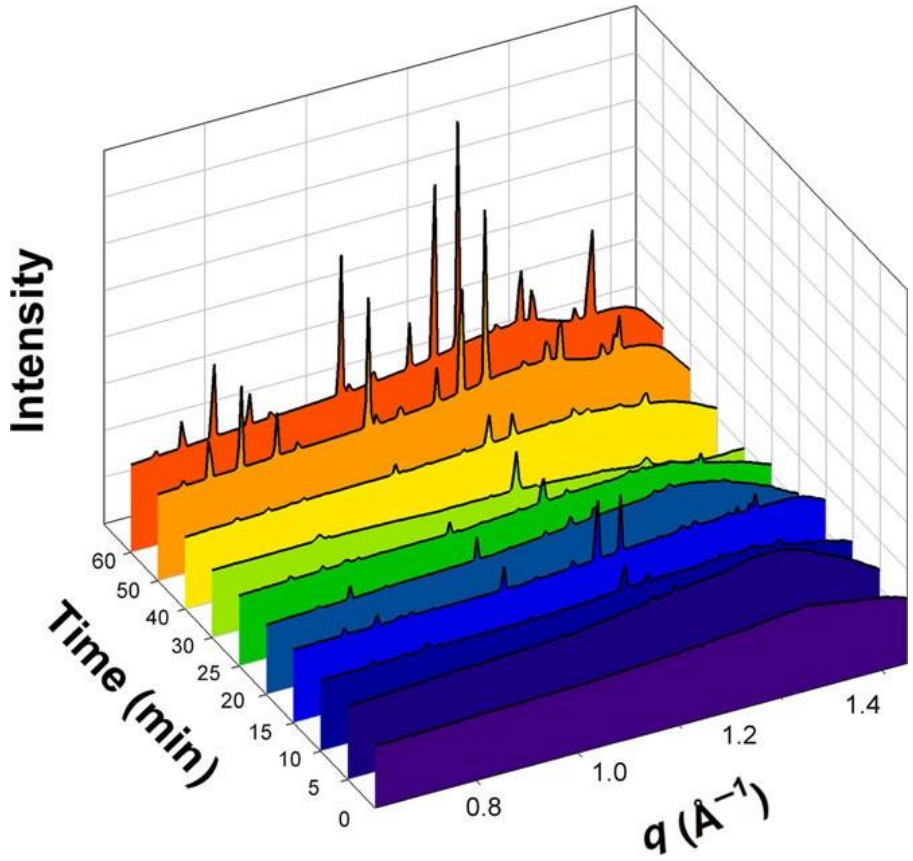

Figure 7: *Ex situ* SAXS profiles during the lipolysis of the MC-SNEDDS formulation containing fenofibrate. Drug precipitation was evident at 5 min after the addition of pancreatic lipase, which was the first time point sample, with the characteristic diffraction peaks for fenofibrate visible.

Precipitated fenofibrate was evident at 5 min, which was the first time point sample collected, centrifuged and analysed. Although the scattering intensity of precipitated fenofibrate increased during the experiment, there was an inconsistency in the scattering data that were not observed with the *in situ*

experiment discussed above, which utilised the flow-through capillary. This inconsistency in the scattering data is proposed to arise from inhomogeneity in the centrifuged pellet phase, and differences in the background scattering from the individual vials. In contrast, during the flow-through method, an average of the volume was irradiated in the fixed capillary yielding more consistent and representative scattering at various time points. The *ex situ* technique, therefore, is not well suited for following the progress of drug precipitation during digestion, as there will inevitably be an element of uncertainty around whether the precipitated drug is present in the section of the pellet phase measured by SAXS, and whether there is a uniform distribution of precipitated drug within the pellet phase. From the *ex situ* data shown in Figure 7, it was apparent that the drug was not uniformly distributed within the pellet phase, as care was taken to ensure the centre of the pellet was measured for each sample. The *ex situ* technique may be useful for obtaining a general qualitative perspective of the solid state of precipitated drugs during lipolysis, but accurate and consistent scattering that can reveal information on the kinetics and progression of precipitation, as well as solid-state transformations requires the use of the *in situ* flow-through SAXS technique discussed above.

Discussion

The *in situ* flow-through approach to studying the kinetics of drug precipitation presents a powerful approach for resolution of fast kinetic processes. Precluding this approach from the study of these types of systems, acquiring a diffractogram for a solid form could take longer than 10 min using regular XRD methods. In contrast, in the *in situ* study here, the scattering data were obtained for 5 sec every 20 sec (for clarity only the data at 1 min intervals was presented in Figure 5a); thus, much

higher time resolution for precipitation could easily be obtained.

The use of a highly supersaturated drug load of fenofibrate, which was 200% of its equilibrium solubility in the formulation, in conjunction with the use of rapidly digesting MC lipids in the formulation, promoted rapid precipitation of fenofibrate shortly after the addition of lipase. The onset of drug precipitation at 4 min into the lipolysis is comparable to the results obtained using *in situ* lipolysis combined with Raman spectroscopy (23), where precipitation of fenofibrate was first detected between 3 and 6 min of initiating digestion. When studied using Raman spectroscopy, this initial 3–6 min prior to the precipitation of fenofibrate was attributed to a brief period of drug supersaturation, and is likely what was observed here as well, using the *in situ* diffraction approach. In contrast, this delayed onset of drug precipitation was not apparent in both studies when centrifugation and HPLC was used, where in both studies fenofibrate was present in the pellet phase after 1 min of commencing lipolysis. The discrepancy between the precipitation kinetics observed with HPLC data and the in situ diffraction technique used here may be attributed to sample handling, with the centrifugation accelerating the precipitation of drug. If centrifugation of the digested samples does accelerate the precipitation of drug, then the kinetics of drug precipitation over the course of *in vitro* digestion determined using HPLC may be misleading.

The limit of detection for the *in situ* lipolysis and SAXS technique for measuring precipitated drug over time may also contribute to the interpretation of the early kinetics of precipitation. The intensity of scattering from fenofibrate increased after the drug was first detected 4 min into the lipolysis experiment (Figure 6a). A clear increase in peak area was observed in the 4–15 min time frame during lipolysis. This increase

in peak area over time corresponded to the trend observed with the increasing concentration of precipitated fenofibrate in the pellet phase measured by HPLC. Therefore, an increase in scattering intensity over time could be attributed to concentration effects, because the amount of precipitated fenofibrate remained constant after 15 min of lipolysis from HPLC data (Figure 3). The data further inform the sensitivity of the method — the peak area at 4 min was 12.3% of that at 30 min, corresponding to an approximate drug concentration of 4 mg/mL, representing a practical limit for detection under the current configuration. Further optimisation of the detector configuration may improve this limitation.

The change in relative peak area for precipitated fenofibrate was also clearly dependent on the extent of formulation digested (Figure 6b). Although the characteristic diffraction peaks for fenofibrate were first observed 4 min into lipolysis, which corresponds to approximately 50% digestion of the MC-SNEDDS, a drastic increase in relative peak area occurred once the formulation was approximately 60% digested (10 min into lipolysis). This finding suggests that the MC-SNEDDS used in this study could keep fenofibrate solubilised until the formulation was 50% digested, and that the formulation drastically lost its solubilisation capacity at approximately 60% digested, at which point fenofibrate crashed out of the system and the relative peak area for precipitated fenofibrate increased significantly. More broadly, this finding alludes to a threshold amount digested inherent in LBFs, above which there is a drastic loss in solubilisation capacity of the formulation and an increased risk of drug precipitation. Moreover, the relationship between the extent of formulation digested and drug precipitation may be utilised in future studies to reveal the colloidal lipid structures present at the time where the majority of drug has precipitated during digestion.

The application of the *in situ* lipolysis and SAXS to drug precipitation studies will be of increasing interest in systems that display polymorphic transitions on precipitation, or exhibit precipitation in different polymorphic forms with changes in formulation. In particular, there is a growing interest in understanding the propensity for precipitation of ionisable drugs in an amorphous form, which may over time crystallise. Ionisable drugs may potentially interact with charged species that are present during digestion, where the charged species may be endogenous components such as bile salts, or products of digestion such as fatty acids (20). The *in situ* formation of these complexes is proposed to induce amorphous solid precipitates that may have high re-dissolution potential compared with precipitation of the drug in a crystalline form.

Conclusion

In situ lipolysis and real-time synchrotron SAXS were used to provide a powerful time-resolved approach for directly elucidating the solid-state form of precipitated drug (fenofibrate) during *in vitro* digestion of LBFs. Although this proof-of concept study utilised a simple MC-SNEDDS formulation where the drug has a high propensity to precipitate and crystallise during digestion, future application of the technique would suit formulations and conditions where variability in the solid-state form upon precipitation of the drug is anticipated. Raman spectroscopy has been proven to provide similar utility in such studies, but the lipid colloidal background and indirect correlation with solid-state form complicate the data analysis. *In situ* lipolysis and synchrotron SAXS as a direct approach therefore offers an unambiguous alternative, and further studies with more complex systems are underway.

Further Optimisation of the *In Situ* Method and Application to Different Drug and Lipid Systems

The journal article above reports the development of the *in situ* lipolysis and synchrotron SAXS method for detecting the precipitation and solid-state form of a drug during *in vitro* digestion experiments. The selected parameters allowed for the detection of precipitated drug, or crystalline material, down to a concentration of 3.99 mg/mL in the flow through capillary. This placed a limitation on the types of lipid systems that could be examined, and in the case of the above study a high drug loading and rapidly digested formulation were chosen to maximise the likelihood of capturing drug precipitation using this technique. Further optimisation was thus necessary with the aim of increasing the sensitivity of the instrument to enable the detection of crystalline material at lower concentrations. By doing so, it was envisioned that lipid formulations prepared at more relevant drug loadings could also be assessed in this way.

To achieve increased sensitivity the X-ray scattering signal from precipitated drug needs to be higher, or background scattering, i.e. the scattering of everything outside of the sample, needs to be lower. The concentration of sample and flux of X-rays are the two determinants in capturing the crystalline diffraction and could not be further optimised, leaving the background as the manageable variable.

The setup of the sample within the beamline is such that on either side of the optics windows, both from the side of the beam to the sample and from the sample to the detector, there is an unavoidable air gap that the beam needs to travel through. This air gap can result in increased background scattering, because the X-ray beam is required to travel through this space and this leads to detection of the scattering of molecules within this air gap, which are outside of the sample. While it is in

theory possible to have the capillary under vacuum, this facility is not common, and is not available yet at the Australian Synchrotron. To bring the background scattering to an absolute minimum, and hence increase the signal of precipitated drug, the air gap on either side of the flow-through capillary was made to be as small as possible.

Following this optimisation of the beamline the same lipid formulation, as used in the above study, was prepared with fenofibrate and a lower total amount of lipid formulation was analysed using the *in situ* technique. The Type-3A MC-SNEDDS was prepared with fenofibrate at 200% of the equilibrium solubility of the drug in the formulation as earlier in this chapter. This time, however, instead of addition of formulation at 25 mg/mL of digest as in earlier experiments, formulation was added to the *in vitro* digestion vessel at 3.3 mg/mL. This means a significantly reduced amount of drug was present during the experiment (0.95 mg/mL digest), when compared with the study above (7.16 mg/mL of digest). Therefore the amount of crystalline fenofibrate flowing through the capillary during the experiment was a little less than 1 mg/mL, assuming all of the fenofibrate precipitated during lipolysis. If fenofibrate did not precipitate completely then the concentration of drug in flow-through would be even further reduced. After 15 min of lipolysis, at which point the formulation was completely digested, by observing a plateau in the titration profile, there were visible diffraction peaks that corresponded to precipitated fenofibrate, as shown in Figure 8. This finding established that the further optimisation performed at the Australian Synchrotron, which included minimising the air gap for the beam to travel through, greatly improved sensitivity to precipitated drug, or crystalline material in flow-through mode.

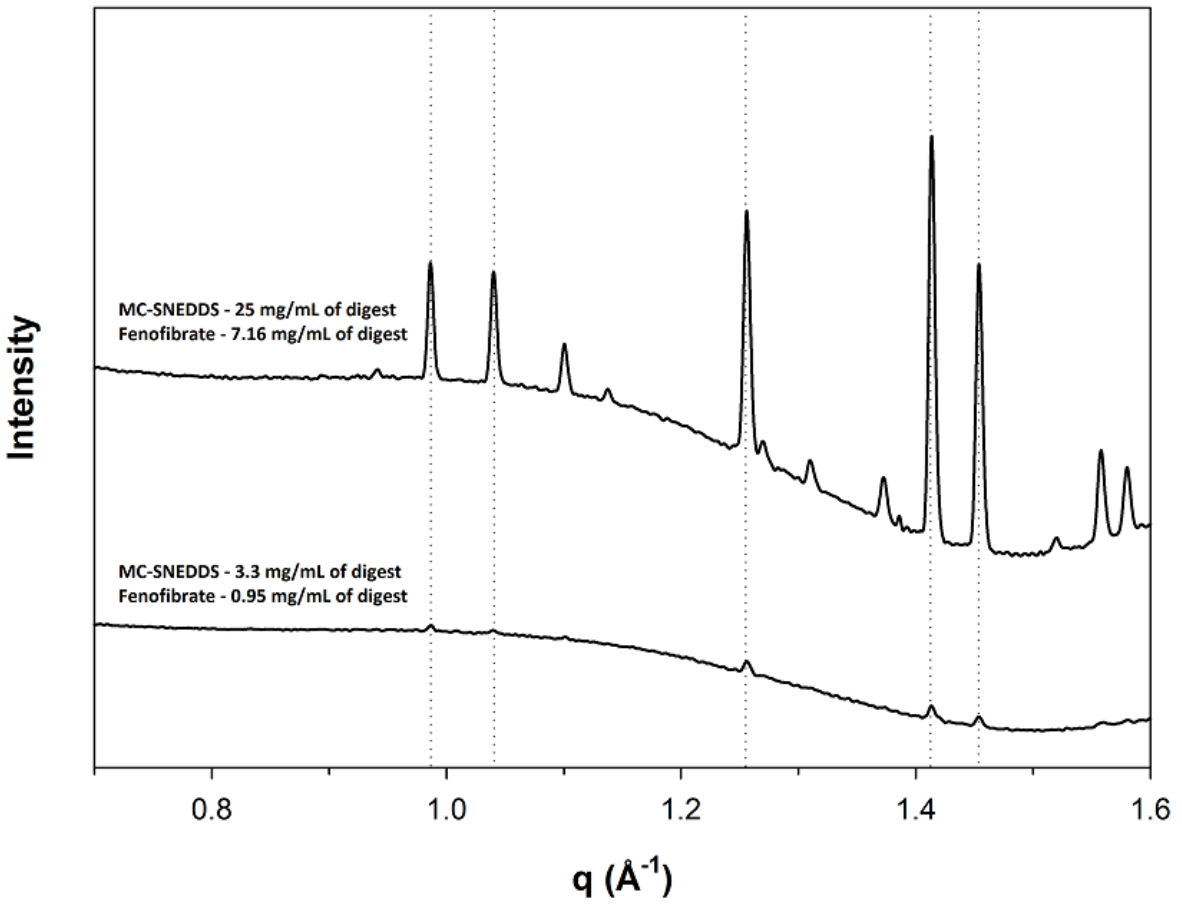

Figure 8: After 15 min of *in vitro* lipolysis, the SAXS profile from the experiment with lower total amount of formulation (3.3 mg/mL of digest) flowing through the capillary showed the presence of diffraction peaks for precipitated fenofibrate at angles identical to that observed with the experiment at the higher loading of formulation (25 mg/mL of digest). The limit of detection for precipitated fenofibrate in flow-through was improved to approximately 0.95 mg/mL (previously 3.99 mg/mL).

After optimising the beamline setup to detect concentrations of precipitated drug down to less than 1 mg/mL, further experiments were performed to investigate drug precipitation behaviour. These experiments examined a different drug (loratadine) from the digestion of the same MC-SNEDDS used in the above study, and the same drug (fenofibrate) from the digestion of a different formulation (LC-SNEDDS). The drug loading was made more relevant to real lipid formulations, where the respective SNEDDS were loaded with drug to 100% equilibrium solubility of the drug in

the formulation, as opposed to 200% in the above study. The main purpose of these follow-on experiments was to probe whether the threshold amount of lipid digestion that caused drug precipitation in the above study, which was 60% of formulation digested, translates to other drugs and lipid systems.

Loratadine, a weakly-basic drug with a pKa of 5.0, was chosen as an alternate drug to test using the improved *in situ* lipolysis and SAXS setup. It has been shown previously that loratadine precipitates extensively in a crystalline form during the digestion of lipid formulations (20). The crystalline precipitation of loratadine can be attributed to the state of ionisation of the drug during digestion. Loratadine has a pKa of 5.0, and remains largely unionised at experimental pH (pH = 6.5) and therefore unable to form ionic interactions with oppositely charged species to drive amorphous-salt precipitation. This concept will be explored in detail in chapter 4.

The Type-3A MC-SNEDDS was loaded with loratadine at 100% equilibrium solubility of the drug in the formulation and subjected to *in situ* lipolysis and SAXS. The resultant scattering data obtained in real-time over the course of the 60 min digestion was summarised and is presented in Figure 9.

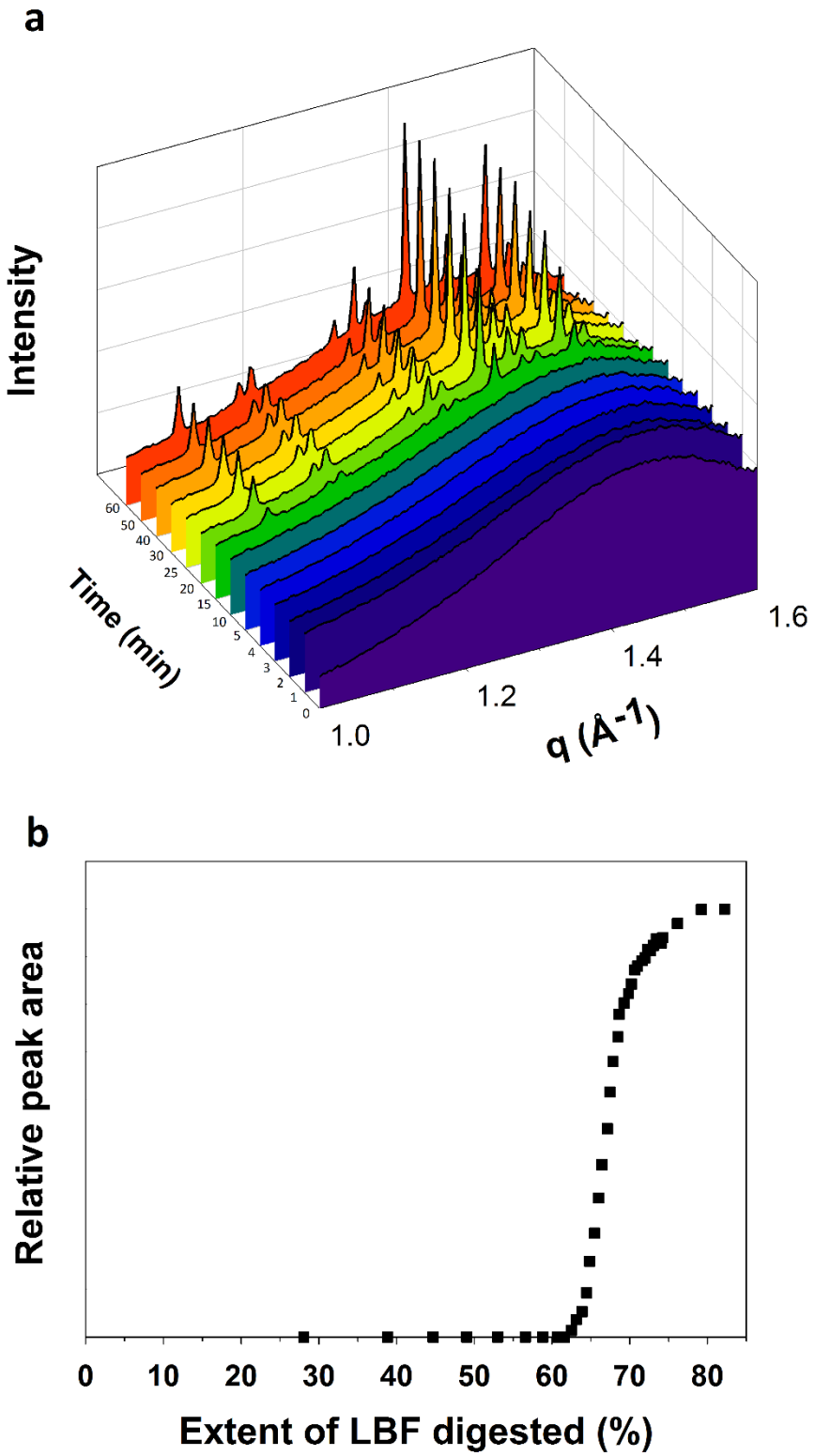

Figure 9: (a) *In situ* SAXS profiles during the lipolysis of the MC-SNEDDS formulation containing loratadine. Drug precipitation was evident at 11 min min after the addition of pancreatic lipase, after which the intensity of diffraction peaks increased over the 60 min lipolysis. (b) The threshold amount of formulation that needs to be digested before precipitation rapidly ensues appears to be 64%.

It can be seen from Figure 9a that loratadine, in comparison to fenofibrate, took longer to precipitate during the digestion of the same MC-SNEDDS. The first signs of loratadine precipitation were observed from the scattering data at around 11 min after initiating lipolysis, whereas fenofibrate started to precipitate after 4 min. From the perspective of drug precipitation with respect to time elapsed during digestion this difference is significant. Loratadine stays solubilised for longer than fenofibrate and would therefore present more opportunity for drug absorption provided a similar precipitation profile was observed *in vivo*.

From a formulation optimisation perspective, however, it is also useful to gauge the ability of the formulation to maintain drug in solution rather than simply conceding that there are inherent differences in precipitation behaviour between different drugs. From Figure 9b it becomes obvious that whilst loratadine takes longer to precipitate during digestion, the MC-SNEDDS displays similar performance with regard to drug solubilisation capacity. The threshold amount of formulation digested prior to rapid precipitation of loratadine was 64%, as compared to 60% for fenofibrate.

This result indicates that drug precipitation during digestion was closely linked to the extent of digestion of the specific formulation used, in this case a Type-3A MC-SNEDDS. Both fenofibrate and loratadine began to precipitate rapidly after 60% of the MC-SNEDDS was digested, which points to a significant loss in solubilisation capacity for the drug once this threshold amount of digestion is reached. From this perspective, across different drugs, the MC-SNEDDS performed similarly in relation to undergoing a loss in solubilisation capacity for the drug and triggering drug precipitation.

The loss in solubilisation capacity after 60% of the formulation was digested could result from a high level of drug supersaturation in the remaining undigested formulation, as well as supersaturation in the colloidal structures formed from lipid digestion products. After 60% of the formulation was digested, for the MC-SNEDDS used in the study, the colloidal structures formed from lipid digestion may have been different to those present during the initial stages of digestion, which could also affect solubilisation capacity for the drug. It has been shown before that MC digestion products intercalate with bile salt and phospholipid micelles to form mixed micelles and vesicles (5). It would be of interest in the future, however, to examine the correlation between colloidal structure formation, extent of digestion and drug precipitation, by examining the scattering from both the lipid digestion products and precipitated drug.

Also worth taking into consideration is the kinetics of digestion for different formulations. It is well established that LC-lipid formulations digest slowly in comparison to MC-lipid formulations, and to a lesser extent. To examine the effect of changing the lipid system on the threshold amount of lipid digestion that needs to occur prior to drug precipitation, a Type 3A LC-SNEDDS containing fenofibrate was prepared and subject to *in situ* lipolysis and SAXS.

The difference between the MC-SNEDDS and LC-SNEDDS was the replacement of all MC lipid components with LC lipid components. The MC triglycerides (Captex 355) and monoglycerides (Capmul MCM) were replaced with LC triglycerides (soybean oil) and monoglycerides (Maisine 35-1) in the same mass ratios to form the LC-SNEDDS. Fenofibrate was added to the LC-SNEDDS at 100% of the equilibrium solubility of the drug in the formulation. The resultant scattering data during *in situ* lipolysis and SAXS was summarised and is presented in Figure 10.

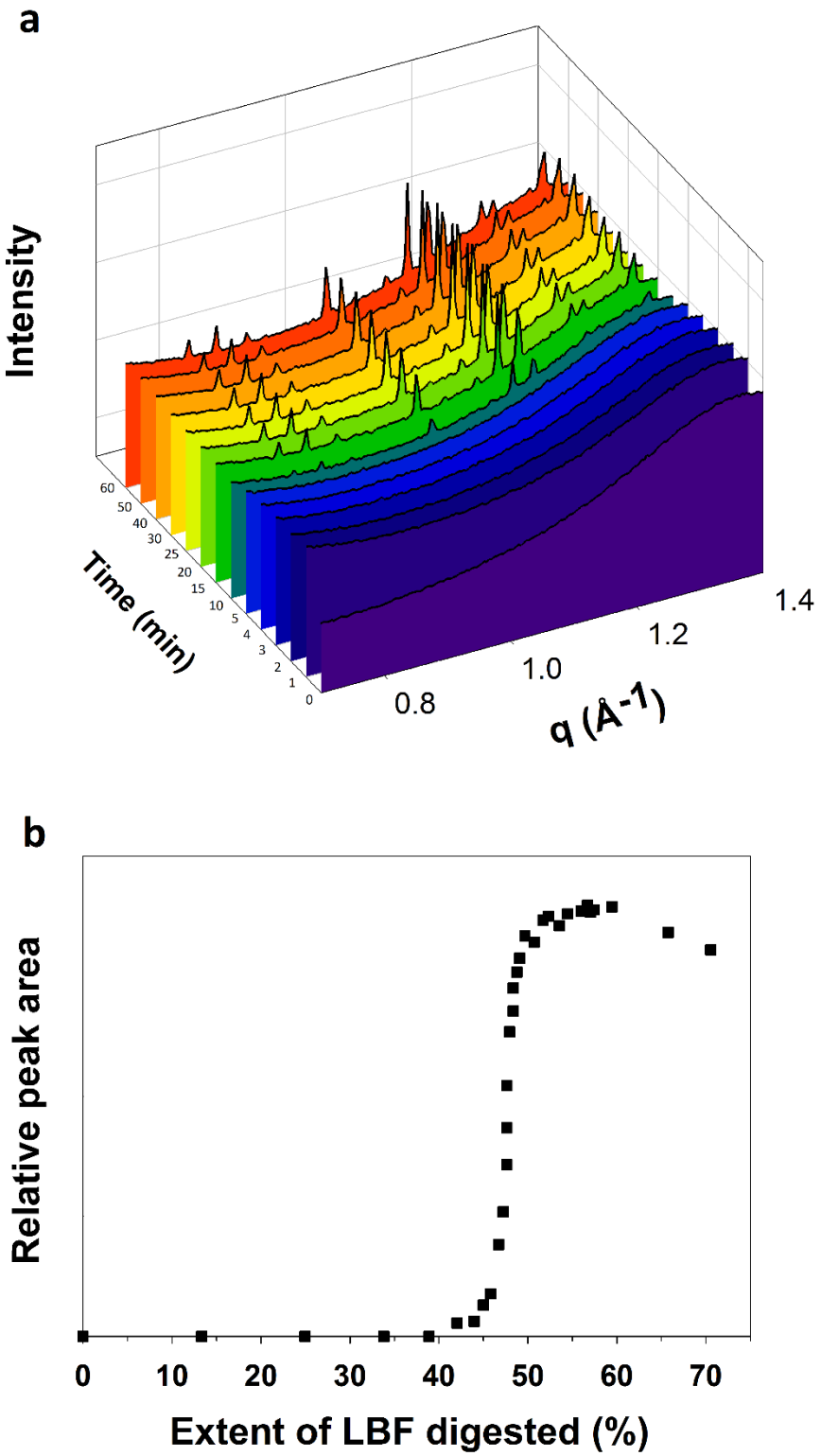

Figure 10: (a) *In situ* SAXS profiles during the lipolysis of the LC-SNEDDS formulation containing fenofibrate. Drug precipitation was evident at 9 min after the addition of pancreatic lipase, after which the intensity of diffraction peaks increased over the 60 min lipolysis. (b) The threshold amount

of formulation that needs to be digested before precipitation rapidly ensues appears to be 46%.

The LC-SNEDDS digested to a lesser extent than the MC-SNEDDS examined thus far. At the end of the 60 min lipolysis period the extent of digestion was calculated to be 70% for the LC-SNEDDS, as opposed to greater than 80% for the experiments that used a MC-SNEDDS above. In addition, the LC-SNEDDS digestion kinetics were slower, when compared to the MC-SNEDDS. The threshold amount of LC-SNEDDS that needed to be digested before fenofibrate precipitation ensued was 46%, as shown in Figure 10b. The slow kinetics of digestion for the LC-SNEDDS, however, meant that fenofibrate stayed in solution for more than double the time period than when the MC-SNEDDS was used (onset of precipitation at 9 min as opposed to 4 min). The rapid digestion of MC lipids leads to rapid drug precipitation during digestion, and a seemingly inferior solubilisation capacity to the LC-SNEDDS.

Therefore, creating an optimal lipid based formulation that can minimise the risk of drug precipitation during digestion may need to be slow to digest, as well as have a high threshold amount of digestion that needs to take place, before triggering drug precipitation. Another interesting aspect of using LC lipid formulations, such as the one used above, is the formation of digestion products with improved solubility for poorly water-soluble compounds in comparison to the digestion products of MC formulations. Unlike MC digestion products, which form predominantly mixed micelles and vesicles, LC digestion products can display more complex phase behaviour, due to an increased lipid chain length, or unsaturation. In addition to mixed micelles and vesicles, LC digestion products have been shown to form bicontinuous and micellar cubic phases, and inverted hexagonal phases (5). These cubic phases, for instance, have been shown to improve the solubility of hydrocortisone compounds, which represented poorly water soluble drugs in the said study (34).

From Figure 10b it can be seen that after 57% of the formulation is digested, the peak area representing precipitated fenofibrate began to decrease. A decrease in peak area was not observed for the MC-SNEDDS formulations examined above, in the case of fenofibrate and loratadine. This decrease in peak area observed with the LC-SNEDDS could be a result of precipitated fenofibrate becoming re-solubilised by the LC digestion products, where the number of colloidal structures formed from the digested formulation increased as the formulation proceeded to digest. A possible change in number or form of the structures formed from the digestion of LC lipids may have improved drug solubility in the aqueous colloidal phase. It would be interesting to examine if the LC-SNEDDS was allowed to digest further, meaning the lipolysis was performed for longer than 60 min, whether this re-solubilisation effect becomes more pronounced. This is another reason why it will be important in future studies to consider both the scattering from the colloidal structures formed during digestion and the scattering from precipitated drug, in an attempt to try and correlate structure formation to drug precipitation. The novel *in situ* lipolysis and SAXS method discussed in this chapter would allow for this important analysis to be performed.

References

1. Amidon GL, Lennernas H, Shah VP, Crison JR. 1995. *A theoretical basis for a biopharmaceutic drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability*. Pharm Res **12**(3):413–420.

2. Porter CJ, Trevaskis NL, Charman WN. 2007. *Lipids and lipid-based formulations: Optimizing the oral delivery of lipophilic drugs*. Nat Rev Drug Discov **6**(3):231–248.

3. Porter CJ, Charman SA, Charman WN. 1996. *Lymphatic transport of halofantrine in the triple-cannulated anesthetized rat model: Effect of lipid vehicle dispersion*. J Pharm Sci **85**(4):351–356.

4. Khoo SM, Shackleford DM, Porter CJ, Edwards GA, Charman WN. 2003. *Intestinal lymphatic transport of halofantrine occurs after oral administration of a unit-dose lipid-based formulation to fasted dogs*. Pharm Res **20**(9):1460–1465.

5. Phan S, Salentinig S, Prestidge C, Boyd BJ. 2014. *Self-assembled structures formed during lipid digestion: Characterization and implications for oral lipid-based drug delivery systems*. Drug Deliv Transl Res **4**(3):275–294.

6. Wilde PJ, Chu BS. 2011. *Interfacial & colloidal aspects of lipid digestion*. Adv Colloid Interface Sci **165**(1):14–22.

7. Kossena GA, Charman WN, Boyd BJ, Porter CJ. 2005. *Influence of the intermediate digestion phases of common formulation lipids on the absorption of a poorly water-soluble drug*. J Pharm Sci **94**(3): 481–492.

8. Yeap YY, Trevaskis NL, Quach T, Tso P, Charman WN, Porter CJ. 2013. *Intestinal bile secretion promotes drug absorption from lipid colloidal phases via induction of supersaturation*. Mol Pharm **10**(5):1874–1889.

9. Holm R, Mullertz A, Mu H. 2013. *Bile salts and their importance for drug absorption*. Int J Pharm **453**(1):44–55.

10. MacGregor KJ, Embleton JK, Lacy JE, Perry EA, Solomon LJ, Seager H, Poton CW. 1997. *Influence of lipolysis on drug absorption from the gastro-intestinal tract*. Adv Drug Deliv Rev **25**(1):33–46.

11. Anby MU, Williams HD, McIntosh M, Benameur H, Edwards GA, Pouton CW, Porter CJ. 2012. *Lipid digestion as a trigger for supersaturation: Evaluation of the impact of supersaturation stabilization on the in vitro and in vivo performance of self-emulsifying drug delivery systems*. Mol Pharm **9**(7):2063–2079.

12. VanEerdenbrugh B, Raina S, Hsieh YL, Augustijns P. 2014. *Classification of the crystallization behavior of amorphous active pharmaceutical ingredients in aqueous environments*. Pharm Res **31**(4): 969–982.

13. Bevernage J, Brouwers J, Brewster ME, Augustijns P. 2013. *Evaluation of gastrointestinal drug supersaturation and precipitation: Strategies and issues*. Int J Pharm **453**(1):25–35.

14. Williams HD, Trevaskis NL, Yeap YY, Anby MU, Pouton CW, Porter CJ. 2013. *Lipid-based formulations and drug supersaturation: Harnessing the unique benefits of the lipid digestion/absorption pathway*. Pharm Res **30**(12):2976–2992.

15. Augustijns P, Brewster ME. 2012. *Supersaturating drug delivery systems: Fast is not necessarily good enough*. J Pharm Sci **101**(1):7–9.

16. Warren DB, Benameur H, Porter CJ, Pouton CW. 2010. *Using polymeric precipitation inhibitors to improve the absorption of poorly water soluble drugs: A mechanistic basis for utility*. J Drug Target **18**(10):704–731.

17. Brouwers J, Brewster ME, Augustijns P. 2009. *Supersaturating drug delivery systems: The answer to solubility-limited oral bioavailability?* J Pharm Sci **98**(8):2549–2572.

18. Feeney OM, Williams HD, Pouton CW, Porter CJ. 2014. *'Stealth' lipid-based formulations: Poly(ethylene glycol)-mediated digestion inhibition improves oral bioavailability of a model poorly water soluble drug.* J Control Release **192**:219–227.

19. Sassene PJ, Knopp MM, Hesselkilde JZ, Koradia V, Larsen A, Rades T, Mullertz A. 2010. *Precipitation of a poorly soluble model drug during in vitro lipolysis: Characterization and dissolution of the precipitate.* J Pharm Sci **99**(12):4982–4991.

20. Stillhart C, Durr D, Kuentz M. 2014. *Toward an improved understanding of the precipitation behavior of weakly basic drugs from oral lipid-based formulations.* J Pharm Sci **103**(4):1194–1203.

21. Thomas N, Holm R, Mullertz A, Rades T. 2012. *In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS).* J Control Release **160**(1):25–32.

22. Williams HD, Sassene P, Kleberg K, Calderone M, Igonin A, Jule E, Vertommen J, Blundell R, Benameur H, Mullertz A, Pouton CW, Porter CJ. 2013. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 3: Understanding supersaturation versus precipitation potential during the in vitro digestion of type I, II, IIIA, IIIB and IV lipid-based formulations.* Pharm Res **30**(12):3059–3076.

23. Stillhart C, Imanidis G, Kuentz M. 2013. *Insights into drug precipitation kinetics during in vitro digestion of a lipid-based drug delivery system using in-line Raman spectroscopy and mathematical modeling.* Pharm Res **30**(12):3114–3130.

24. Boetker J, Rades T, Rantanen J, Hawley A, Boyd BJ. 2012. *Structural elucidation of rapid solution-mediated phase transitions in pharmaceutical solids using in situ synchrotron SAXS/WAXS*. Mol Pharm **9**(9):2787–2791.

25. Warren DB, Anby MU, Hawley A, Boyd BJ. 2011. *Real time evolution of liquid crystalline nanostructure during the digestion of formulation lipids using synchrotron small-angle X-ray scattering*. Langmuir **27**(15):9528–9534.

26. Thomas N, Richter K, Pedersen TB, Holm R, Mullertz A, Rades T. 2014. *In vitro lipolysis data does not adequately predict the in vivo performance of lipid-based drug delivery systems containing fenofibrate*. AAPS J **16**(3):539–549.

27. Persson LC, Porter CJ, Charman WN, Bergstrom CA. 2013. *Computational prediction of drug solubility in lipid based formulation excipients*. Pharm Res **30**(12):3225–3237.

28. Thomas N, Holm R, Garmer M, Karlsson JJ, Mullertz A, Rades T. 2013. *Supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS) enhance the bioavailability of the poorly water soluble drug simvastatin in dogs*. AAPS J **15**(1):219–227.

29. Williams HD, Sassene P, Kleberg K, Bakala-N'Goma JC, Calderone M, Jannin V, Igonin A, Partheil A, Marchaud D, Jule E, Vertommen J, Maio M, Blundell R, Benameur H, Carriere F, Mullertz A, Porter CJ, Pouton CW. 2012. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 1: Method parameterization and comparison of in vitro digestion profiles across a range of representative formulations*. J Pharm Sci **101**(9):3360–3380.

30. Larsen AT, Sassene P, Mullertz A. 2011. *In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems*. Int J Pharm **417**(1–2):245–255.

31. Gargouri Y, Moreau H, Verger R. 1989. *Gastric lipases: Biochemical and physiological studies*. Biochim Biophys Acta **1006**(3):255–271.

32. Dressman JB, Berardi RR, Dermentzoglou LC, Russell TL, Schmaltz SP, Barnett JL, Jarvenpaa KM. 1990. *Upper gastrointestinal (GI) pH in young, healthy men and women*. Pharm Res **7**(7):756–761.

33. Kirby NM, Mudie ST, Hawley AM, Cookson DJ, Mertens HDT, Cowieson N, Samardzic-Boban V. 2013. *A low-background-intensity focusing small-angle X-ray scattering undulator beamline*. J Appl Crystallogr **46**(6):1670–1680.

34. *Kossena GA, Charman WN, Boyd BJ, Dunstan DE, Porter CJ. 2004 Probing drug solubilization patterns in the gastrointestinal tract after administration of lipid-based delivery systems: a phase diagram approach. Journal of pharmaceutical sciences **93**(2):332-48*

Chapter 4:
The Precipitation Behaviour of Weakly-Basic Drugs During Lipid Digestion

Declaration

This chapter consists of a published journal article that closely examines the trend of the precipitation of some weakly-basic drugs in an amorphous form during the digestion of lipid formulations. Specifically, it examines potential ionic interactions between the weakly-basic drug cinnarizine and oppositely charged fatty acids present during digestion, as a driver of previously observed amorphous precipitation. The article was published as: Khan J, Rades T, Boyd B. *Lipid-based formulations can enable the model poorly water-soluble weakly basic drug cinnarizine to precipitate in an amorphous-salt form during in vitro digestion*. Mol. Pharm. 2016, 13 (11): 3783-3793.

Declaration by candidate:

For chapter 4, the nature and extent of my contribution to the work was as follows:

Nature of contribution	**Extent of contribution**
Perform experiments, preparation of manuscript	90%

The following co-authors contributed to the work:

Name	**Nature of contribution**
Thomas Rades	Co-supervisor, preparation of manuscript
Ben Boyd	Main supervisor, preparation of manuscript

The undersigned hereby declare that the above declaration correctly reflects the nature and extent of candidate and co-author contributions:

Candidate's signature: [redacted] Date: 20-04-2017

Main supervisor's signature: [redacted] Date: 20-04-2017

Lipid-Based Formulations Can Enable the Model Poorly Water-Soluble Weakly Basic Drug Cinnarizine To Precipitate in an Amorphous-Salt Form During *In vitro* Digestion

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Abstract

The tendency for poorly water-soluble weakly basic drugs to precipitate in a noncrystalline form during the *in vitro* digestion of lipid-based formulations (LBFs) was linked to an ionic interaction between drug and fatty acid molecules produced upon lipid digestion. Cinnarizine was chosen as a model weakly basic drug and was dissolved in a medium-chain (MC) LBF, which was subject to *in vitro* lipolysis experiments at various pH levels above and below the reported pKa value of cinnarizine (7.47). The solid-state form of the precipitated drug was analyzed using X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and crossed polarized light microscopy (CPLM). In addition, the phase distribution of cinnarizine upon lipolysis was analyzed using high-performance liquid chromatography (HPLC). Cinnarizine precipitated in a noncrystalline form during lipolysis experiments at pH 6.5, pH 5.5, and pH 4.0 but precipitated in a crystalline form at pH 8.0 according to XRD measurements

on the pellets. Differences were also observed in the FTIR spectra of the pellet phases at pH 8.0 and pH 6.5, with the absorption bands in the C–N stretch region of the IR spectra supporting a shift from the starting free base crystalline material to the hydrochloride salt, thus supporting the case that ionic interactions between weak bases and fatty acid molecules during digestion are responsible for producing amorphous-salts upon precipitation. The conclusion has wide implications for understanding past *in vitro* and *in vivo* data for lipid-based formulations of basic drugs, as well as future formulation design and optimization.

Introduction

A common approach to increase the oral bioavailability of poorly water-soluble lipophilic drugs is to use lipid-based formulations (LBFs). These formulations aim to maintain lipophilic drugs in a dissolved state before and during digestion of the formulation throughout gastrointestinal transit, circumventing the issue of slow dissolution often observed with such drugs (1). Lipid digestion leads to the formation of self-assembled colloidal structures in the small intestine, formed through interactions between lipid digestion products and amphiphilic endogenous molecules (2). These colloidal structures, such as mixed micelles and vesicles, are believed to play an integral role in maintaining the drug in solution, by enabling the effective partitioning of drug between these structures during gastrointestinal transit (3).

Drug precipitation, however, becomes a possibility if LBFs lose their solubilization capacity upon dilution and digestion in gastrointestinal fluid. This loss in solubilization capacity becomes more significant as lipid digestion proceeds, as the colloidal structures formed become increasingly hydrophilic, potentially resulting in drug supersaturation within the colloidal structures and subsequent drug precipitation (4–6). There has

been a paradigm in the field that if drug precipitation occurs during the digestion of LBFs then the total amount of absorbed drug would be adversely affected, and this would likely be true if the drug precipitated in its poorly water-soluble crystalline form in every case. On the basis of this assumption, formulation strategies have focused heavily on trying to inhibit, or prolong the onset of drug precipitation during the digestion of LBFs for as long as possible. The thermodynamic driver for absorption provided by a supersaturated system is another key reason for aiming to maintain the supersaturation of drug during the digestion of LBFs, while avoiding drug precipitation (7). Polymeric materials are often used for this purpose, where their mechanism of action affords a gradual decrease in the concentration of dissolved drug toward equilibrium solubility, as opposed to the rapid decrease in the concentration of dissolved drug seen without polymers (6, 8).

A number of studies, however, have recently emerged suggesting a link between the solid-state form of the precipitated drug and its potential effect on redissolution of drug *in vivo*. A key finding in this regard was the apparent precipitation of cinnarizine in a noncrystalline form during the digestion of a long chain (LC) LBF and the 10-fold higher dissolution rate of this precipitated form of the drug in simulated intestinal fluid when compared to the reference crystalline form (9). There appears to be a trend that weakly-basic drugs more readily produce noncrystalline precipitates during digestion in comparison to neutral (e.g., fenofibrate (10–13) and danazol (14)) and acidic drugs such as tolfenamic acid (13). It had previously been suggested that weak bases precipitate in a noncrystalline form due to interactions with oppositely charged species present during digestion.15 Liberated fatty acids from the digestion of lipids might interact with weakly basic drugs during the digestion of LBFs. A key finding that supports this proposition comes from a study that observed a difference in the solid-state form of the

precipitate between two weakly basic drugs with differing pKa values upon digestion of model LBFs. The weakly basic drug carvedilol precipitated in a noncrystalline form upon digestion of the LBFs, whereas loratadine precipitated in its crystalline form (15). This difference in the solid-state form of the precipitate for the two weakly basic drugs tested could be a consequence of their state of ionization, which is dependent on the pKa values of the drug relative to the pH of the digesting medium. Specifically, carvedilol has a literature pKa value of 7.8 (16) and would be predominantly ionized at pH 7.5 (pH of the digesting medium used in that study) and therefore available for ionic interactions with oppositely charged species (e.g fatty acids). Loratadine, however, precipitated in a crystalline form during digestion of the same LBF, which might be due to it having a literature pKa value of 5.0 (17) and being present predominantly in its un-ionized form during digestion (pH 7.5 of digesting medium). As a result, unlike carvedilol, loratadine would have been unavailable for ionic interactions with fatty acids present within the digesting medium. Further evidence of drug-fatty acid interactions during digestion has emerged when cinnarizine was loaded into a LC-LBF and solid-state NMR was performed on the precipitated material (18) The results suggest that there was an interaction between the drug and fatty acids, though it is unclear whether this interaction was a result of interactions with fatty acids liberated upon digestion of the LBF, or due to the fatty acid (oleic acid) included in the starting formulation.

Therefore, while there is circumstantial evidence for the ionization state of weakly basic drugs to be a critical determinant of the propensity for ionic interactions with anionic components in the digestion medium, and thereby driving the solid-state form of the precipitated drug, the need to directly confirm the hypothesis remains. The current study thus aims to determine clearly whether the solid-state form of the drug is driven by the pH of digestion medium relative to the pKa of the drug. A

model medium chain (MC) length lipid formulation was used for these studies, due to the likelihood of precipitation of the model drug cinnarizine, and digestion performed above and below the pKa of the drug with interrogation of the precipitate by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The MC lipids also afforded the possibility to determine whether the lipid chain-length influences the solid state of precipitated cinnarizine (as depicted in Figure 1), as previous work utilized a LC self-microemulsifying drug delivery system (LC-SMEDDS), where cinnarizine was shown to precipitate in a noncrystalline form during digestion (9,18). In that instance, it was proposed that the oleic acid, both in the starting formulation and the oleic acid liberated from the digestion of the long chain lipids in the formulation, formed interactions with the weakly basic drug cinnarizine during digestion.

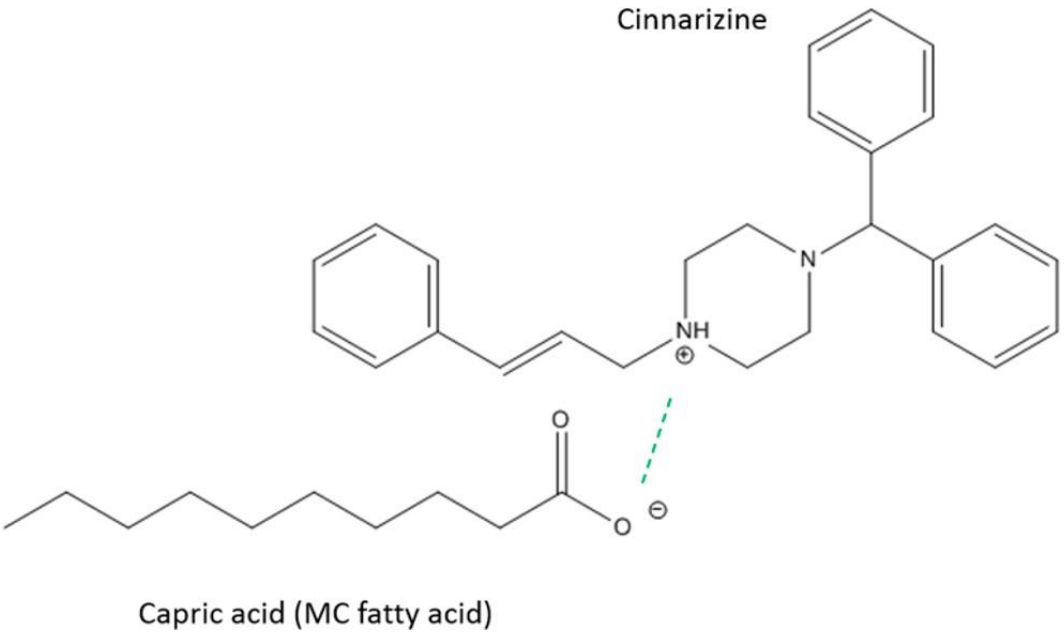


Figure 1: Schematic of the ionic interaction that takes place between the weakly basic drug cinnarizine (pK_a = 7.47) and MC fatty acid molecules (pK_a = 4.89) upon digestion of the model LBF to affect the solid-state form of precipitated drug

Materials

Captex 355 [medium chain triglyceride mixture composed of 59% caprylic acid (C_8), 40% capric acid (C_{10}), < 1% lauric acid (C_{12}) as stated in the product information], and Capmul MCM [mono/di-glycerides composed of caprylic acid (C_8) in glycerol], were obtained from Abitec Corporation (Janesville, Wisconsin). Cremophor EL was purchased from BASF Corporation (Washington, New Jersey). Cinnarizine, Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate (NaTDC), > 95%), and 4-bromophenylboronic acid (4-BPBA, > 95%) were purchased from Sigma-Aldrich (St. Louis, Missouri). Cinnarizine hydrochloride was isolated from the free base and the hydrochloride salt, which was confirmed by FTIR. Calcium chloride dihydrate (> 99%) and ammonium dihydrogen orthophosphate were obtained from Ajax Finechem (Seven Hills, New South Wales, Australia). Sodium chloride (> 99%) was purchased from Chem Supply (Gillman, South Australia, Australia). HPLC grade methanol and acetonitrile were purchased from Merck (MA, U.S.A.). Phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) was obtained from Trapeze Associates Pty. Ltd. (Clayton, Victoria, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia).

Methods

Preparation of LBF

The composition of the model LBF chosen for this study was a MC self-nanoemulsifying drug delivery system (MC-SNEDDS), consisting of 0.3 g of Captex 355, 0.3 g of Capmul, 0.3 g of Cremophor EL, and 0.1 g of ethanol, based on previous studies (19). The fatty acids present and available for interactions with cinnarizine in the current study are a product of lipid digestion alone, as the starting formulation contained no additional fatty acid. The excipients that formed the MC-SNEDDS were weighed into

a glass scintillation vial and vortexed for 1 min to ensure the components had mixed prior to adding cinnarizine. The amount of cinnarizine added to the MC-SNEDDS was 50 mg/g of formulation in all instances, and it was selected to match the study conducted by Sassene *et al.* (9, 18). It is important to note, however, that the degree of drug saturation in the formulation in the current study would be slightly different to that of the study by Sassene *et al.*, as there are differences in the composition of the formulation, namely, the chain length of the lipids used. The MC-SNEDDS-containing drug was further vortexed for 1 min and then placed in a roller oven at 40 °C overnight to allow the drug to dissolve completely. The formulation was visually inspected for solid drug particles prior to performing the digestion experiments, and it was placed in a 60 °C oven for a short time in instances where the drug was not fully dissolved.

Preparation of Digestion Medium and Pancreatic Lipase

The digestion medium and pancreatic lipase solution were prepared using digestion buffer, which consisted of 2 mM Tris–maleate, 1.4 mM $CaCl_2 \cdot 2H_20$, and 150 mM NaCl. The digestion buffer was adjusted to pH 6.5 using NaOH and HCl solutions. Digestion medium was then made up using the above buffer solution, supplemented with 5 mM of NaTDC (bile salt) and 1.25 mM of DOPC (phospholipid) to represent fasted state simulated conditions. A round-bottom flask was used to dissolve the DOPC in an appropriate amount of chloroform, and then the chloroform was removed under vacuum to leave an evenly coated film of lipid at the bottom of the flask. Digestion buffer and NaTDC were then added, and the flask was placed in a sonicator bath to dissolve any remaining solid particles. The digestion medium was kept refrigerated at 4 °C before use. Pancreatin extract was weighed (2 g) into a small glass beaker, before 5 mL of digestion buffer was added to form a suspension. The suspension was stirred for 5

min, transferred to a 12 mL plastic centrifuge tube, and centrifuged at 2205g at 4 °C for 15 min. The process yielded pancreatic lipase solution as the supernatant (1000 TB units/mL of digest), which was collected and stored at 4 °C prior to use. Each lipolysis experiment was initiated by adding 4 mL of this pancreatic lipase solution.

In vitro Lipolysis

Lipolysis experiments were performed according to previously established protocols (1, 20). In summary, a thermostated glass vessel, set to maintain a temperature of 37 °C, was attached to a stirring unit, which was attached to a Metrohm titrator, 5 mL dosing unit with autoburet and an iUnitrode pH probe (Metrohm AG, Herisau, Switzerland). The digestion medium (36 mL) was added to the vessel and stirred, before the MC-SNEDDS containing cinnarizine was also added to the vessel and allowed to disperse for 5 min. During this 5 min dispersion period, the pH was adjusted to the experimental pH value using NaOH and HCl. Lipolysis experiments were performed above and below the pKa value of cinnarizine, as a way of probing the link between the solid-state form of precipitated cinnarizine and the state of ionization of cinnarizine. The literature pKa value of cinnarizine is 7.47 (21, 22) and therefore, a lipolysis experiment with the digesting medium set to a pH above 7.47 was expected to force un-ionized cinnarizine at this pH to precipitate in a crystalline form, as the drug would not be able to ion pair with fatty acids. However, at a pH below 7.47, it was expected the ionized cinnarizine would ion pair with fatty acids and produce a non-crystalline precipitate. As a result, the pH values of the digestion medium chosen for analyzing the solid state of cinnarizine were pH 8.0 and pH 6.5, but selected experiments were also performed at pH 7.47, pH 5.5, and pH 4.0.

After the 5 min dispersion period, the digestion was initiated by adding 4 mL of the previously prepared pancreatic lipase

solution (to achieve an overall activity of 1000 TB units/mL of digest). As the lipidic components in the formulation were digested, the fatty acids liberated were titrated with 0.6 M NaOH solution, which maintained the specific experimental pH level over the course of the experiment. The NaOH solution titrated only ionized fatty acids at the specific pH of the lipolysis experiment. Consequently, at the end of the 60 min digestion period, for all experiments, the pH was rapidly increased to pH 9.0 to ionize all fatty acids. This allowed the un-ionized fatty acid molecules present during lipolysis to be quantified as well by taking into account the amount of NaOH needed to increase the pH to 9.0 (23). This increase to pH 9.0 at the end of lipolysis was done only for experiments concerned with calculating the extent of digestion and not for experiments where the solid state of the pellet phase was evaluated. The extent of digestion was calculated according to Equation 1.

$$Extent\ of\ LBF\ digested\ (\%) = \frac{Ionised\ fatty\ acid\ (mol) + Unionised\ fatty\ acid\ (mol)}{Theoretical\ fatty\ acid\ in\ LBF\ (mol)} \times 100$$

Equation 1

HPLC Quantification of Drug and Sample Treatment

A previously reported method for analyzing cinnarizine in digestion samples was followed to determine the phase distribution of the drug upon digestion of the MC-SNEDDS (24). The HPLC system included a Shimadzu CBM-20A system controller, LC-20AD solvent delivery module, SIL-20A auto sampler and a CTO-20A column oven set at 40 °C, coupled to an SPD-20A UV-detector and an RF-10AXL fluorescence detector (Shimadzu Corporation, Kyoto, Japan). A reverse-phase C_{18} column was used (4.6 × 75 mm, 3.5 µm; Waters Symmetry, MA, U.S.A.).

Aliquots (200 µL) from the lipolysis experiments were taken at specific time points before and after adding the pancreatic

lipase solution (t = 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min). The aliquots were transferred to a 1.75 mL Eppendorf tube containing 20 µL of lipase inhibitor (0.05 M 4-BPBA) and centrifuged at 7708g for 1 h. After centrifuging for 1 h, the supernatant (aqueous colloidal phase) was separated from the pellet phase (precipitated drug and calcium-fatty acid soaps). The aqueous colloidal phase was diluted with mobile phase until the concentration of drug fell in the range of the standard curve. The pellet phase was first dissolved in 200 µL of methanol and then diluted in mobile phase until the drug concentration fell within the range of the standard curve. Lipolysis experiments and HPLC analysis at pH 8.0, pH 6.5 and pH 4.0 were performed in triplicate.

The mobile phase was an isocratic premix composed of acetonitrile and Milli Q water (50:50) with ammonium dihydrogen orthophosphate at 20 mM and pH adjusted to 4.2. The flow rate used was 1 mL/min, and the injection volume was 50 µL. The concentration of drug in the digested samples was determined by comparison to a standard curve.

Polarised Light Microscopy – Morphology of Precipitated Drug

The isolated pellet phase, from the lipolysis experiments, was visualized under crossed-polarized light to determine whether crystalline drug had precipitated during digestion of the model MC-SNEDDS. A Nikon ECLIPSE Ni–U upright microscope fitted with crossed polarizing filters and a DS-U3 digital camera control unit (Nikon, Tokyo, Japan) was used for CPLM of pellet phases. Precipitated cinnarizine in its thermodynamically stable crystalline form has been shown previously to exhibit birefringence (9), whereas amorphous materials do not, due to the lack of long-range order in the sample (25). For the CPLM studies, the

pellet phase was isolated after performing the lipolysis experiment and placed onto a glass microscope slide, where it was allowed to air-dry before analysis.

X-ray Diffraction – Determination of the Solid State Form of Precipitated Cinnarizine

The pellet phase was isolated and analyzed by XRD to identify the solid state of the precipitated drug. A Bruker D8 Advance X-ray diffractometer with Ni-filtered Cu Kα radiation (1.54 Å) at 40 kV and 40 mA was used to collect XRD data. The samples were analyzed in the range of 5–50° in 2θ, with a step size of 0.02° and a scan rate of 0.5 s per step. Characteristic peaks for cinnarizine in the pellet phase of digested formulations were compared to the reference crystalline free base; the presence of the same peaks in the pellet phase indicated cinnarizine had precipitated in its crystalline free base form during digestion. Conversely, a lack of crystalline peaks in the diffractogram and the presence of a large halo region signified the material was amorphous, or noncrystalline (26).

FTIR – Probing Drug-Fatty Acid Interactions

The pellet phase of the digested MC-SNEDDS formulations at selected pH levels was left to air-dry on a microscope slide for a few hours, collected, and then analyzed using a PerkinElmer Frontier FTIR with attenuated total reflectance (ATR) (Waltham, Massachusetts). A small amount of sample was carefully placed onto the ATR crystal, and the pressure valve was used to improve the uniformity of contact between the sample and the ATR crystal of the instrument. Samples were analyzed using 32 scans at a resolution of 4 cm^{-1}. A background scan was collected initially, and the sample spectrum was collected subsequently with the Spectrum software automatically subtracting the background.

Results

Upon addition of the MC-SNEDDS to the simulated digestion fluid, the formulation immediately dispersed and was left to stir for 5 min, before pancreatic lipase solution was added to initiate digestion. MC-SNEDDS formulations have been shown previously to digest to completion under similar lipolysis conditions at pH 6.5 and pH 7.5 (13, 15). The MC-SNEDDS in the current study was loaded with cinnarizine at 50 mg/g and lipolysis was performed in triplicate at pH 8.0, pH 6.5 and pH 4.0. As described above, the pH of the digesting medium was rapidly increased to pH 9.0 at the end of the 60 min digestion period, as a way to quantify un-ionized and ionized fatty acid molecules, and the overall extent of formulation digested. The resultant lipolysis titration profiles at pH 8.0 and 6.5 are presented in Figure 2.

The lipid components of the MC-SNEDDS appeared to digest at a slightly faster rate at pH 8.0 than at pH 6.5, as can be seen from Figure 2. Within 26 min of initiating lipolysis at pH 8.0, the MC-SNEDDS had digested to about 88.5%, which was the same extent to which the MC-SNEDDS had digested at pH 6.5 after 60 min. The overall extent of digestion for the MCSNEDDS at pH 8.0 was around 95.8%. This discrepancy in the extent of formulation digested at pH 8.0 and pH 6.5 may be attributed to the effect of pH on lipase activity. The optimal pH for pancreatic lipase activity has been reported to be closer to pH 7.5 (27), but pH 6.5 is generally employed for lipolysis experiments, as this value affords a more suitable compromise between lipase activity and duodenal pH (28). A change in the initial rate of lipolysis as a function of pH has been reported previously with MC lipids, where it was shown that the rate of lipolysis increases as pH is increased from pH 4.0 up to a maximum rate of lipolysis at pH 7.5, before decreasing again (29). The rate of lipolysis at pH 8.0, however, was still higher than at pH 6.5 in the above study, as was also observed with the current study.

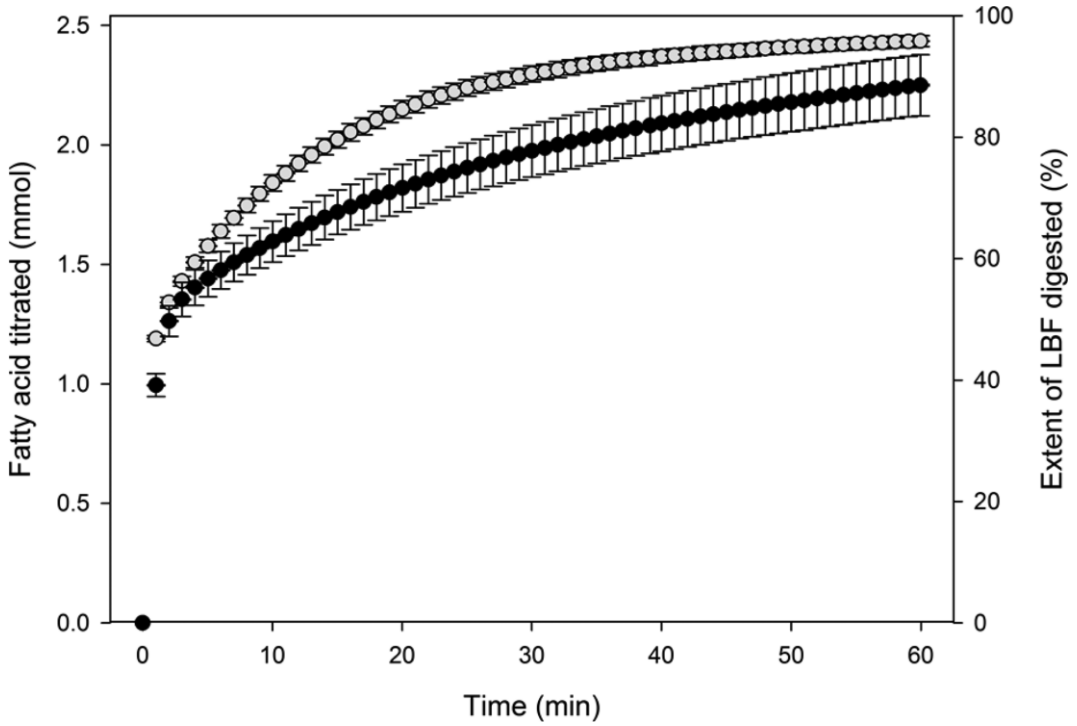


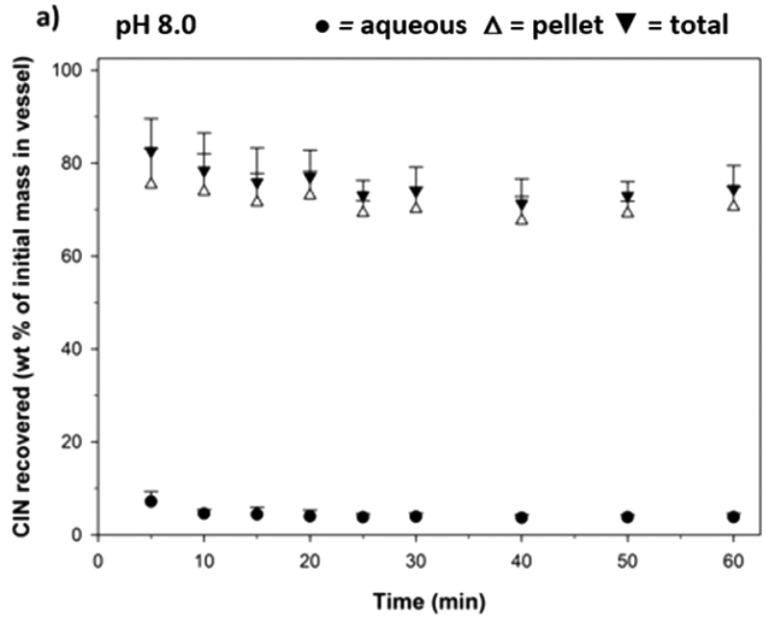
Figure 2: Titration profiles of ionized and un-ionized fatty acid produced during *in vitro* lipolysis of the MC-SNEDDS at pH 8.0 (gray circles) and pH 6.5 (black circles) (mean ± SD, *n* = 3 different digestions). The profile also shows the extent of LBF digested over the 60 min lipolysis. The titration profile for the lipolysis at pH 4.0 over the 60 min period could not be presented due to the impact of lipase addition on pH preventing return of the pH to below pH 4.0, which would trigger addition of titrant, but the extent of digestion is discussed in the text.

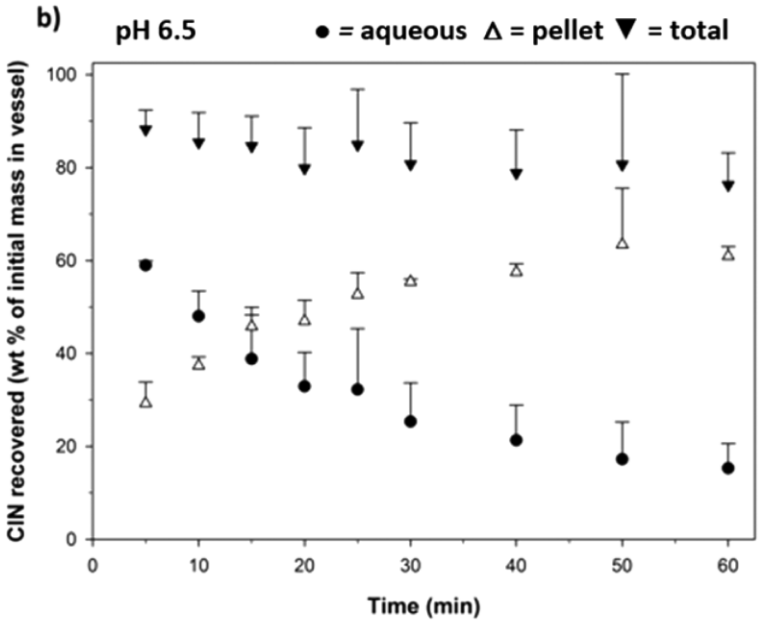
The digestion profile of the MC-SNEDDS at pH 4.0 could not be presented in Figure 2. This is because during the experiment, the lipase added to initiate digestion increased the pH of the digesting medium from a starting pH of 4.0 to around pH 4.5. The liberation of fatty acids slowly decreased the pH of the system back toward pH 4.0, which indicated digestion of lipids was occurring, but NaOH was not added to the digestion vessel over the course of the 60 min digestion experiment. Therefore, only the extent of digestion for the MC-SNEDDS could be determined from the back-titration volume at pH 4.0. In the end, the MC-SNEDDS at pH 4.0 was found to digest to 65.8%. The extent of digestion for the MC-SNEDDS increased significantly from a lipolysis performed at pH 4.0 to a lipolysis at pH 6.5 and pH 8.0.

This can again be attributed to the pH of the digesting environment being far removed, in the case of pH 4.0, from the optimal pH of pancreatic lipase activity. The extent and kinetics of lipid digestion were somewhat different between the experiments at pH 8.0 and pH 6.5, and it was expected that this small difference may affect the rate and extent of precipitation of cinnarizine. The difference in precipitation behavior with regard to solid-state form, however, at pH 6.5 and pH 8.0, was expected to be a result of the pH environment and therefore the ionization state of cinnarizine. Notably, the extent of digestion at pH 4.0 was not comparable to the other two pH settings. The MCSNEDDS was digested to a much lesser degree, and therefore, the driving force for precipitation would be lower at pH 4.0 than at pH 6.5 and pH 8.0. This intuitively points toward the likelihood that cinnarizine remained largely solubilized within the undigested lipid phase during lipolysis at pH 4.0, as the MC-SNEDDS would not lose its solubilization capacity to the same extent when compared to lipolysis experiments at pH 6.5 and pH 8.0.

Phase Distribution of Cinnarizine During Lipolysis

Samples were taken from the lipolysis experiments at pH 8.0, pH 6.5, and pH 4.0, at 5 min intervals up until 30 min after initiating digestion, and then at 10 min intervals until the end of the experiments at 60 min. The distribution of cinnarizine between the aqueous colloidal phase and the precipitated pellet phase in the samples was analyzed by HPLC. The phase distribution of cinnarizine upon lipolysis of the MC-SNEDDS at the different pH levels is presented in Figure 3.


a)
pH 8.0
● = aqueous Δ = pellet ▼ = total
CIN recovered (wt % of initial mass in vessel)
Time (min)


b)
pH 6.5
● = aqueous Δ = pellet ▼ = total
CIN recovered (wt % of initial mass in vessel)
Time (min)

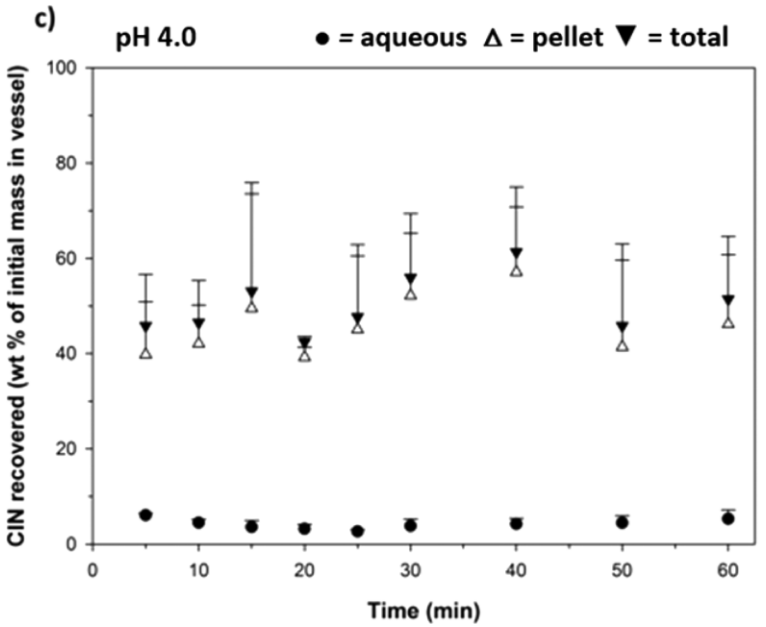

c)
pH 4.0
● = aqueous Δ = pellet ▼ = total
CIN recovered (wt % of initial mass in vessel)
Time (min)

Figure 3: Distribution of cinnarizine in the aqueous colloidal phase and pellet phase during lipolysis at (a) pH 8.0; (b) pH 6.5 and (c) pH 4.0. Where (●) = wt % cinnarizine in the aqueous colloidal phase; (Δ) = wt % cinnarizine in the pellet phase and (▼) = wt % cinnarizine recovered relative to the initial mass of drug recovered from the start of the experiment. Samples taken at the various time points throughout lipolysis were diluted with mobile phase and then analyzed using HPLC. Experiments were performed in triplicate (n = 3 different digestions, mean ± SD).

Around 1 g of MC-SNEDDS containing cinnarizine was carefully transferred to the digestive medium to start the lipolysis experiments using a consistent pipetting method. The viscous and sticky nature of the formulation, however, meant that a proportion of the formulation was lost on transfer, as it would inevitably stick to the sides of the glass vial in which it was prepared, and also be lost to some degree during pipetting. As a result, the actual amount of MC-SNEDDS added to the vessel was slightly less than 1 g, and therefore, the amount of cinnarizine recovered during the dispersion phase (t = 0) was treated as the initial mass for subsequent time point recovery values. For t = 0, at pH 8.0, the amount of cinnarizine recovered of the initial mass loaded in the 1 g of MC-SNEDDS was 69.0 ± 1.68%, at pH 6.5 the recovery was 75.9 ± 5.89%, and at pH 4.0 the recovery was 71.9 ± 4.04%.

The lipids in the MC-SNEDDS were digested rapidly by pancreatic lipase at pH 8.0 and pH 6.5, with the majority of lipid digestion occurring within the first 10 min as can be seen in Figure 2. As a result, the MC-SNEDDS rapidly lost its solubilization capacity, and around 30 wt % of cinnarizine was recovered at t = 5 min in the pellet phase for the experiment at pH 6.5 (Figure 3b). The amount of cinnarizine recovered from the pellet phase slowly increased as the digestion continued, with around 60 wt % of cinnarizine ending up in the pellet phase by the end of the lipolysis (t = 60 min). Although the drug was precipitating

throughout digestion of the MCSNEDDS, the concentration of cinnarizine recovered from the aqueous colloidal phase decreased over time as expected.

In contrast, while the degree and kinetics of lipid digestion were only slightly different for the lipolysis at pH 8.0, compared to the lipolysis at pH 6.5, the phase distribution of cinnarizine was markedly different (Figure 3a). The majority of drug precipitation at pH 8.0 occurred within the first 5 min of lipolysis, with around 75 wt % of cinnarizine being recovered from the pellet phase at t = 5 min, with the level of drug in the pellet phase remaining relatively constant throughout the rest of the lipolysis at pH 8.0. This much faster rate of precipitation at pH 8.0 in comparison to the lipolysis at pH 6.5 could be a result of the faster rate of lipolysis of the MC-SNEDDS (Figure 2), or the difference in pH of the digesting medium relative to the experimental pKa of cinnarizine. At pH 8.0, which is above the experimental pKa of cinnarizine, the majority of drug would have been in its unionized form and therefore was not solubilized to the same extent in the aqueous colloidal phase, thus driving precipitation of drug to occur rapidly. It was possible that at pH 8.0 during the dispersion phase, prior to adding pancreatic lipase to the dispersed SNEDDS, some of the cinnarizine may have precipitated, given the subsequently observed phase distribution. To test for this, the sample taken from the dispersion period (t = 0) was centrifuged, and no precipitated drug was observed visually in the form of a pellet within the Eppendorf tube, as a secondary test the dispersion was repeated with the MC-SNEDDS at pH 8.0, and the contents were filtered through a piece of 0.45 µm filter paper under vacuum; however, no solid material or precipitated drug was recovered from the surface of the filter paper.

The extent of lipolysis of the MC-SNEDDS was much less at pH 4.0 than at pH 6.5 and pH 8.0, as discussed above. As a result, the

amount of cinnarizine recovered in the aqueous colloidal phase and pellet phase was relatively low (Figure 3c). This suggests that the majority of cinnarizine was still solubilized by the undigested lipid during the lipolysis experiment at pH 4.0. The amount of cinnarizine recovered in the pellet phase was lowest at pH 4.0 and highest at pH 8.0. The relatively low amount of cinnarizine recovered in the pellet at pH 4.0, however, was likely due to the difference in the extent of lipid digestion, rather than the ability of the aqueous colloidal phase to solubilize the drug.

Solid-State Analysis of Pellet Phase

The pellet phase was isolated at the end point of lipolysis experiments and visualized under crossed polarized light. The image presented in Figure 4 indicates that cinnarizine precipitated in a crystalline form during the lipolysis performed at pH 8.0, with the presence of needle like drug particles exhibiting birefringence. The pellet phase from the lipolysis experiments performed at a pH below the literature pKa value of cinnarizine, however, did not present the appearance of needle shaped crystals (see Appendix).

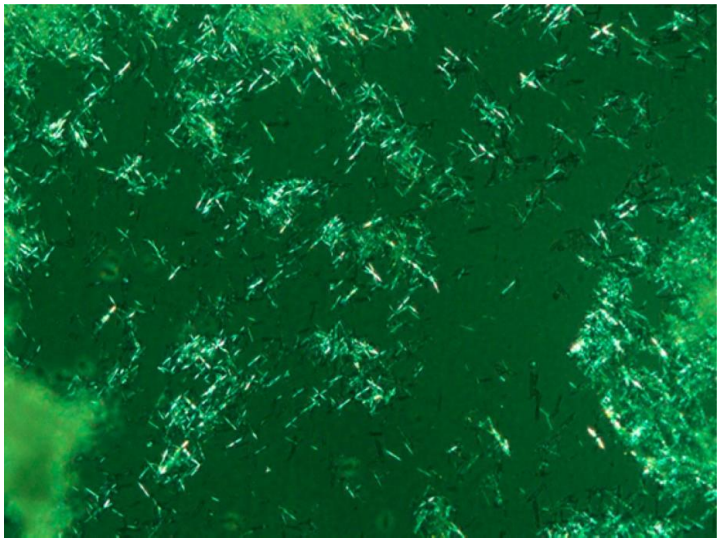


Figure 4: Crossed polarized light microscopy image of the pellet phase obtained from the lipolysis experiment at pH 8.0. Crystalline cinnarizine was observed as many needle-like structures.

The pellet phase was further analyzed by XRD to determine the solid state of precipitated cinnarizine after lipolysis of the MC-SNEDDS. In addition to the lipolysis experiments at pH 8.0, pH 6.5, and pH 4.0, lipolysis experiments were performed at pH 7.47 (literature pKa value of cinnarizine) and pH 5.5, and the pellet phase from digestions at these five different pH levels were evaluated. The XRD data is shown in Figure 5.

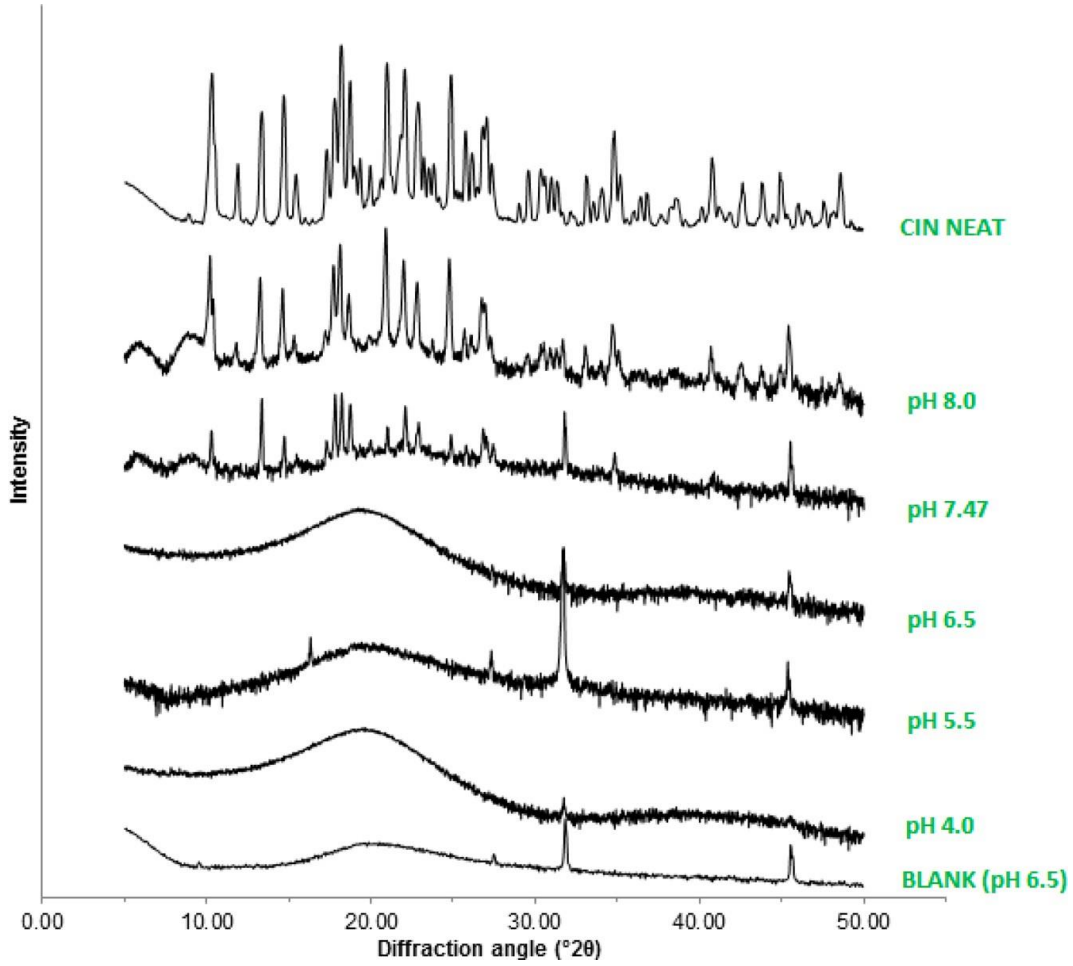


Figure 5: Diffraction patterns from XRD experiments performed on the pellet phase from the lipolysis of the MC-SNEDDS containing cinnarizine conducted at various pH levels, the pellet phase from the lipolysis of drug-free MC-SNEDDS at pH 6.5 (BLANK), and the neat crystalline cinnarizine free base (CIN neat).

It has been shown previously that the lipolysis of a LCSMEDDS with cinnarizine at the same drug loading (50 mg/g) produced a pellet phase containing noncrystalline cinnarizine (9). This was deduced after determining that cinnarizine was present in the pellet phase following lipolysis of the LC-SMEDDS, via HPLC, and that the X-ray diffractogram for the pellet phase showed an absence of peaks corresponding to crystalline cinnarizine. A halo region in the diffractogram, generally corresponding to amorphous material, was observed in place of the sharp peaks

that represent crystalline cinnarizine. The lipolysis of the MC-SNEDDS in the current study was expected to produce either crystalline, or noncrystalline, precipitated cinnarizine depending on the pH of the digesting medium relative to the reported pKa value of cinnarizine (7.47). The X-ray diffractograms of pellet phases collected from the lipolysis experiments performed at a pH below the pKa of cinnarizine showed an absence of peaks inherent to the reference crystalline cinnarizine free base. For these pellet samples, collected from lipolysis experiments at pH 6.5, pH 5.5, and pH 4.0, a halo region was observed in the diffractograms (Figure 5), which indicated the presence of non-crystalline material. In contrast, the pellet phase obtained from the lipolysis experiments at pH 7.47 and pH 8.0 showed a diffraction pattern that matched the reference crystalline cinnarizine starting material. The few diffraction peaks observed for the pellet phases from experiments at pH 6.5, pH 5.5, and pH 4.0 did not correspond to the cinnarizine reference material. A lipolysis was carried out at pH 6.5 on the MC-SNEDDS formulation free of cinnarizine, and it was observed that those particular diffraction peaks were likely due to the soaps and other precipitated material associated with the enzyme and not related to drug (Figure 5).

When lipolysis was performed on the MC-SNEDDS at pH 8.0, it was assumed that the majority of cinnarizine would be present during digestion in its neutral form. As such, cinnarizine was not available to ion-pair with oppositely charged fatty acid molecules upon digestion of the MC-SNEDDS causing cinnarizine to precipitate in a crystalline form.

At pH 7.47, which is the reported pKa value of cinnarizine, it was expected that roughly equal amounts of ionized cinnarizine and neutral cinnarizine would be present, and therefore, a fraction of the precipitated drug would be ion-paired to fatty acid molecules forming the amorphous-salt; however, the rest of the

drug would precipitate in a crystalline form. From the diffractogram, it appeared that the lipolysis at pH 7.47 produced predominantly crystalline cinnarizine, albeit with an apparently reduced intensity relative to the baseline. The assumption that 50% of the cinnarizine would be ionized and 50% un-ionized at pH 7.47 was in hindsight unreasonable, as the literature pKa value for a tertiary amine structure like cinnarizine carries a degree of uncertainty, and the actual pKa value of the drug in the digesting medium could be quite different, or could change as the drug comes into close proximity to surfaces (22). Further analysis of the diffractogram at pH 7.47, however, indicated that the ratio of maximum height of the amorphous halo region to maximum peak height of crystalline cinnarizine was 2-fold larger than that calculated for the diffractogram obtained for the lipolysis at pH 8.0. This suggested that at pH 7.47 cinnarizine did precipitate to some degree in the amorphous-salt form.

Overall, the XRD data generally supports the concept that when the weakly basic drug cinnarizine is present during digestion predominantly in its ionized form (below a pH of 7.47), and the drug does not precipitate in its crystalline form, which implies that ionization was responsible for the noncrystalline precipitation observed. To determine whether an interaction between cinnarizine and oppositely charged fatty acid molecules during lipolysis had taken place to produce the amorphous-salt upon precipitation, FTIR was used.

Examining the Interactions between Cinnarizine and Fatty Acids on Lipolysis Using FTIR

The pellet phases from the lipolysis experiments at pH 8.0 and pH 6.5 were analysed using FTIR, along with the cinnarizine free base starting material and cinnarizine hydrochloride. The spectra obtained from the FTIR analysis are presented in Figure 6.

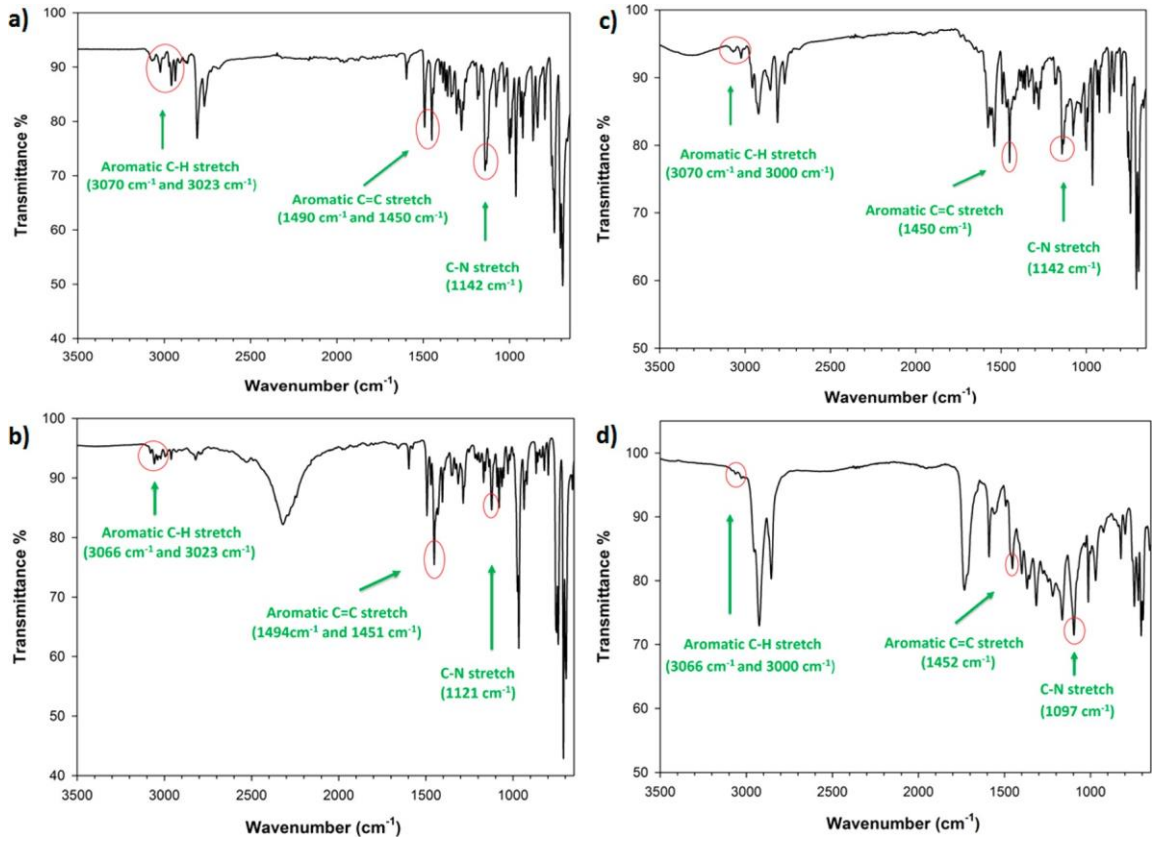


Figure 6: Infrared absorption spectra for (a) cinnarizine free base starting material; (b) cinnarizine hydrochloride; (c) pellet phase from the lipolysis experiment at pH 8.0; and (d) pellet phase from the lipolysis experiment at pH 6.5. A change in the C–N environment was observed for cinnarizine hydrochloride and the pellet phase obtained at pH 6.5, which is likely due to an ionic interaction of the tertiary amine in cinnarizine.

Cinnarizine has two tertiary amines in its structure, which makes identifying ionic interactions more difficult than for structures with primary or secondary amines. This is due to the absence of characteristic N–H stretch bands around 3400– 3250 cm^{-1} for tertiary amines otherwise observed for primary and secondary amines (30). To complicate matters, a characteristic band should exist for amine salts with the $N–H^{+}$ band usually appearing around 3000–2800 cm^{-1}. In this case, however, the absorption bands for aromatic cinnarizine C–H bonds and aliphatic C–H bonds ranging from 3040–2808 cm^{-1} overlap this region, and a clear deduction with regard to the amorphous salt could not be made.

Therefore, a change in the aliphatic C–N stretch environment between the reference cinnarizine free base and the precipitated amorphous-salt of cinnarizine was the only available evidence to suggest whether an ionic interaction around this bond had occurred during lipolysis experiments at pH 6.5. The FTIR spectrum for the reference cinnarizine free base (Figure 6a) showed some key absorption bands including the aromatic C–H stretch at 3070 and 3023 cm^{-1}, the aromatic C=C stretch at 1490 and 1450 cm^{-1}, and the C–N stretch, which was observed at 1142 cm^{-1}. Cinnarizine hydrochloride was also analyzed to identify differences in the absorption spectrum that a salt form with ionic interactions around the tertiary amine would produce.

The FTIR spectrum for cinnarizine hydrochloride showed the aromatic C–H and aromatic C=C bands to be in a similar position to cinnarizine free base, as can be seen in Figure 6b. The band for the C–N stretch, however, appeared at 1121 cm^{-1} instead of at 1142 cm^{-1}, where the same C–N band was observed for the free base form of cinnarizine. This difference can be attributed to the ionic interaction between the protonated tertiary amine of cinnarizine and deprotonated Cl^- ions, effectively changing the environment of the C–N bond to cause a noticeable shift in the wavenumber for absorption around this bond. The spectrum for the pellet phase obtained from the lipolysis experiment at pH 8.0 (Figure 6c) also showed aromatic C–H stretch absorption bands at 3070 and 3000 cm^{-1}, aromatic C=C stretch absorption at 1450 cm^{-1}, but importantly, the C–N stretch was observed at 1142 cm^{-1}, which was at the same wavenumber as the reference cinnarizine free base. This suggested that the lipolysis experiment at pH 8.0 caused cinnarizine to precipitate in its reference crystalline form and that the C–N environment remained unchanged under these conditions, due to cinnarizine remaining neutral and unable to form ionic interactions, complementing the XRD data above.

In contrast, the pellet phase from the lipolysis experiment at pH 6.5 produced an FTIR spectrum (Figure 6d) that showed the aromatic C–H stretch at 3066 and 3000 cm^{-1} and the aromatic C=C stretch at 1452 cm^{-1}, but the C–N stretch was observed at 1097 cm^{-1}, rather than at 1142 cm^{-1}. Both cinnarizine hydrochloride and the pellet phase from the lipolysis at pH 6.5 showed differences in the C–N stretch band, and it is reasonable to propose that this was a result of ionic interactions. While the C–N stretch appears at a different wavenumber for cinnarizine hydrochloride and the pellet at pH 6.5 relative to cinnarizine free base, they also differ from each other in this respect (1121 cm^{-1} for cinnarizine hydrochloride and 1097 cm^{-1}). The ionic interactions are between different species for cinnarizine hydrochloride and the pellet at pH 6.5, and these ionic interactions contribute to unique C–N environments, thus the interaction between the tertiary amine of cinnarizine and the deprotonated carboxylic acid group from MC fatty acids present during lipolysis appeared to produce an absorption band for the C–N stretch at 1097 cm^{-1}.

Discussion

Poorly water-soluble weak bases have been identified in the pellet phase of digested lipid-based formulations in a noncrystalline form previously. It was suggested that this trend is due to the noncrystalline precipitation of weakly basic drugs as a result of ionic interactions with oppositely charged species present during digestion (15). Using a model LC-SMEDDS formulation and cinnarizine as a model weakly basic drug, this proposed mechanism of ion-pairing upon precipitation of drug during lipolysis was investigated recently with solid-state NMR techniques (18). The results from the aforementioned study strongly suggest there were interactions between cinnarizine and the carboxylic acid group of the oleic acid present in the pellet, thus affecting the solid-state form of the precipitated cinnarizine.

Additional differential scanning calorimetry data also confirmed that cinnarizine was not present in the pellet phase of the digested formulation as a separate amorphous component, as the glass transition properties of the drug containing pellets differed from purely amorphous cinnarizine, further suggesting interactions between cinnarizine and pellet constituents. The LBF in the above case, however, contained oleic acid as a starting material, and it was unclear whether the oleic acid produced upon lipid digestion contributed to the subsequent effect observed with the solid state of the drug upon precipitation.

The current study looked to complement the findings of the study above by using an alternative spectroscopic technique to show ionic interactions between cinnarizine and fatty acid upon lipolysis of an LBF; additionally, the purpose was to determine whether MC fatty acids affect the solid state of cinnarizine in the same way as oleic acid and to examine whether fatty acid molecules produced exclusively from the digestion of lipids can have the same effect as a formulation that has fatty acid as a starting component. From the current study, cinnarizine precipitated in its starting crystalline form during a lipolysis performed on the MC-SNEDDS at pH 8.0 and pH 7.47, and in a noncrystalline form at pH 6.5, pH 5.5, and pH 4.0, as can be seen from the XRD data (Figure 5). These results consolidate the idea that the pH of the digesting medium in relation to the ionization state of cinnarizine plays a critical role in determining the solid-state form of the precipitated drug during the digestion of lipid formulations. It was interesting, however, that when the lipolysis experiment on the MC-SNEDDS was performed at pH 4.0, cinnarizine still precipitated in a noncrystalline form. At pH 4.0, cinnarizine would be ionized almost completely and available to form ionic interactions, but MC fatty acids (capric and caprylic acid) have a reported pKa value of around 4.89 (31). The amorphous-salt was not expected to form at pH 4.0, due to the expectation that MC fatty acids would be largely un-ionized

and unavailable for ion-pairing in this environment. The amount of fatty acid, however, relative to drug is far greater even with only 65% of the MC-SNEDDS being digested at pH 4.0. Moreover, the actual pH of the digesting medium during this experiment was closer to pH 4.5, as the addition of pancreatic lipase solution caused an initial increase in the experimental pH, as mentioned above. Therefore, a proportion of fatty acid may have been ionized, and this was sufficient for the drug to precipitate as an amorphous-salt.

The rapid onset of drug precipitation in the current study during lipolysis at pH 6.5 (Figure 3b) in comparison to previous phase distribution data for cinnarizine obtained from the lipolysis of a LC-SMEDDS at the same pH 9 can be attributed to the slower rate of lipolysis generally observed with LC lipids and the increased solubility of lipophilic drugs in LC digestion products (32–34). MC lipids, such as those used in the current study, are rapidly digested and form more hydrophilic structures that lack the solubilization capacity of structures formed upon digestion of LC lipids. The difference in lipid chain length, therefore, plays a critical role in determining the kinetics of drug precipitation upon digestion of LBFs. In addition, the nature of the aqueous colloidal phase would vary depending on the pH of the digesting medium (35). The lipolysis experiments performed at pH 4.0 would produce a greater proportion of unionized MC fatty acid molecules and favour the formation of vesicular or emulsified phases, whereas the increased electrostatic repulsion between fatty acid head groups at pH 6.5 and pH 8.0 would produce micelles (36). These types of colloids formed at low pH have been shown previously to have a greater solubilization capacity for highly lipophilic drugs, such as fenofibrate and cinnarizine (36) Fatty acid protonation at lower pH conditions, such as pH 4.0, has also been reported to produce more oil-rich digestion phases. Therefore, the task of separating and isolating digested phases after a lipolysis at pH 4.0

becomes difficult and could lead to an overestimation of the amount of drug that had precipitated, as the oil-rich phases may contaminate the pellet phase during analysis. This is possibly what has been observed from the HPLC data above at pH 4.0 (Figure 3c), where the variability in the phase distribution data was considerably greater than for the analyses carried out from lipolysis experiments at pH 8.0 and pH 6.5.

Other weakly basic drugs, such as halofantrine (37) and carvedilol (15), have been shown previously to produce amorphous precipitates during similar *in vitro* digestion experiments. Both of these drugs were likely to be predominantly ionized in the studies conducted, as the pH of the digesting medium was lower than their respective pKa values, and therefore, the basic compounds likely interacted with fatty acid molecules during digestion to produce amorphous-salts. Loratadine, on the other hand, precipitated in a crystalline form upon digestion of model LBFs (15), which actually supports the logic that interactions between weakly basic drugs and fatty acid drives amorphous precipitation, because loratadine has a pKa of 5.0 (17) and would have been largely present in its neutral form during digestion and unable to form this interaction. Furthermore, poorly water-soluble neutral compounds that are routinely tested in LBFs, fenofibrate and danazol, have been repeatedly shown to precipitate in a crystalline form during *in vitro* digestion experiments (10–14) The one exception to this trend appears to be the neutral compound simvastatin, which precipitated as an amorphous form during the *in vitro* digestion of a supersaturated-SNEDDS formulation (38). While the current study has consolidated the importance of ionic interactions between weakly basic compounds and fatty acids during digestion as a driver for amorphous precipitation, it is clearly not the only factor governing the formation of amorphous precipitates on digestion, as the extent and kinetics of supersaturation can play a major role as well (34,39). The supersaturation behaviour of simvastatin during

digestion may have therefore been the reason why an amorphous precipitate formed in that particular study.

Ultimately, the formation of amorphous-salts during the digestion of LBFs containing weakly basic drugs may favor their redissolution *in vivo*. This has perhaps already been shown indirectly when cinnarizine was delivered *in vivo* to beagle dogs in a LBF that showed amorphous precipitation *in vitro*, yet performed just as well as formulations that did not show any precipitation during *in vitro* experiments (40). It is important to note, however, that the presence of an absorptive sink *in vivo* complicates the task of finding genuine correlations with *in vitro* experiments (12), as the latter is a closed system with non-sink conditions. It remains unclear whether drug precipitation occurs *in vivo* for highly lipophilic compounds, as for highly permeable drugs the rapid absorptive flux across the small intestine would alleviate the highly supersaturated state otherwise formed *in vitro*. In this way, the extent of drug precipitation from the conventional *in vitro* lipolysis model may be overestimated, and ultimately misleading, which has led to efforts to develop more sophisticated lipolysis models that take into account the absorption of drug *in vivo* (41). Additionally, the role of calcium *in vitro* is to remove liberated fatty acid molecules from the surface of the oil droplet, by complexing and precipitating the fatty acids as soaps, so that pancreatic lipase can continue to digest the formulation unhindered. This effectively reduces the solubilization capacity of the colloidal structures formed upon lipid digestion, due to the removal of fatty acid, thus also increasing the risk of drug precipitation (42). However, the proportions of fatty acid far exceed those of drug, such that sufficient fatty acid is expected to be available also to interact with the drug even in the presence of calcium.

In some ways, the formation of amorphous-salts upon the precipitation of weakly basic drugs during the digestion of LBFs is analogous to the recent efforts to deliver similar compounds as ionic liquids in lipid vehicles. Transforming weakly basic drugs into ionic liquids has been effective in increasing the amount of drug that can be dissolved into an LBF, and ionic liquids are also effective at minimizing precipitation, due to favourable dissolution properties. Cinnarizine was recently transformed into an ionic liquid and was dissolved to a 7-fold higher degree in an LBF compared to the crystalline free base with insignificant precipitation of the drug observed during *in vitro* lipolysis (43). Therefore, weakly basic compounds that are ionically paired to another species have improved solubility and dissolution properties during digestion, and generating amorphous-salts of weak bases during the digestion of lipid formulations may be a simple and effective way of drawing on this knowledge.

Conclusion

Cinnarizine was shown to precipitate as an amorphous-salt form during the *in vitro* lipolysis of a MC-SNEDDS formulation. It was established that the tertiary amine within cinnarizine underwent a change when lipolysis was performed at pH 6.5 (below the pKa of cinnarizine (7.47)), where the drug would be predominantly ionized and available for interactions with oppositely charged species. Specifically, ionized MC fatty acid molecules liberated upon digestion of the formulation are proposed to interact with cinnarizine, changing the C–N environment within cinnarizine, as was observed with the IR data above (Figure 6). It has previously been shown that oleic acid present in the starting formulation and oleic acid formed upon lipolysis of LC lipids interact with cinnarizine to form a noncrystalline precipitate, and the current work confirms that MC fatty acid molecules produced exclusively from the digestion of a MC-SNEDDS formulation also have the same effect of altering the solid-state form of

cinnarizine upon precipitation. Therefore, through the careful selection of excipients, LBFs have the potential to drive weakly basic drugs to precipitate as high energy amorphous-salt forms during digestion, which could allow for the redissolution of these amorphous-salt forms in an absorptive environment *in vivo*. Optimising the formulation of lipid systems in this way is expected to enhance the overall fraction of drug that is absorbed from a single dose.

References

1. Porter, C. J.; Trevaskis, N. L.; Charman, W. N. *Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs*. Nat. Rev. Drug Discovery 2007, **6** (3), 231–48.

2. Phan, S.; et al. *Self-assembled structures formed during lipid digestion: characterization and implications for oral lipid-based drug delivery systems*. Drug Delivery Transl. Res. 2014, **4** (3), 275–294.

3. Phan, S.; et al. *Structural aspects of digestion of medium chain triglycerides studied in real time using sSAXS and Cryo-TEM*. Pharm. Res. 2013, **30** (12), 3088–100.

4. Williams, H. D.; et al. *Lipid-based formulations and drug supersaturation: harnessing the unique benefits of the lipid digestion/absorption pathway*. Pharm. Res. 2013, **30** (12), 2976–92.

5. Anby, M. U.; et al. *Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilization on the in vitro and in vivo performance of self-emulsifying drug delivery systems*. Mol. Pharmaceutics 2012, **9** (7), 2063–79.

6. Warren, D. B.; et al. *Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility*. J. Drug Target 2010, **18** (10), 704–31.

7. Brouwers, J.; Brewster, M. E.; Augustijns, P. *Supersaturating drug delivery systems: the answer to solubility-limited oral bioavailability?* J. Pharm. Sci. 2009, **98** (8), 2549–72.

8. Augustijns, P.; Brewster, M. E. *Supersaturating drug delivery systems: fast is not necessarily good enough*. J. Pharm. Sci. 2012, **101** (1), 7–9.

9. Sassene, P. J.; et al. *Precipitation of a poorly soluble model drug during in vitro lipolysis: characterization and dissolution of the precipitate*. J. Pharm. Sci. 2010, **99** (12), 4982–91.

10. Khan, J.; Hawley, A.; Rades, T.; Boyd, B. J. *In Situ Lipolysis and Synchrotron Small-Angle X-ray Scattering for the Direct Determination of the Precipitation and Solid-State Form of a Poorly Water- Soluble Drug During Digestion of a Lipid-Based Formulation*. J. Pharm. Sci 2016, **105**, 2631–2639.

11. Stillhart, C.; Imanidis, G.; Kuentz, M. *Insights into drug precipitation kinetics during in vitro digestion of a lipid-based drug delivery system using in-line raman spectroscopy and mathematical modeling*. Pharm. Res. 2013, **30** (12), 3114–30.

12. Thomas, N.; Richter, K.; Pedersen, T. B.; Holm, R.; Mullertz, A.; Rades. *In vitro Lipolysis Data Does Not Adequately Predict the In vivo Performance of Lipid-Based Drug Delivery Systems Containing Fenofibrate*. AAPS J. 2014, **16**, 539.

13. Williams, H. D.; et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 3: understanding supersaturation versus precipitation potential during the in vitro digestion of type I, II, IIIA, IIIB and IV lipid-based formulations*. Pharm. Res. 2013, **30** (12), 3059–76.

14. Larsen, A. T.; Sassene, P.; Mullertz, A. *In vitro lipolysis models as a tool for the characterization of oral lipid and*

surfactant based drug delivery systems. Int. J. Pharm. 2011, **417** (1–2), 245–55.

15. Stillhart, C.; Durr, D.; Kuentz, M. *Toward an Improved Understanding of the Precipitation Behavior of Weakly Basic Drugs from Oral Lipid-Based Formulations*. J. Pharm. Sci. 2014, **103**, 1194.

16. Shewale, B. D.; et al. *Effect of hydroxylpropyl-β-cyclodextrin on Solubility of Carvedilol*. Indian Journal of Pharmaceutical Sciences 2008, **70** (2), 255–257.

17. Ramulu, G.; et al. *A New Validated Liquid Chromatographic Method for the Determination of Loratadine and its Impurities. Sci*. Pharm. 2011, **79** (2), 277–291.

18. Sassene, P. J.; et al. *Elucidating the Molecular Interactions Occurring during Drug Precipitation of Weak Bases from Lipid-Based Formulations: A Case Study with Cinnarizine and a Long Chain Self-Nanoemulsifying Drug Delivery System*. Mol. Pharmaceutics 2015, **12** (11), 4067–4076.

19. Thomas, N.; et al. *Influence of Lipid Composition and Drug Load on the In vitro Performance of Self-Nanoemulsifying Drug Delivery Systems*. J. Pharm. Sci. 2012, **101** (5), 1721–1731.

20. Williams, H. D.; et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 1: method parameterization and comparison of in vitro digestion profiles across a range of representative formulations*. J. Pharm. Sci. 2012, **101** (9), 3360–80.

21. Peeters, J. *Determination of Ionization-Constants in Mixed Aqueous Solvents of Varying Composition by a Single Titration*. J. Pharm. Sci. 1978, **67** (1), 127–129.

22. Prankerd, R. J. *Critical Compilation of pKa Values for Pharmaceutical Substances*. Profiles Drug Subst., Excipients, Relat. Methodol. 2007, **33**, 1–726.

23. Fernandez, S.; et al. *Lipolysis of the semi-solid self-emulsifying excipient Gelucire 44/14 by digestive lipases*. Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 2008, **1781** (8), 367–75.

24. Lee, K. W. Y.; Porter, C. J. H.; Boyd, B. J. *The effect of administered dose of lipid-based formulations on the In vitro and In vivo performance of cinnarizine as a model poorly water-soluble drug*. J. Pharm. Sci. 2013, **102** (2), 565–578.

25. Carlton, R. A. *Chapter 2: Polarized Light Microscopy. Pharmaceutical Microscopy*; Springer: New York,2011; p 321, DOI: 10.1007/978-1-4419-8831-7

26. Harris, K. D. *Powder diffraction crystallography of molecular solids*. Top. Curr. Chem. 2011, **315**, 133–77.

27. Gargouri, Y.; Moreau, H.; Verger, R. *Gastric lipases: biochemical and physiological studies*. Biochim. Biophys. Acta, Lipids Lipid Metab. 1989, **1006** (3), 255–71.

28. Dressman, J. B.; Berardi, R. R.; Dermentzoglou, L. C.; Russell, T. L.; Schmaltz, S. P.; Barnett, J. L.; Jarvenpaa, K. M. *Upper gastrointestinal (GI) pH in young, healthy men and women*. Pharm. Res. 1990, **7** (7), 756–61.

29. Pouton, C. W. *Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system*. Eur. J. Pharm. Sci. 2006, **29** (3-4), 278-87.

30. Coates, J. *Interpretation of Infrared Spectra, A Practical Approach*. Encyclopedia of Analytical Chemistry; John Wiley & Sons, Ltd.: Hoboken, NJ, 2006; DOI: 10.1002/9780470027318.a5606.

31. Naupert, C.; Rommel, K. *Absorption of short and medium chain fatty acids in the jejunum of the rat*. Clin. Chem. Lab. Med. 1975, **13** (12), 553-562.

32. Pouton, C. W. *Self-emulsifying drug delivery systems: assessment of the efficiency of emulsification*. Int. J. Pharm. 1985, **27** (2), 335-348.

33. Sek, L.; Porter, C. J.; Charman, W. N. *Characterisation and quantification of medium chain and long chain triglycerides and their in vitro digestion products, by HPTLC coupled with in situ densitometric analysis*. J. Pharm. Biomed. Anal. 2001, **25** (3-4), 651-61.

34. Feeney, O. M.; et al. *50 years of oral lipid-based formulations: Provenance, progress and future perspectives*. Adv. Drug Delivery Rev. 2016, **101**, 167-94.

35. Salentinig, S.; et al. *pH-responsive micelles based on caprylic acid*. Langmuir 2014, **30** (25), 7296-303.

36. Williams, H. D.; et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 4: proposing a new lipid formulation performance classification system*. J. Pharm. Sci. 2014, **103** (8), 2441-55.

37. Thomas, N.; et al. *In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS)*. J. Controlled Release 2012, **160** (1), 25–32.

38. Thomas, N.; et al. *Supersaturated self-nanoemulsifying drug delivery systems (Super-SNEDDS) enhance the bioavailability of the poorly water-soluble drug simvastatin in dogs*. AAPS J. 2013, **15** (1), 219–27.

39. Hsieh, Y. L.; Box, K.; Taylor, L. S. *Assessing the Impact of Polymers on the pH-Induced Precipitation Behavior of Poorly Water Soluble Compounds using Synchrotron Wide Angle X-Ray Scattering*. J. Pharm. Sci. 2014, **103** (9), 2724–35.

40. Larsen, A. T.; et al. *Oral bioavailability of cinnarizine in dogs: relation to SNEDDS droplet size, drug solubility and in vitro precipitation*. Eur. J. Pharm. Sci. 2013, **48** (1–2), 339–50.

41. Crum, M. F.; et al. *A new in vitro lipid digestion – in vivo absorption model to evaluate the mechanisms of drug absorption from lipid-based formulations*. Pharm. Res. 2016, **33** (4), 970–82.

42. Devraj, R.; et al. *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*. Int. J. Pharm. 2013, **441** (1–2), 323–33.

43. Sahbaz, Y.; et al. *Transformation of poorly water-soluble drugs into lipophilic ionic liquids enhances oral drug exposure from lipid based formulations*. Mol. Pharmaceutics 2015, **12** (6), 1980–91.

Appendix

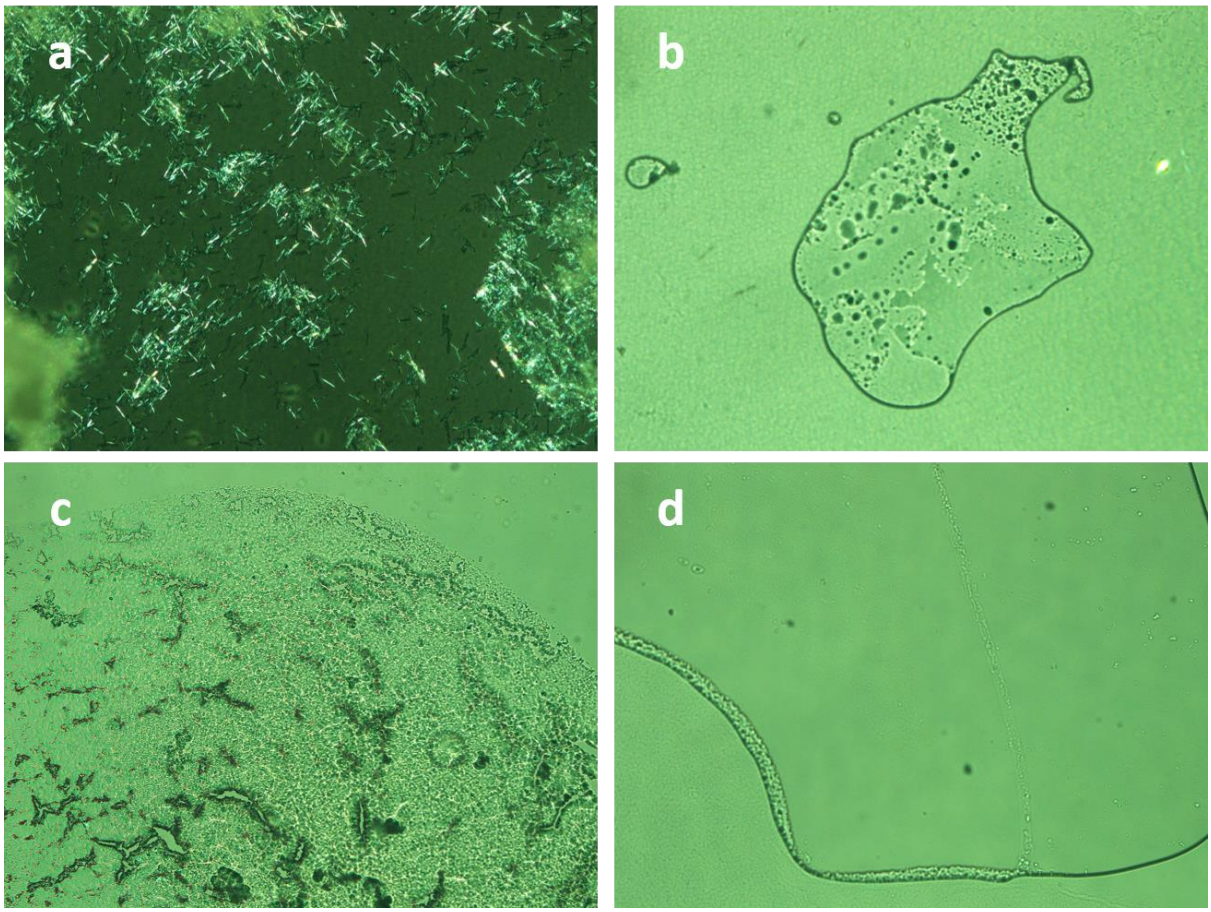


Figure 7: Pellet obtained from the *in vitro* lipolysis experiment at pH 8.0 shows precipitated crystalline cinnarizine as needle-like structures that exhibit birefringence under crossed-polarised light (a), whereas the pellets obtained from the *in vitro* lipolysis experiments at pH 6.5 (b), pH 5.5 (c) and pH 4.0 (d) do not show crystalline cinnarizine under crossed-polarised light, and instead show non-birefringent structures without crossed-polarisers on the microscope, which could be the amorphous-salt precipitates containing cinnarizine.

Chapter 5:
The Precipitation Behaviour of Weakly-Acidic Drugs During Lipid Digestion

Declaration

This chapter consists of a manuscript in preparation that examines the precipitation behaviour of the model weakly-acidic drug, tolfenamic acid, with regard to the solid-state form of the precipitate. The cationic surfactant, DDAB, was included in the starting lipid formulation with the intention of affecting the solid-state form of the precipitated drug through ionic interactions during *in vitro* digestion. The manuscript will be submitted as: Khan J, Rades T, Boyd B. *Proof of Concept for the Use of Cationic Surfactants to Alter the Solubility Profile of Poorly Water Soluble Acidic Drugs and the Solid-State Form of the Precipitate upon In Vitro Lipolysis*.

Declaration by candidate:

For chapter 5, the nature and extent of my contribution to the work was as follows:

Nature of contribution	**Extent of contribution**
Perform experiments, preparation of manuscript	90%

The following co-authors contributed to the work:

Name	**Nature of contribution**
Thomas Rades	Co-supervisor, preparation of manuscript
Ben Boyd	Main supervisor, preparation of manuscript

The undersigned hereby declare that the above declaration correctly reflects the nature and extent of candidate and co-author contributions:

Candidate's signature:  Date: 20-04-2017

Main supervisor's signature:  Date: 20-04-2017

Proof of Concept for the Use of Cationic Surfactants to Alter the Solubility Profile of Poorly Water Soluble Acidic Drugs and the Solid-State Form of the Precipitate upon *In Vitro* Lipolysis

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Manuscript in preparation.

Abstract

It has been previously shown that the interaction of some weakly-basic drugs with oppositely-charged fatty acids during digestion can influence the solid-state form of the drug if it precipitates. The current study hypothesised the opposite effect for weakly-acidic drugs. Tolfenamic acid and an oppositely charged cationic surfactant, didodecyldimethylammonium bromide (DDAB), were combined in a model medium chain lipid formulation. The phase distribution upon *in vitro* lipolysis was determined using high performance liquid chromatography and the solid-state form of precipitated tolfenamic acid determined using X-ray diffraction and crossed polarized light microscopy. Tolfenamic acid precipitated in a different polymorphic crystalline form to the starting reference material in the absence of DDAB, but precipitated in an amorphous form when DDAB was included in the same formulation. The solubility of tolfenamic acid upon dispersion and digestion of the formulation was considerably higher in the presence of DDAB. The findings point to ionic interactions between tolfenamic acid and DDAB as the cause of the improved drug

solubility throughout digestion, and precipitation of drug in an amorphous-salt form, analogous to what has been observed in the past for some poorly-water soluble weakly basic drugs.

Introduction

Lipid based formulations (LBFs) can enable the oral delivery of poorly water soluble lipophilic drugs by circumventing the need for drug dissolution in the gastrointestinal tract (1). The digestion of lipids by gastric lipase and pancreatic lipase produces free fatty acids, which interact with endogenous amphiphilic molecules to form different self-assembled colloidal structures (2). Lipophilic drugs are transported to sites of absorption in the small intestine via these colloidal structures in a dissolved state.

An issue with LBFs, however, is the reduction in solubilisation capacity of the formulation upon dilution in gastrointestinal fluid (3). The solubilisation capacity can be further reduced upon lipid digestion, as the digestion products formed become increasingly hydrophilic. A decrease in the ability of the LBF to maintain drug in a dissolved state during digestion often leads to supersaturation of drug within the formulation and digestion products, and ultimately to the precipitation of drug (4), which can decrease the total amount of absorbed drug and thereby limit bioavailability. Polymeric precipitation inhibitors have been used previously to attempt to delay the onset of drug precipitation (5, 6). Polymers have been effective for this purpose, and in turn afford a high absorptive flux of drug across the membrane from a supersaturated state (7-9). The inhibitory properties of polymers are clearly useful, however, drug precipitation is not always confined to producing poorly water-soluble crystalline forms.

Recent studies have shown variation in the solid-state form of precipitated drug during the *in vitro* lipolysis of LBFs containing certain poorly water-soluble drugs (10-13). Specifically, it has been established that at appropriate pH, lipophilic weakly basic drugs, such as cinnarizine, interact with oppositely charged fatty acid molecules during *in vitro* digestion to form non-crystalline precipitates (14, 15). These amorphous-salt forms have a significantly higher dissolution rate in gastrointestinal media compared to low-energy crystalline forms (10-12). Using LBFs to form ionic interactions between weakly basic drugs and fatty acid provides a level of control over drug precipitation, and we hypothesize that the analogous approach would apply for weakly acidic drugs with LBFs containing cationic additives.

Weakly acidic drugs generally dissolve adequately in the gastrointestinal tract and are subsequently absorbed to a great extent, due to favourable pH conditions in the small intestine, however, there are some acidic drugs currently in the development pipeline where bioavailability is limited by solubility. The lipophilic weakly-acidic drug tolfenamic acid has been studied previously in LBFs with regard to the solid-state form of the precipitate (chemical structure shown in Figure 1) (16). It was shown that during the *in vitro* digestion of different types of LBFs, tolfenamic acid precipitated either in its thermodynamically stable crystalline form, or as its ‘yellow’ polymorphic crystalline form. The high energy polymorph is expected to have different dissolution behaviour in the gastrointestinal tract compared to the thermodynamically stable crystalline form of tolfenamic acid. An approach to drive precipitation of the drug to an amorphous form may further improve dissolution characteristics.

The combination of a weakly acidic drug and oppositely charged surfactant with regard to the effect on the solid state form of

precipitated drug upon *in vitro* digestion of LBFs has not been studied to our knowledge. The primary aim of the current study was to investigate whether the apparent ionic interactions that occur between fatty acid and weakly basic drugs to affect precipitation behaviour carries over to weakly acidic drugs and oppositely charged surfactants, and whether this interaction between the two species allows for a level of control over the precipitation of weakly acidic drugs. The cationic surfactant didodecyldimethylammonium bromide (DDAB) (chemical structure shown in Figure 1) was chosen as the oppositely charged species to include in the starting LBF to influence the precipitation of tolfenamic acid. This combination acts as a proof-of-concept to assess the ability of cationic components to influence the solid state characteristics of weakly acidic drugs upon lipid digestion, as outlined in Figure 1.

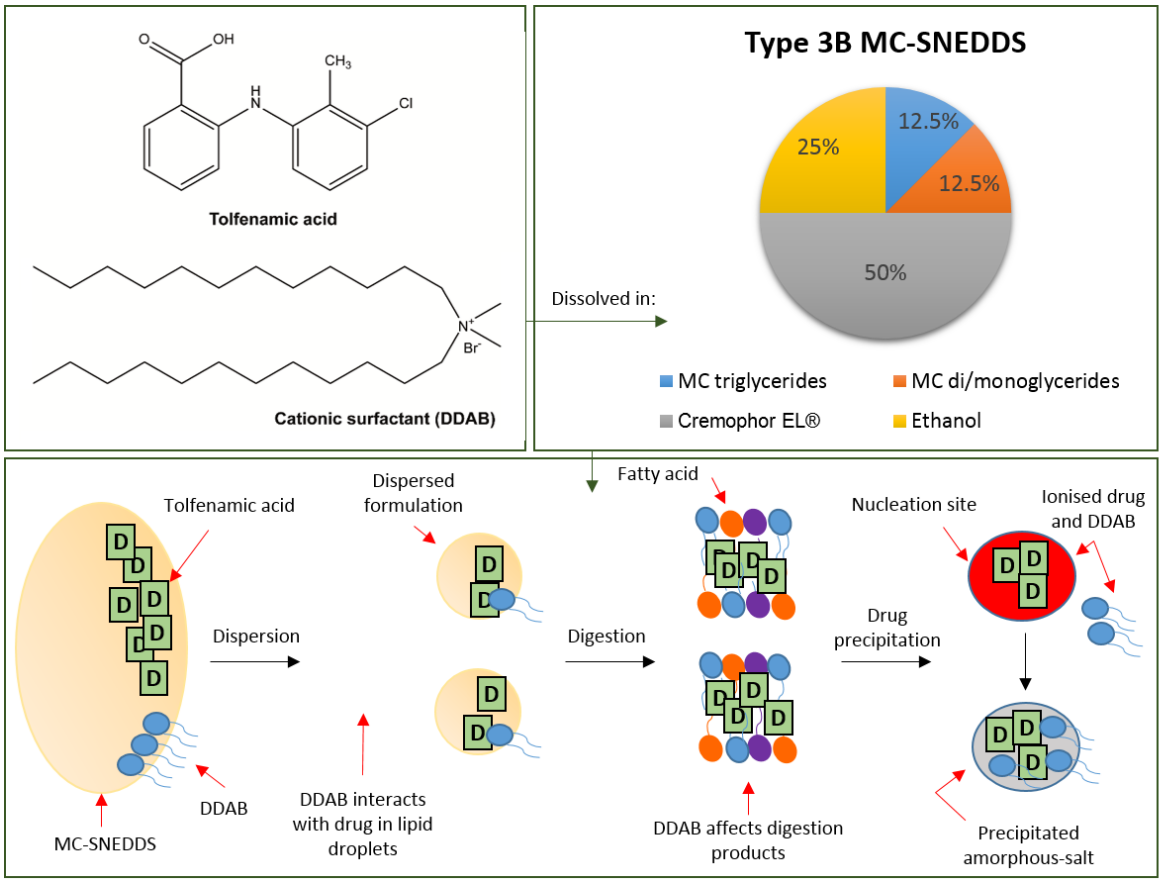

Figure 1: The chemical structures of tolfenamic acid and DDAB. The composition of the model Type 3B MC-SNEDDS used in this study and a prospective

overview of the fate of drug, lipid and surfactant during *in vitro* dispersion and digestion.

Materials

Captex 355® [medium chain triglyceride mixture composed of 59% caprylic acid (C_8), 40% capric acid (C_{10}), <1% lauric acid (C_{12}) as stated in the product information], and Capmul MCM® [mono/di-glycerides composed of caprylic acid (C_8) in glycerol], were obtained from Abitec Corporation (Janesville, Wisconsin). Cremophor EL® was purchased from BASF Corporation (Washington, New Jersey). Didodecyldimethylammonium bromide (DDAB), Tris maleate (reagent grade), bile salt (sodium taurodeoxycholate (NaTDC), >95%), and 4-bromophenylboronic acid (4-BPBA, >95%) were purchased from Sigma-Aldrich (St. Louis, Missouri). Tolfenamic acid was purchased from AK Scientific (Union City, CA). Calcium chloride dihydrate (>99%) was obtained from Ajax Finechem (Seven Hills, New South Wales, Australia). Sodium chloride (>99%) was purchased from Chem Supply (Gillman, South Australia, Australia). HPLC grade acetonitrile was purchased from Merck (MA, USA). Phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) was obtained from Trapeze Associates Pty. Ltd. (Clayton, Victoria, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia).

Methods

Preparation of LBF

A Type-3B MC self nano-emulsifying drug delivery system (SNEDDS) was chosen for this particular study (17). It has been shown previously that tolfenamic acid precipitates upon dispersion of this relatively hydrophilic LBF at drug loadings of 80 – 100%

saturation, whereas upon dispersion and digestion of more lipophilic Type 1 - 3A formulations tolfenamic acid remains mostly in solution (16). The Type 3B MC-SNEDDS consisted of 0.125 g Captex 355, 0.125 g Capmul MCM, 0.5 g Cremophor EL and 0.25 g ethanol based on previous studies, and was prepared in 1 g batches (18). The above excipients were weighed into a glass scintillation vial and vortexed for 1 min to mix the different components. Tolfenamic acid was weighed (54 mg) into a separate vial, before the 1 g of formulation prepared above was added to give a final drug loading of 51 mg/g in the Type 3B MC-SNEDDS that was used. The MC-SNEDDS was then added to the vial containing tolfenamic acid and allowed to mix in an oven set to 37°C overnight before use. In instances where solid drug was visually observed after mixing overnight in the 37°C oven the formulation was placed in a 60°C oven until all solid particles were dissolved. The second formulation contained the cationic surfactant DDAB at a 1:1 drug/surfactant mol ratio. For these DDAB containing formulations, DDAB (95 mg) was weighed and added to the vial with the tolfenamic acid, before the MC-SNEDDS was finally added and mixed in a 37°C oven as above.

Preparation of Digestion Medium and Pancreatic Lipase

Digestion buffer was prepared and used to make up both the digestion medium and pancreatic lipase solution. The digestion buffer was comprised of 2 mM Tris-maleate, 1.4 mM $CaCl_2 \cdot 2H_20$, 150 mM NaCl and adjusted to pH 6.5 using NaOH and HCl solutions. Fasted state simulated digestion medium was then prepared by adding 5 mM NaTDC and 1.25 mM DOPC to the digestion buffer. To make the digestion medium, the required amount of DOPC was weighed into a round-bottom flask and dissolved in an appropriate amount of chloroform, before the chloroform was removed under vacuum to leave an evenly coated lipid film at the bottom of the flask. The required amount of NaTDC was then added to the flask and dissolved in digestion buffer under sonication. The

digestion medium was kept refrigerated at 4 °C before use. To make the pancreatic lipase solution the raw pancreatin extract was weighed (2 g) into a small glass beaker and suspended in 5 mL of digestion buffer. This mixture was stirred for 5 min, transferred to a 12 mL plastic centrifuge tube and centrifuged at 2205 g at 4 °C for 15 min. The resultant supernatant was used as the pancreatic lipase solution (1000 TB units/mL of digest), which was collected and stored at 4 °C before use. A fresh batch of lipase solution was prepared as required, and each lipolysis experiment was initiated by adding 4 mL of this pancreatic lipase solution.

In vitro Lipolysis

The lipolysis experiments followed previously established protocols (19). Briefly, a Metrohm titrator with 5 mL dosing unit, autoburette and an iUnitrode pH probe (Metrohm AG, Herisau, Switzerland) was attached to a thermostatted glass vessel set to 37 °C. The MC-SNEDDS formulation containing tolfenamic acid was added to 36 mL of digestion medium in the glass vessel and allowed to disperse for 5 min. Adjustments to pH were made as necessary using HCl and NaOH solutions during this 5 min dispersion period to reach the experimental pH value of 6.5. Tolfenamic acid has a pKa of 3.7 (20) and therefore is mostly ionised at the experimental pH of 6.5, thus the drug could potentially participate in ionic interactions with oppositely charged DDAB molecules. Immediately following the 5 min dispersion period, lipolysis was initiated by adding 4 mL of the previously prepared pancreatic lipase solution (to achieve an overall activity of 1000 TB units/mL of digest).

Upon addition of the lipase the MC-SNEDDS was digested and the enzymatic hydrolysis of lipids produced free fatty acids. This led to a decrease in the pH of the digestion medium, and 0.6 M NaOH was used as the titrant solution to restore the pH to 6.5. Upon digestion at pH 6.5, the liberated fatty acid molecules

were partly ionised and partly unionised, only ionised fatty acid molecules were titrated.

To account for the unionised fatty acids at the end of lipolysis the pH of the digestion medium was rapidly shifted to pH 9.0, which ionised all fatty acids (21). The amount of titrant required to shift to pH 9.0 at the end of the 60 min digestion period indicated the amount of unionised fatty acid present during lipolysis. This shift to pH 9.0 was performed only for experiments concerned with calculating the extent of lipolysis of the formulations, and was not performed for experiments where the solid-state of precipitated drug was analysed. The extent of digestion was calculated according to Equation 1.

Equation 1:

$$Extent\ of\ LBF\ digested\ (\%) = \frac{Ionised\ fatty\ acid\ (mol) + unionised\ fatty\ acid\ (mol)}{Theoretical\ fatty\ acid\ in\ LBF\ (mol)} \times 100$$

Quantification of Drug and Sample Treatment for HPLC

The phase distribution of tolfenamic acid was assessed upon the *in vitro* digestion of the Type 3B MC-SNEDDS formulation in the presence and absence of DDAB. The HPLC system included a Shimadzu CBM-20A system controller, LC-20AD solvent delivery module, SIL-20A auto sampler and a CTO-20A column oven set at 40°C, coupled to an SPD-20A UV-detector (Shimadzu Corporation, Kyoto, Japan). A reverse-phase C_{18} column was used (4.6 × 75 mm, 3.5 µm; Waters Symmetry®, MA, USA).

During the *in vitro* digestion experiments, aliquots (200 µL) of the digesting formulation were taken at specific time points (t = 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min). These aliquots were transferred to 1.75 mL Eppendorf tubes prepared with 20 µL of lipase inhibitor (0.05 M 4-BPBA), which were then centrifuged at 7708 x g for 1 hr. The resultant aqueous colloidal phase (supernatant) was separated from the precipitated drug (pellet phase) for analysis. Mobile phase was used to dilute the aqueous

colloidal phase until the concentration of drug fell within the concentration range of the standard curve. For analysis of the pellet phase the samples were first dissolved in 200 µL of acetonitrile, before diluting with mobile phase appropriately as described above. Lipolysis experiments and HPLC quantification of drug within the digested phases were performed in triplicate for the MC-SNEDDS with and without DDAB.

The mobile phase was prepared with acetonitrile and water at a v/v ratio of 80:20, with 0.1% formic acid added to the aqueous phase, using isocratic elution. The flow rate was 1 mL/min and the injection volume was 50 µL. The concentration of drug in the digested samples was determined by comparison to a standard curve.

Determination of Morphology and Birefringence of Precipitated Drug using Polarised Light Microscopy

Crossed polarised light was used to visualise the precipitated drug from the lipolysis experiments. Tolfenamic acid in its crystalline forms is birefringent under crossed polarised light, whereas amorphous materials do not exhibit birefringence, due to the lack of long range order in their molecular arrangement (22). Therefore, CPLM was used to indicate the crystallinity and morphology of precipitated drug. A Nikon ECLIPSE Ni-U upright microscope fitted with crossed polarising filters and a DS-U3 digital camera control unit (Nikon, Tokyo, Japan) was used. Following the lipolysis experiments the pellet phases were collected and placed onto glass microscope slides to air dry before analysis.

Determination of the Solid State Form of Precipitated Tolfenamic Acid using X-ray Diffraction

The solid state form of the precipitated drug was determined using XRD. Pellet phases from the lipolysis experiments were

isolated and a Bruker D8 Advance X-ray diffractometer with Ni-filtered Cu Kα radiation (1.54 Å) at 40 kV and 40 mA was used to collect XRD data. The samples were analysed in the range of 5–50° in 2θ, with a step size of 0.02° and a scan rate of 0.5 seconds per step. The diffraction peaks from the pellet samples were compared to the reference crystalline tolfenamic acid starting material. The presence of diffraction peaks in the samples confirmed the precipitation of tolfenamic acid in a crystalline form, whilst a halo region and a lack of diffraction peaks indicated the presence of amorphous material.

Results

The Type-3B MC-SNEDDS formulation immediately dispersed upon addition to the digestion medium. Visually there was an immediate increase in opacity of the medium during dispersion, changing from a transparent micellar solution to a cloudy oil-in-water emulsion upon contact of the formulation with the medium. The dispersion period was set to 5 min to allow for complete mixing and to determine any initial propensity for precipitation, and necessary pH adjustments were made during this time, before pancreatic lipase solution was added to initiate digestion.

Lipid formulations containing MC lipids have been shown previously to digest completely under the lipolysis conditions used in this study (11, 16). Moreover, the Type-3B MC-SNEDDS used in the current study contains only 30% w/w lipids with the remainder consisting of surfactant and co-solvent. Rapid and complete digestion of the glyceride components was therefore expected, however, it was unknown how the inclusion of the charged non-digestible surfactant DDAB might influence the rate and extent of lipolysis for the DDAB containing formulations. Consequently, the *in vitro* lipolysis experiments were performed for the Type-3B MC-SNEDDS containing tolfenamic acid with and without addition of DDAB. The resultant lipolysis titration profiles for the two formulations tested are presented in Figure 2.

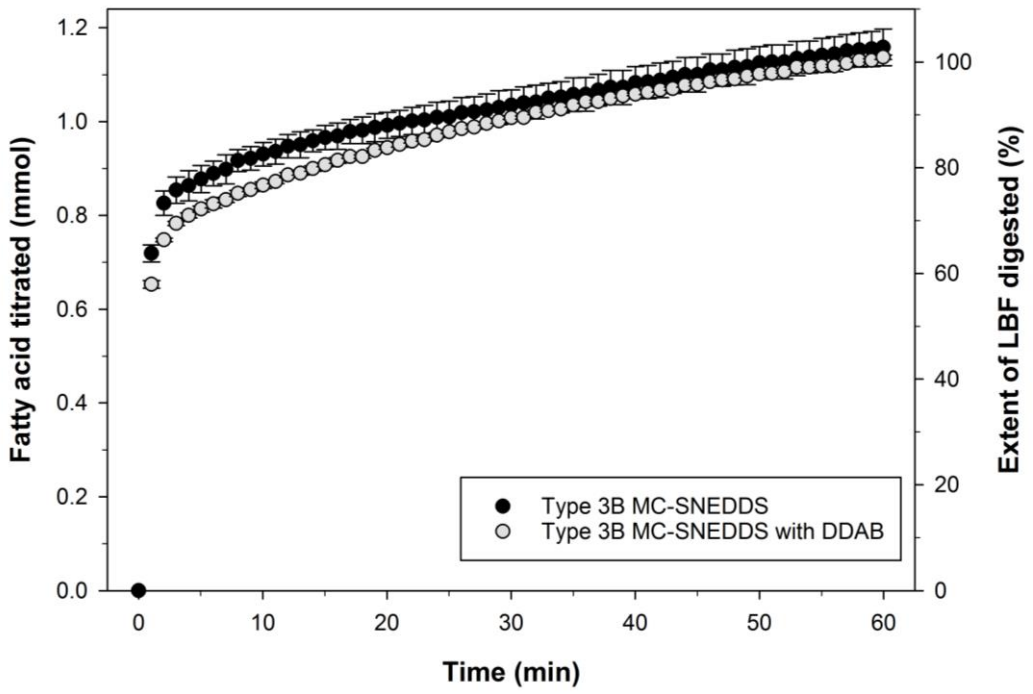

Figure 2: Titration profile of ionised and unionised fatty acid produced during *in vitro* lipolysis of the Type 3B MC-SNEDDS at pH 6.5 without DDAB (black circles) and with the addition of DDAB in the starting formulation (grey cirlces) (mean ± SD, n = 3 different digestions). The profile also shows the extent of LBF digested over the 60 min lipolysis.

The rate and extent of lipolysis for the Type 3B MC-SNEDDS were similar for both formulations tested, as can be seen from the lipolysis profiles from Figure 2. The MC-SNEDDS rapidly undergoes digestion upon addition of the pancreatic lipase and digests to completion within the 60 min period of lipolysis. The addition of DDAB in the formulation did not affect the overall extent of formulation digested during the experiment, and appeared to only slightly affect the rate of lipolysis. The similar digestion profiles obtained for the two different formulations means that the rate and extent of lipid digestion would not be an important factor in any differences in precipitation behaviour.

Phase Distribution of Tolfenamic Acid During Lipolysis

At the beginning of the lipolysis experiments approximately 1 g of formulation was added to the digestion medium. The viscous nature of the formulation precluded accurate addition, so the amount of drug recovered from the samples taken at various time points throughout lipolysis was calculated relative to the initial amount of drug in the dispersion prior to the addition of lipase.

For the MC-SNEDDS without DDAB it was evident upon dispersion that a fraction of the tolfenamic acid had precipitated, as a pellet could be visually observed after centrifuging the sample. The pellet from t = 0 min was analysed and it was found that 30.1 ± 6.43% w/w of the drug had precipitated during this dispersion period. The precipitation of drug upon dispersion of the formulation is generally believed to be indicative of a poorly-performing formulation. Relatively hydrophilic lipid formulations, such as the Type 3B MC-SNEDDS used in this study, are less able to maintain lipophilic drugs in solution as the formulation is dispersed in comparison to Type 1 – 3A formulations.

However upon initiation of lipolysis of the DDAB-free formulation, tolfenamic acid continued to precipitate as the lipid components were hydrolysed (Figure 3a). A gradual increase in the amount of tolfenamic acid recovered from the pellet phase was observed throughout lipolysis, with 51% w/w of the initial tolfenamic acid sedimented in the pellet after 60 min.

In contrast, the MC-SNEDDS with DDAB presented a very different phase distribution profile for tolfenamic acid upon *in vitro* dispersion and digestion. Notably, there was no drug precipitation during the dispersion phase when DDAB was included in the formulation. This could be a result of ionic interactions between tolfenamic acid and DDAB in the starting formulation, which may have in turn improved the solubility of tolfenamic

acid in the dispersed lipid droplets, as outlined in Figure 1. The above scenario is highly likely, as the mol amount of DDAB added to the formulation was equal to the mol amount of tolfenamic acid.

In addition, upon during lipolysis there was a significant increase in the amount of drug retained in the aqueous colloidal phase throughout digestion in comparison to the formulation without DDAB, as shown in Figure 3b. The precipitation of tolfenamic acid from the formulation containing DDAB was gradual and began only after 15 min of lipolysis. This means close to 80% of the formulation needed to be digested before drug precipitation ensued. This difference in behaviour between the two formulations tested is significant given the formulation without DDAB was unable to maintain drug in solution during dispersion, yet with the addition of DDAB at a 1:1 mol ratio of drug to surfactant the tolfenamic acid remained solubilised throughout dispersion, and remained solubilised up to a point where 80% of the formulation was digested.

After 60 min of lipolysis the formulation with the DDAB had digested completely, and only 28% w/w of the initial tolfenamic acid was in the pellet phase. Therefore, the final amount of precipitated tolfenamic acid was much less for the formulation with DDAB. It was clear from the phase distribution study that DDAB was having a significant effect on the precipitation behaviour of tolfenamic acid during *in vitro* digestion, as the addition of DDAB was the only variable between the two formulations examined. It remained unknown at this stage, however, whether DDAB was also affecting the solid-state form of the precipitated tolfenamic acid during the experiment.

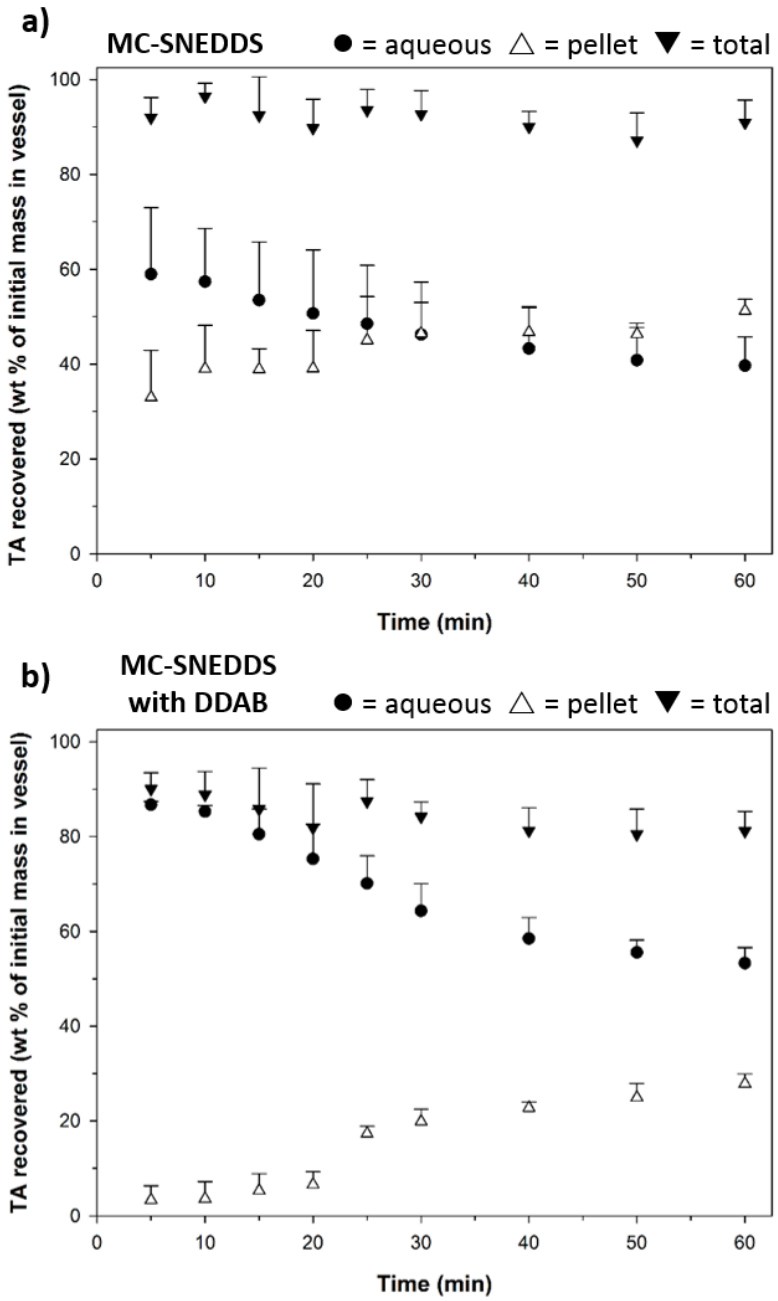

Figure 5: Distribution of tolfenamic acid (TA) in the aqueous colloidal phase and pellet phase during lipolysis of the model (a) Type 3B MC-SNEDDS without DDAB and (b) the Type 3B MC-SNEDDS with DDAB, conducted at pH 6.5. Where (●) = wt % tolfenamic acid in the aqueous colloidal phase; (Δ) = wt % tolfenamic acid in the pellet phase and (▼) = wt % tolfenamic acid recovered relative to the initial mass of drug in the digestion vessel. Samples taken at the various time points throughout lipolysis were diluted with mobile phase and then analysed using HPLC. Experiments were performed in triplicate (n = 3 different digestions, mean ± SD).

Solid-State Analysis of Pellet Phase

After performing *in vitro* digestion on both formulations (with and without DDAB) the pellet phase was isolated and viewed under a crossed polarized light microscope. The pellet obtained from the formulation without DDAB was bright yellow in colour. This gave a strong indication that tolfenamic acid had precipitated during the experiment in its 'yellow' polymorphic form, which has previously been reported (16, 23). The pellet obtained from the digestion of the formulation with DDAB, however, did not display this distinct yellow colour, but was brown, much like what is normally seen from pellets obtained from similar drug-free digestion experiments. From Figure 4 it can be seen that the formulation without DDAB gave rise to crystalline tolfenamic acid upon precipitation. This was evident in the form of sharp needle-like structures observed under the crossed polarized light. The pellet obtained from the *in vitro* digestion experiment performed on the formulation with DDAB did not display the same birefringence under crossed polarized light. There were no thin needle-like structures observed for the formulation with DDAB, indicating the absence of crystalline drug in the precipitated components.

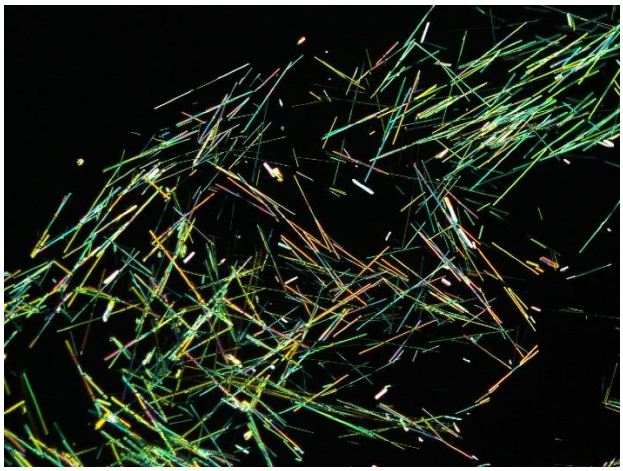

Figure 4: Crossed polarized light microscopy image of the pellet phase obtained after *in vitro* digestion of the tolfenamic acid containing Type 3B MC-SNEDDS without DDAB. The 'yellow' polymorphic form of tolfenamic acid was observed upon precipitation as many needle-like structures.

The pellet phases from the lipolysis of both formulations were also analysed using XRD to identify the solid-state form of the precipitated tolfenamic acid. It has been shown previously that tolfenamic acid precipitates in two different crystalline forms depending on the type of lipid formulation. The 'yellow' polymorphic form, observed in the current study, was also observed previously when XRD was performed on the pellet phase from the *in vitro* digestion of a Type 4 lipid formulation (16). The thermodynamically stable crystalline form, however, was identified upon precipitation during the lipolysis of a similar Type 3B lipid formulation to the one used in this study. The XRD data from the current study, presented in Figure 5, reveals that the 'yellow' polymorphic form of tolfenamic acid was formed during digestion of the model Type 3B MC-SNEDDS, indicated by different peak positions to the reference crystalline form, but the same diffraction pattern previously reported for this polymorph by Williams *et al.*(16). The components that made up the Type 3B MC-SNEDDS varied slightly between the current study and the study conducted by Williams *et al.*, although the drug loading was similar (51 mg/g). This minor adjustment in the formulation composition may have led to different drug supersaturation behaviour during dispersion, which caused the tolfenamic acid to precipitate as the 'yellow' polymorphic form in the current study.

For the formulation containing DDAB there was a clear lack of diffraction peaks in the XRD data. This reflected the absence of precipitated drug in the crystalline form, and the presence of a halo region in the diffractogram suggested that the tolfenamic acid had precipitated in an amorphous form (24). It is worth noting that the amount of tolfenamic acid that precipitated after lipolysis of the formulation with DDAB was around 20% w/w less than from the formulation without DDAB.

This absence of crystalline drug with DDAB present was not a result of a lack of sensitivity with the XRD instrument. The amount of material required for XRD measurements is several mg, which is sufficient for the current study given that 30% w/w of the initial tolfenamic acid precipitated during lipolysis of the formulation with DDAB. Therefore, if tolfenamic acid had precipitated in a crystalline form it would have been detected.

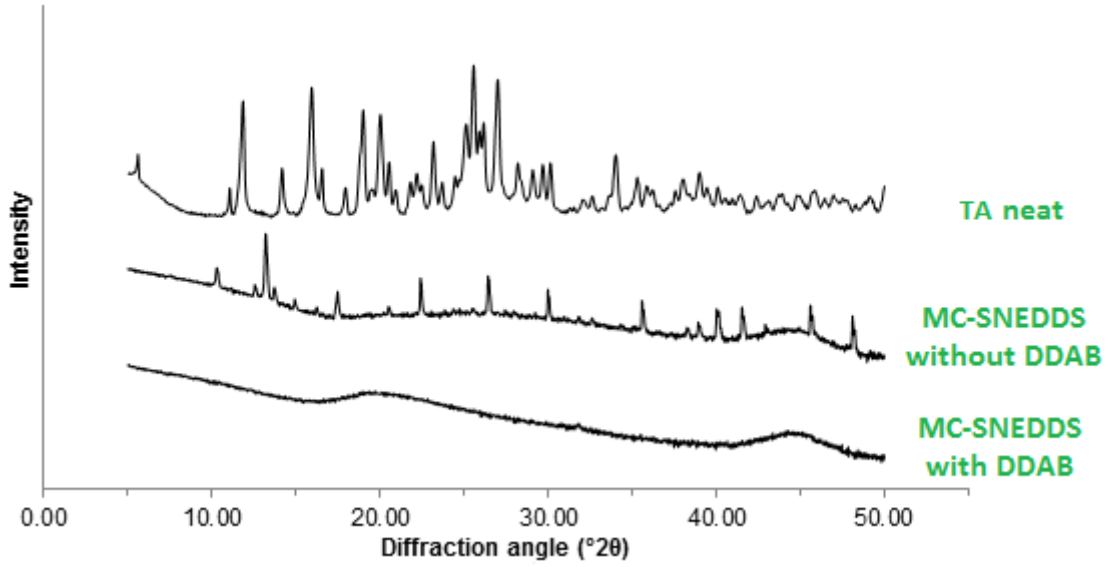

Figure 5: Diffraction patterns from XRD experiments performed on the pellet phase from the lipolysis of the Type 3B MC-SNEDDS containing tolfenamic acid with and without DDAB conducted at pH 6.5, and the neat crystalline tolfenamic acid starting material (TA neat).

Discussion

The effects of LBFs on the precipitation behaviour of poorly water soluble drugs have been examined in recent times, in particular with regard to the solid-state form of the precipitated drug (11-13, 16, 25-28). It is now established that some weakly basic drugs appear to precipitate in an amorphous form during *in vitro* digestion experiments, due to ionic interactions with oppositely charged fatty acids present in the digestion medium (14, 15). These fatty acids are liberated upon the enzymatic

hydrolysis, or digestion, of the lipids in the formulation. At pH 6.5, which is the pH that lipolysis experiments are routinely conducted and representative of the upper small intestinal contents, the majority of fatty acids present are ionised and available for ionic interactions with oppositely charged species, such as weakly basic drugs that are also ionised at this pH. In addition, fatty acid molecules are complexed with calcium ions to form precipitated soaps, and this allows for digestion to proceed in the closed *in vitro* model where absorption of digestion products is not an aspect (29, 30). By this mechanism the fatty acid molecules are removed from the surface of the digesting lipid droplet, allowing lipases continued access to the lipid substrate.

Analogous to the ionic interactions that occur between fatty acid and oppositely charged species during digestion, the weakly acidic drug tolfenamic acid was hypothesised to interact with the oppositely charged surfactant DDAB included in the Type 3B MC-SNEDDS examined in this study. As shown in Figure 3, tolfenamic acid precipitated upon dispersion of the MC-SNEDDS without DDAB, and continued to precipitate after lipase was added to the digestion vessel. Notably, and in contrast, upon dispersion of the MC-SNEDDS with DDAB there was no evidence of crystalline tolfenamic acid, and the extent of drug precipitation over the course of the *in vitro* digestion experiment was comparatively less than in the absence of DDAB.

The DDAB was dissolved in the starting MC-SNEDDS and therefore likely formed ionic interactions with tolfenamic acid, forming a lipophilic ion pair. This drug-surfactant ion pair had a much greater solubility in the lipid upon dispersion of the formulation compared to tolfenamic acid alone, to the point where no drug precipitation was evident after the 5 min dispersion period for the MC-SNEDDS with DDAB. DDAB was added to the formulation in equal mol amount to tolfenamic acid, and ionic interactions

between the two species likely maintained the drug in a dissolved state within the droplets formed from the emulsified MC-SNEDDS upon contact with the dispersion medium.

Although the ion pair was not isolated to determine its solid state properties, transforming drugs to ionic liquid forms through similar ion pairing has been shown previously to improve the solubility of drug in lipid excipients, which allows for a greater initial drug loading in the formulation (31, 32). Acidic lipophilic counterions were used to turn the weakly basic drug cinnarizine into an ionic liquid for this purpose, which resulted in a 7-fold increase in the solubility of cinnarizine as an ionic liquid in the starting lipid formulation (31, 34). A similar approach was used to enhance the loading of the anticancer drug irinotecan into nanostructured lipid carrier particles (33).

It is also worth noting that DDAB inherently self-aggregates in water at low concentrations (critical micelle concentration = 0.05 – 0.15 mM) to form vesicles (35). The high hydrophobicity of DDAB, however, and the presence of lipid components from the formulation means it is unlikely for there to be any free DDAB during dispersion, as the DDAB had already interacted with tolfenamic acid within the dispersed oil droplets.

After the dispersion period, during lipolysis, it was clear that a decrease in the amount of drug precipitation, when DDAB was added to the formulation, coincided with a high initial solubility of tolfenamic acid in the aqueous colloidal phase, as described by the phase distribution data in Figure 3. This high solubilisation capacity of the aqueous colloidal phase for tolfenamic acid throughout lipolysis was likely a result of DDAB affecting the nature of the colloidal structures formed in the aqueous phase.

The inclusion of DDAB in the formulation did not negatively impact the rate and extent of digestion of the formulation, as can be seen from the *in vitro* lipolysis profiles in Figure 2. This is in contrast to what has previously been reported for the effect of a similar cationic surfactant, dodecyl trimethyl ammonium bromide (DTAB), on the digestion of corn oil (36). It was shown that at concentrations greater than 1.4% w/w a noticeable inhibitory effect was observed on lipid digestion. The inhibitory effect of DTAB on lipid digestion was attributed to its ability to displace lipase from the surface of the digesting lipid droplet, or due to DTAB induced denaturing of the lipase. These findings, however, did not appear to translate to the current study, where the effect of DDAB on the rate and extent of lipolysis was studied. The extent of drug precipitation and the solid state form of the precipitate was therefore exclusive of the effect of DDAB on the lipolysis of lipids in the formulation.

In addition to tolfenamic acid, which appears to interact with DDAB, there are other species present during the *in vitro* digestion of the MC-SNEDDS that could interact with DDAB, such as fatty acids and bile salt. Vesicles composed of fatty acids can be stabilised by interactions between the ionised head-group of the fatty acid molecules and DDAB (37). In a similar fashion it has been shown previously that DDAB self-assembly in excess water results in different types of vesicle structures, and this phase behaviour is affected by the addition of sodium taurodeoxycholate (38). Interactions between the ionised head-groups of the anionic (sodium taurodeoxycholate) and cationic (DDAB) surfactants and the complex geometric packing of the sodium taurodeoxycholate increased the size of the vesicles in the two-component system. Sodium taurodeoxycholate incorporates within the DDAB vesicle aggregates in this way up to an addition of 20 mol%, beyond which leads to a phase separation.

From the current work, however, it was proposed that DDAB ion paired with tolfenamic acid in the starting formulation, and remained ion paired throughout dispersion and digestion. Thus improving drug solubility in the formulation during dispersion and in both the formulation and aqueous colloidal phase during digestion. Having said this, examining the self-assembly behaviour of lipids and lipid digestion products in the presence of DDAB during dispersion and digestion is certainly warranted. This could help establish whether DDAB, fatty acids and bile salt interact when combined in a single system, and whether the presence of tolfenamic acid affects these interactions. The potential interactions between DDAB, fatty acid, and bile salt do not necessarily preclude an increase in tolfenamic acid solubility during dispersion and digestion. The DDAB-fatty acid-bile salt structures that could make up the aqueous colloidal phase in the above scenario would provide a solubilising environment for tolfenamic acid. Further work is necessary to understand the structural aspects of the aqueous colloidal phase in this system and how this correlates to drug solubility during the *in vitro* digestion experiment.

It is of course conceded that such use of cationic surfactants in lipid formulations administered to humans may not be suitable due to toxicity reasons, however, alternative, less toxic, compounds may be explored in the future that could be adopted for development of an actual product. One potential alternative to surfactants is lipopeptides, which combine the structural properties of lipids (aliphatic chains) and amino acids and have been shown in some cases to be non-toxic to human cell lines (39). They also offer high versatility in potential structures, where the amino acid sequence and the number and length of the aliphatic chains can be customised to achieve a desired structure. Ultimately peptidases are likely to break down these components in the gut, potentially after they have provided their formulation function. Another option is to explore the use of

'soft' cations, such as pyridinium and guanidinium. One study showed that the toxicity of ionic liquids based on guanidinium was largely determined by the nature of the anionic components, where the majority of combinations used exhibited a low toxicity level (40).

In addition to exploring the use of less toxic cationic excipients for LBFs, there is also a need to study the performance of these formulations from a bioavailability perspective to test the proposed hypothesis. This appears especially true for highly lipophilic drugs that are proposed to rapidly permeate the intestinal membrane from the free fraction, keeping the level of drug saturation below the point after which precipitation occurs. Such studies, however, have been limited to using animal models such as mice and beagle dogs. Therefore, it remains unclear whether drug precipitation, and to what extent, from LBFs occurs in humans. Moreover, the primary focus of *in vivo* studies on LBFs in the past has been placed on quantifying the amount of absorbed drug and using this measured value as an indication of the extent of drug precipitation. Only recently has a study examined the precipitation behaviour and solid-state form of two poorly water-soluble drugs, fenofibrate and danazol, during the *in vivo* digestion of LBFs (41). The above study examined the gastric and intestinal contents of mice administered with LBFs using benchtop XRD. A more suitable experimental model for the purpose of following the fate of drug and lipid during digestion might be to adapt the recently developed *in vitro* digestion – *in vivo* absorption model (42). This model combines the standard *in vitro* lipolysis experiment coupled to an intestinal perfusion experiment in an anaesthetised rat. Further combining this setup with *in situ* synchrotron small angle x-ray scattering (SAXS) could potentially inform on formulation digestion, the formation of digestion products (self-assembled structures), which is especially important when the phase behaviour is expected to change due to excipients like DDAB used in the current study,

drug precipitation behaviour and the solid-state form of the precipitate, and drug absorption, all in real-time.

Conclusion

The weakly-acidic drug tolfenamic acid was more soluble in a lipid-based MC-SNEDDS formulation containing DDAB due to ion pair formation. Dispersion of the formulation containing DDAB in aqueous media did not result in precipitation, whereas dispersion of the DDAB-free formulation induced precipitation of >20% crystalline drug. In addition, the solid state form of tolfenamic acid upon precipitation during lipolysis was amorphous according to XRD data and CPLM images when DDAB was included in the MC-SNEDDS, whereas the drug precipitated in a polymorphic crystalline form in the absence of DDAB. The results obtained suggest ionic interactions had occurred between tolfenamic acid and DDAB to form a lipophilic ion pair in the starting MC-SNEDDS, and this complex significantly improved the solubility of the drug during dispersion and in the aqueous colloidal phase during digestion.

References

1. Porter CJ, Trevaskis NL, Charman WN. *Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs*. Nature reviews Drug discovery. 2007;**6**(3):231-48.

2. Phan S, Salentinig S, Prestidge C, Boyd B. *Self-assembled structures formed during lipid digestion: characterization and implications for oral lipid-based drug delivery systems*. Drug Deliv and Transl Res. 2014;**4**(3):275-94.

3. Williams HD, Trevaskis NL, Yeap YY, Anby MU, Pouton CW, Porter CJ. *Lipid-based formulations and drug supersaturation: harnessing the unique benefits of the lipid digestion/absorption pathway*. Pharmaceutical research. 2013;**30**(12):2976-92.

4. Anby MU, Williams HD, McIntosh M, Benameur H, Edwards GA, Pouton CW, et al. *Lipid digestion as a trigger for supersaturation: evaluation of the impact of supersaturation stabilization on the in vitro and in vivo performance of self-emulsifying drug delivery systems*. Molecular pharmaceutics. 2012;**9**(7):2063-79.

5. Warren DB, Benameur H, Porter CJ, Pouton CW. *Using polymeric precipitation inhibitors to improve the absorption of poorly water-soluble drugs: A mechanistic basis for utility*. Journal of drug targeting. 2010;**18**(10):704-31.

6. Warren DB, Bergstrom CA, Benameur H, Porter CJ, Pouton CW. *Evaluation of the structural determinants of polymeric precipitation inhibitors using solvent shift methods and principle component analysis*. Molecular pharmaceutics. 2013;**10**(8):2823-48.

7. Yeap YY, Trevaskis NL, Porter CJ. *Lipid absorption triggers drug supersaturation at the intestinal unstirred water layer and promotes drug absorption from mixed micelles*. Pharmaceutical research. 2013;**30**(12):3045-58.

8. Augustijns P, Brewster ME. *Supersaturating drug delivery systems: fast is not necessarily good enough*. Journal of pharmaceutical sciences. 2012;**101**(1):7-9.

9. Brouwers J, Brewster ME, Augustijns P. *Supersaturating drug delivery systems: the answer to solubility-limited oral bioavailability?* Journal of pharmaceutical sciences. 2009;**98**(8):2549-72.

10. Sassene PJ, Knopp MM, Hesselkilde JZ, Koradia V, Larsen A, Rades T, et al. *Precipitation of a poorly soluble model drug during in vitro lipolysis: characterization and dissolution of the precipitate*. Journal of pharmaceutical sciences. 2010;**99**(12):4982-91.

11. Stillhart C, Durr D, Kuentz M. *Toward an Improved Understanding of the Precipitation Behavior of Weakly Basic Drugs from Oral Lipid-Based Formulations*. Journal of pharmaceutical sciences. 2014.

12. Thomas N, Holm R, Mullertz A, Rades T. *In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS)*. Journal of controlled release : official journal of the Controlled Release Society. 2012;**160**(1):25-32.

13. Thomas N, Holm R, Garmer M, Karlsson JJ, Mullertz A, Rades T. *Supersaturated self-nanoemulsifying drug delivery systems (Super-SNEDDS) enhance the bioavailability of the poorly water-*

soluble drug simvastatin in dogs. The AAPS journal. 2013;**15**(1):219-27.

14. Sassene PJ, Mosgaard MD, Lobmann K, Mu H, Larsen FH, Rades T, et al. *Elucidating the Molecular Interactions Occurring during Drug Precipitation of Weak Bases from Lipid-Based Formulations: A Case Study with Cinnarizine and a Long Chain Self-Nanoemulsifying Drug Delivery System*. Molecular pharmaceutics. 2015;**12**(11):4067-76.

15. Khan J, Rades T, Boyd BJ. *Lipid-Based Formulations Can Enable the Model Poorly Water-Soluble Weakly Basic Drug Cinnarizine To Precipitate in an Amorphous-Salt Form During In vitro Digestion*. Molecular pharmaceutics. 2016;**13**(11):3783-93.

16. Williams HD, Sassene P, Kleberg K, Calderone M, Igonin A, Jule E, et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 3: understanding supersaturation versus precipitation potential during the in vitro digestion of type I, II, IIIA, IIIB and IV lipid-based formulations*. Pharmaceutical research. 2013;**30**(12):3059-76.

17. Pouton CW. *Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system*. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2006;**29**(3-4):278-87.

18. Thomas N, Mullertz A, Graf A, Rades T. *Influence of lipid composition and drug load on the In vitro performance of self-nanoemulsifying drug delivery systems*. Journal of pharmaceutical sciences. 2012;**101**(5):1721-31.

19. Williams HD, Sassene P, Kleberg K, Bakala-N'Goma JC, Calderone M, Jannin V, et al. *Toward the establishment of standardized in vitro tests for lipid-based formulations, part 1: method parameterization and comparison of in vitro digestion profiles across a range of representative formulations*. Journal of pharmaceutical sciences. 2012;**101**(9):3360-80.

20. Velkov T, Horne J, Laguerre A, Jones E, Scanlon MJ, Porter CJ. *Examination of the role of intestinal fatty acid-binding protein in drug absorption using a parallel artificial membrane permeability assay*. Chemistry & biology. 2007;**14**(4):453-65.

21. Fernandez S, Rodier JD, Ritter N, Mahler B, Demarne F, Carriere F, et al. *Lipolysis of the semi-solid self-emulsifying excipient Gelucire 44/14 by digestive lipases*. Biochimica et biophysica acta. 2008;**1781**(8):367-75.

22. R.A. C. Chapter 2: *Polarized Light Microscopy*. *Pharmaceutical Microscopy* 2011. p. 321p. 139 illus, 02 illus. in color.

23. Surov AO, Szterner P, Zielenkiewicz W, Perlovich GL. *Thermodynamic and structural study of tolfenamic acid polymorphs*. Journal of pharmaceutical and biomedical analysis. 2009;**50**(5):831-40.

24. Harris KD. *Powder diffraction crystallography of molecular solids*. Topics in current chemistry. 2012;**315**:133-77.

25. Stillhart C, Imanidis G, Kuentz M. *Insights into drug precipitation kinetics during in vitro digestion of a lipid-based drug delivery system using in-line raman spectroscopy and mathematical modeling*. Pharmaceutical research. 2013;**30**(12):3114-30.

26. Khan J, Hawley A, Rades T, Boyd BJ. *In Situ Lipolysis and Synchrotron Small-Angle X-ray Scattering for the Direct Determination of the Precipitation and Solid-State Form of a Poorly Water-Soluble Drug During Digestion of a Lipid-Based Formulation*. Journal of pharmaceutical sciences. 2015.

27. Larsen AT, Sassene P, Mullertz A. *In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems*. International journal of pharmaceutics. 2011;**417**(1-2):245-55.

28. Thomas N, Richter K, Pedersen TB, Holm R, Mullertz A, Rades T. *In vitro Lipolysis Data Does Not Adequately Predict the In vivo Performance of Lipid-Based Drug Delivery Systems Containing Fenofibrate*. The AAPS journal. 2014.

29. MacGregor KJ, Embleton JK, Lacy JE, Perry EA, Solomon LJ, Seager H, et al. *Influence of lipolysis on drug absorption from the gastro-intestinal tract*. Advanced drug delivery reviews. 1997;**25**(1):33-46.

30. Devraj R, Williams HD, Warren DB, Mullertz A, Porter CJ, Pouton CW. *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. 2013;**441**(1-2):323-33.

31. Sahbaz Y, Williams HD, Nguyen TH, Saunders J, Ford L, Charman SA, et al. *Transformation of poorly water-soluble drugs into lipophilic ionic liquids enhances oral drug exposure from lipid based formulations*. Molecular pharmaceutics. 2015;**12**(6):1980-91.

32. Williams HD, Sahbaz Y, Ford L, Nguyen TH, Scammells PJ, Porter CJ. *Ionic liquids provide unique opportunities for oral drug delivery: structure optimization and in vivo evidence of utility*. Chemical communications (Cambridge, England). 2014;**50**(14):1688-90.

33. Boyd BJ, Whittaker DV, Khoo SM, Davey G. *Hexosomes formed from glycerate surfactants--formulation as a colloidal carrier for irinotecan*. International journal of pharmaceutics. 2006;**318**(1-2):154-62.

34. Feeney OM, Crum MF, McEvoy CL, Trevaskis NL, Williams HD, Pouton CW, et al. *50 years of oral lipid-based formulations: Provenance, progress and future perspectives*. Advanced drug delivery reviews. 2016;**101**:167-94.

35. Griffin LR, Browning KL, Truscott CL, Clifton LA, Webster J, Clarke SM. *A comparison of didodecyldimethylammonium bromide adsorbed at mica/water and silica/water interfaces using neutron reflection*. Journal of colloid and interface science. 2016;**478**:365-73.

36. Li Y, McClements DJ. *Inhibition of lipase-catalyzed hydrolysis of emulsified triglyceride oils by low-molecular weight surfactants under simulated gastrointestinal conditions*. European journal of pharmaceutics and biopharmaceutics. 2011;**79**(2):423-31.

37. Suga K, Yokoi T, Kondo D, Hayashi K, Morita S, Okamoto Y, et al. *Systematical characterization of phase behaviors and membrane properties of fatty acid/didecyldimethylammonium bromide vesicles*. Langmuir : the ACS journal of surfaces and colloids. 2014;**30**(43):12721-8.

38. Marques EF, Khan A. Effect of a bile salt on the aggregation behavior of a double-chained cationic surfactant - the cationic-rich dilute region of the didodecyldimethylammonium bromide-sodium taurodeoxycholate-water system. In: Nylander T, Lindman B, editors. Lipid and Polymer-Lipid Systems. Progress in Colloid and Polymer Science. 120. Berlin: Springer-Verlag Berlin; 2002. p. 83-91.

39. Azmi F, Elliott AG, Marasini N, Ramu S, Ziora Z, Kavanagh AM, et al. *Short cationic lipopeptides as effective antibacterial agents: Design, physicochemical properties and biological evaluation*. Bioorganic & medicinal chemistry. 2016;**24**(10):2235-41.

40. Yu J, Zhang S, Dai Y, Lu X, Lei Q, Fang W. *Antimicrobial activity and cytotoxicity of piperazinium- and guanidinium-based ionic liquids*. Journal of hazardous materials. 2016;**307**:73-81.

41. Sassene PJ, Michaelsen MH, Mosgaard MD, Jensen MK, Van Den Broek E, Wasan KM, et al. *In vivo Precipitation of Poorly Soluble Drugs from Lipid-Based Drug Delivery Systems*. Molecular pharmaceutics. 2016;**13**(10):3417-26.

42. Crum MF, Trevaskis NL, Williams HD, Pouton CW, Porter CJ. *A new in vitro lipid digestion - in vivo absorption model to evaluate the mechanisms of drug absorption from lipid-based formulations*. Pharmaceutical research. 2016;**33**(4):970-82.

Chapter 6:
Summary and Outlook

It is widely established that a high proportion of newly discovered drugs are poorly water-soluble and therefore ill-suited to conventional oral dose forms. Advancements in the understanding of lipid digestion have led to the use of oral drug delivery systems comprising of lipidic excipients, as a way to enable the delivery of poorly water-soluble, lipophilic drugs. Lipid based formulations aim to maintain lipophilic drugs in a dissolved state throughout gastrointestinal transit, by making use of the lipid digestion mechanisms inherent to humans. The digestion of lipids allows drug to partition between the different colloidal structures that are formed. This effectively removes the rate-limiting dissolution step otherwise associated with other oral drug delivery systems, such as tablet and suspension formulations.

Significant enhancement of drug absorption can be obtained via the use of lipid formulations, however, they are currently far from ideal and remain in need of optimisation to make full use of their potential benefits. One issue with lipid formulations, and the focus of this thesis, is the precipitation of drug that is experienced upon dilution and digestion conditions. This precipitation of drug has been traditionally thought to decrease the overall fraction of absorbed drug, and the work presented in this thesis suggests that the solid-state form of the precipitate largely determines whether this decrease in absorbed drug actually takes place.

As mentioned in chapter 1, crystalline forms of drug are not always produced upon precipitation when digesting lipid formulations, as evident by a number of studies (1-4). Where drug has been observed to precipitate in an amorphous form during *in vitro* digestion experiments, a higher dissolution rate in simulated intestinal fluids was also found. These findings are important to the performance of lipid formulations, yet at the

time these findings were published it was not well understood why different solid-state forms of drug were precipitating during *in vitro* digestion, and whether this was a phenomenon that could be controlled. In order to better understand drug precipitation during digestion from the perspective of the solid-state form, it was necessary to develop a technique that determined structure in real-time whilst giving an indication of precipitation kinetics.

In Chapter 3, a novel technique that combined *in vitro* digestion experiments and synchrotron SAXS was developed to elucidate the solid-state form of precipitated drug in real-time. The poorly water-soluble neutral drug, fenofibrate, was used as a model and was incorporated into a rapidly digesting MC-SNEDDS. The kinetics of precipitation of fenofibrate were observed and it was shown that the drug first appeared to precipitate 4 min after initiating lipolysis. The drug precipitated in the same crystalline form as the reference drug material, as determined by the scattering angle of the observed diffraction peaks. This was the first example of time resolved SAXS being used to study such precipitation phenomena from lipid based formulations.

Importantly, with further optimisation of the experimental setup at the synchrotron, a significant increase in sensitivity for detection of precipitated drug was achieved, which allowed for the detection of fenofibrate down to concentrations of 1 mg/mL in the flow-through setup. This optimisation allows for many lipid systems to be tested in the future with relevant drug loadings, provided the drug displays a characteristic diffraction peak in the measurable q-range (0.04 – 2.00 $Å^{-1}$, equivalent to approximately 5 - 20° in 2θ space).

A key finding from this work was the determination of the critical amount of lipid digestion that needs to occur before drug precipitation rapidly ensues. This was determined by plotting

the change in peak area of the most intense diffraction peak, representing precipitated fenofibrate, against the extent of formulation digested. The resultant curve showed a sharp increase in peak area at 60% of formulation digested, indicating the critical amount of digestion required to trigger fenofibrate precipitation for the MC-SNEDDS. Subsequently, it was shown that a different drug (loratadine) also precipitated close to the same critical amount of digestion (64%) that was required for fenofibrate (60%). The MC-SNEDDS therefore underwent a similar loss in solubilisation capacity during digestion regardless of the drug used. This loss in solubilisation capacity differed to that observed when a LC-SNEDDS was examined, which showed a critical amount of digestion required for fenofibrate precipitation to be 46%. The LC-SNEDDS was, however, slower to digest than the MC-SNEDDS. Therefore, to optimise the effectiveness of lipid formulations and to minimise drug precipitation an ideal lipid formulation may need to be slow to digest, and have a high solubilisation capacity during digestion to push the critical amount of digestion required to trigger drug precipitation to higher values. Both of these aspects can be assessed with the *in situ* lipolysis and synchrotron SAXS method outlined in chapter 3.

Chapter 4 probed the recently observed trend associated with weakly-basic drugs precipitating in an amorphous form during *in vitro* digestion experiments. The purpose of this chapter was to examine the mechanism behind this amorphous precipitation and to determine whether this outcome was dependent on the formulation. From XRD measurements, the weakly-basic drug, cinnarizine, was shown to precipitate in an amorphous form when *in vitro* digestion experiments were performed at pH levels below the pKa of the drug (pKa = 7.47), whereas it precipitated in a crystalline form at pH levels above the pKa. This indicated a link between the state of ionisation of cinnarizine and the final

solid-state form of the precipitated drug, where unionised cinnarizine produced crystalline precipitation and ionised cinnarizine gave rise to amorphous-salt precipitation.

Ionic interactions between cinnarizine and oppositely charged fatty acid molecules were hypothesised to be the driver for amorphous-salt precipitation. After probing the environment of the tertiary amine present in cinnarizine using FTIR, it was observed that for the precipitated drug from the digestion experiment at pH 6.5, the wavenumber at which the C-N stretch appeared was different to that of the precipitate from the experiment at pH 8.0 and the reference crystalline drug. The C-N stretch region for the cinnarizine hydrochloride salt form was different to both the reference material and the precipitated drug from the experiments conducted at pH 6.5 and pH 8.0. An ionic interaction between cinnarizine and the liberated MC fatty acids present during digestion of the MC-SNEDDS was established as responsible for causing the drug to precipitate in a noncrystalline, amorphous-salt form. Thus, lipid formulations can enable weakly-basic drugs with favourable ionisation properties to precipitate in high-energy amorphous-salt forms, which may redissolve in the gastrointestinal tract given the dynamic conditions encountered during lipid digestion. The findings from Chapter 4 also highlight the importance of ensuring that non-misleading terminology is not adopted in the field. Amorphous precipitation of cinnarizine is different to cinnarizine precipitating in an amorphous form. The amorphous-salt that is produced upon *in vitro* digestion of the MC-SNEDDS is an amorphous form of drug combined with fatty acid, however, it is important to differentiate this from amorphous precipitation of cinnarizine, which implies cinnarizine molecules exclusively make up the amorphous precipitate. It is likely that cases where 'amorphous precipitation' have been observed thus far with digesting lipid based formulations and ionisable drugs, the actual form of these

precipitates is amorphous-salts.

The precipitation behaviour of weakly-acidic drugs was then examined in chapter 5, and the concept of ion-pairing between drug and oppositely charged species, as outlined in chapter 4, was applied and tested. Tolfenamic acid was used as a model weakly-acidic drug, and the cationic surfactant DDAB was included in a MC-SNEDDS formulation at a 1:1 drug/surfactant mol ratio. The digestion experiments were performed at pH 6.5, as a result the majority of tolfenmaic acid present during lipolysis was in the ionised form (pKa 3.7) and therefore able to form ionic interactions with oppositely charged species. Crystalline precipitation of tolfenamic acid, in a different polymorphic form than the starting material, was observed upon digesting the MC-SNEDDS that did not contain the cationic surfactant DDAB. Upon adding the DDAB to the formulation, however, the absence of diffraction peaks, from the XRD data, correlating to crystalline tolfenamic acid and the presence of a halo region suggested tolfenamic acid had precipitated in an amorphous form. Ionic interactions between tolfenamic acid and DDAB were considered the driver for the change in solid-state form of the precipitated drug.

Additionally, it was observed from the phase distribution data that a significantly larger amount of tolfenamic acid remained in solution for a longer period of time when DDAB was included in the formulation, as opposed to the DDAB-free MC-SNEDDS. This was likely due to the intercalation of DDAB within the aqueous colloidal phase formed from MC lipid digestion products and the increased solubility of tolfenamic acid within these structures. Analogous to weakly-basic drugs precipitating as amorphous-salts during lipid digestion, this chapter showed that through adding a cationic excipient in the starting formulation, the solid-state form of weakly-acidic drugs can also be controlled.

The above findings advance the current level of knowledge around the precipitation behaviour of poorly-water soluble drugs during lipid digestion on three main fronts. First, real-time detection of precipitated drug and the solid-state form can now be evaluated using the *in situ* lipolysis and SAXS technique, which provides insight into, and is able to track, precipitation kinetics and loss of solubilisation capacity of the formulation during digestion. Second, it was established that weakly-basic drugs with suitable ionisation properties undergo ionic interactions with fatty acids during *in vitro* digestion to drive precipitation in an amorphous-salt form. Third, by including oppositely charged cationic excipients in the lipid formulation, weakly-acidic drugs also undergo ionic interactions and precipitate as amorphous-salt forms.

The optimisation of the *in situ* lipolysis and synchrotron SAXS technique for capturing drug precipitation took place towards the end of candidature. This optimisation resulted from a number of previous attempts, which assisted in understanding the capabilities and limitations of the SAXS beamline at the Australian Synchrotron. As a result, only a small number of drugs and lipid systems were examined with the optimised *in-situ* technique and presented in Chapter 3. Future studies should seek to expand on these findings to provide an in-depth assessment of the risk of precipitation from different types and compositions of lipid formulations with a number of different drugs. The current work largely focused on using model systems and drugs, but in the future a wide range of formulations and drugs that fall under the non-ionisable, weakly-basic, and weakly-acidic categories should be assessed for optimisation and comparison purposes. For instance, to optimise lipid formulations with regard to minimising the risk of drug precipitation, a number of formulations with varying amounts of lipidic components should be assessed using the *in situ* lipolysis and SAXS technique to compare where the critical amount of digestion to trigger drug precipitation

occurs, and how to improve this property in tandem with the rate of digestion of the formulation. A single lipase enzyme activity (1000 TB units/mL of digest) was used throughout this work, in the future this may also be varied to modulate the kinetics of lipid digestion. It was demonstrated using cinnarizine that weakly-basic drugs interact with fatty acids to form amorphous-salts upon precipitation, and it would be of benefit to test a number of different weakly-basic drugs with different physico-chemical properties, with a number of different types of lipid systems, to examine the effects on solid-state form of the precipitate and how lipid chain-length affects the properties of the precipitated amorphous-salt. The same can be said for weakly-acidic drugs and cationic excipient combinations.

Ultimately, this knowledge needs to be translatable to *in vivo* studies with appropriate methods available to test for correlations with the *in vitro* work. Drug precipitation needs to be shown to occur *in vivo* during the digestion of lipid formulations, for the control gained over the solid-state form during *in vitro* experiments to be of benefit. Currently, an experimental technique to monitor drug precipitation and solid-state form in real-time during *in vivo* digestion experiments does not exist. Recently, however, an *in vitro* lipolysis and *in vivo* perfusion model was developed to allow analysis of lipid digestion and drug absorption in the same experiment (5). The findings from this study with regard to the extent of fenofibrate precipitation *in vitro* and the lack of correlation with absorption *in vivo* were interesting in itself. It would be of great benefit to combine the approaches of the in situ lipolysis and synchrotron SAXS technique with the *in vitro* lipolysis and *in vivo* perfusion model, for a comprehensive analysis of lipid formulation digestion, evolution of colloidal structures formed by digestion products, drug absorption and drug precipitation from a single experiment.

Overall, advancements were made in understanding drug precipitation during lipid digestion from the work presented in this thesis. This came in the form of a novel experimental method that provides insight into real-time precipitation behaviour and solid-state form of the drug during digestion, and an indication of formulation performance and loss in solubilisation capacity. The mechanism behind the amorphous-salt precipitation of weakly-basic drugs was revealed, and ways to try and drive a similar outcome with weakly-acidic drugs were examined. Future work is clearly necessary, as discussed above, especially in relation to translatability studies *in vivo* to further consolidate the project's hypotheses and to provide a comparative framework that could inform of expected precipitation behaviour for a number of different drugs and types of lipid formulations. It is envisioned that acquiring this level of knowledge will assist in optimising the manufacture of lipid formulations with respect to the concern of drug precipitation upon digestion.

References

1. Sassene PJ, Knopp MM, Hesselkilde JZ, Koradia V, Larsen A, Rades T, et al. *Precipitation of a poorly soluble model drug during in vitro lipolysis: characterization and dissolution of the precipitate*. J Pharm Sci. 2010;**99**(12):4982-91.

2. Thomas N, Holm R, Mullertz A, Rades T. *In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS)*. J Control Release. 2012;**160**(1): 25-32.

3. Stillhart C, Durr D, Kuentz M. *Toward an improved understanding of the precipitation behavior of weakly basic drugs from oral lipid-based formulations*. J Pharm Sci. 2014; **103**(4):1194-203

4. Thomas N, Holm R, Garmer M, Karlsson JJ, Mullertz A, Rades T. *Supersaturated self-nanoemulsifying drug delivery systems (Super-SNEDDS) enhance the bioavailability of the poorly water-soluble drug simvastatin in dogs*. AAPS J. 2013;**15**(1):219-27.

5. Crum MF, Trevaskis NL, Williams HD, Pouton CW, Porter CJ. *A new in vitro lipid digestion - in vivo absorption model to evaluate the mechanisms of drug absorption from lipid-based formulations*. Pharmaceutical research. 2016;**33**(4):970-82.