RESEARCH ARTICLE

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

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ABSTRACT: The Lipid Formulation Classification System Consortium is an industry–academia collaboration, established to develop standardized *in vitro* methods for the assessment of lipid-based formulations (LBFs). In this first publication, baseline conditions for the conduct of digestion tests are suggested and a series of eight model LBFs are described to probe test performance across different formulation types. Digestion experiments were performed *in vitro* using a pH-stat apparatus and danazol employed as a model poorly water-soluble drug. LBF digestion (rate and extent) and drug solubilization patterns on digestion were examined. To evaluate cross-site reproducibility, experiments were conducted at two sites and highly consistent results were obtained. In a further refinement, bench-top centrifugation was explored as a higher throughput approach to separation of the products of digestion (and compared with ultracentrifugation), and conditions under which this method was acceptable were defined. Drug solubilization was highly dependent on LBF composition, but poorly correlated with simple performance indicators such as dispersion efficiency, confirming the utility of the digestion model as a means of formulation differentiation. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

The issue of low aqueous drug solubility continues to hinder the robust testing of new chemical entities during drug discovery and development. Of the many formulation strategies that have been used to address the obstacles associated with low solubility, lipid-based formulations (LBFs) have generated significant interest.[1] The composition of LBFs can vary widely, although common design features include the presence of molecularly dispersed drug within a blend of various polar and nonpolar oils with/without surfactant and cosolvent.[2,3] As the drug is presented to the gastrointestinal (GI) tract in solution, although in an oily liquid, the use of LBFs circumvents the limitations to solubility introduced by solute–solute interactions in the crystalline solid.[4] LBFs also promote drug solubilization in the GI fluids via the provision of surfactants and lipids (and their digestion products) that collectively supplement the inherent solubilization capacity of the endogenous GI fluids (i.e., that provided by bile salts, phospholipids, and cholesterol secreted in bile).

Unlike many traditional formulations, the physical and chemical nature of most LBFs is dramatically changed after oral administration by the interaction with biliary and pancreatic secretions in the small intestine, in a process analogous to the digestion of food-based lipids.[5] The major mechanism of chemical change is that of lipid digestion. Lipid digestion is mediated by pancreatic lipases and esterases that are secreted into the upper small intestine in response to the ingestion of exogenous lipid, and to a lesser extent by acid-stable lipases in the stomach.[6] The lipid digestion products generated are subsequently solubilized by bile salt–phospholipid–cholesterol-mixed micelles secreted in bile, resulting in the formation of a range of colloidal species in the GI fluids. The resulting colloidal structures support the solubilization of exogenously administered lipids and coadministered poorly water-soluble drugs. LBFs, therefore, exploit the lipid digestion and absorption cascade, such that drug incorporated into the administered lipid vehicle is transferred into the colloidal phases produced during lipid digestion and these species shuttle digestion products and drug from the lipid substrate to the intestinal wall for absorption.[5,7–10] Current understanding suggests that a critical aspect of this process is the avoidance of drug precipitation during processing of lipid formulations because regeneration of the solid state results in a reversion to a situation consistent with administration of a crystalline drug suspen-sion (where dissolution rate is typically a limitation to drug absorption for poorly water-soluble drugs). One caveat to this overarching suggestion is the recent realization that drug precipitation from LBFs may, for some compounds, result in the amorphous drug being formed.[11] In this case, the process of redissolution of precipitated drug is expected to be faster than if the drug were to precipitate in the crystalline form.

The basic mechanisms by which LBFs promote drug absorption are therefore reasonably well developed. The specific determinants of *in vivo* performance, however, are not fully understood, and robust approaches to probe *in vivo* performance using *in vitro* tests remain poorly defined. The determinants of *in vitro* and *in vivo* performance are likely to include (1) the capacity of the formulation to maintain solvent capacity on dilution and digestion, (2) the rate of formulation digestion [which dictates the rate and quantity of lipid (or surfactant) digestion products and drug that partitions from the oil reservoir into the intestinal milieu], and (3) the solubilization capacity of the localized GI environment when enriched with the products of the digested formulation.[12–15] These determinants are applicable to a wide range of LBF as digestion will inevitably impact on the solubilization capacity of all formulations that contain digestible lipids or surfactants. Digestion of poorly dispersed lipid-rich LBF (such as simple lipid solutions) is typically beneficial to drug absorption because digestion leads to the generation of more amphiphilic lipid digestion products that are more readily incorporated into bile salt–phospholipid-mixed micelles. Digestion therefore catalyzes the *in situ* assembly of highly dispersed colloidal phases with high drug solubilization capacities. In contrast, self-emulsifying formulations and formulations with high proportions of surfactant and cosolvent do not require digestion to reduce particle size because initial dispersion of the formulation commonly leads to the production of nanometer-sized colloidal droplets. Formulation digestion remains unavoidable, however, and may be detrimental to absorption if digestion leads to a decrease in solubilization capacity and drug precipitation.

In an attempt to better understand the performance of a wide range of LBFs, *in vitro* lipid digestion models have emerged as a possible mechanism by which the complex series of *in vivo* interactions that underpin utility may be modeled and predicted *in vitro*.[16–24] In these models, the lipid formulation (containing incorporated drug) is dispersed in a digestion medium that is broadly representative of the contents of the upper small intestine, and digestion of the

formulation is initiated by addition of a porcine-derived pancreatic extract containing pancreatic lipase and other pancreatic enzymes. Formulation digestion results in the liberation of fatty acid (FA) from either glyceride lipids or surfactant FA esters, and this leads to a drop in the pH of the digest. By conducting digests in a pH-stat titrator, the transient drop in pH is monitored and neutralized by equimolar titration of sodium hydroxide, thereby maintaining pH at a set point. Quantification of the rate of addition of base therefore provides an indirect measure of the rate and extent of lipid digestion, on the condition that FAs are ionized at the pH of the assay. Samples may be removed at intervals during the digestion test and mixed with a lipolysis inhibitor prior to separation by high-speed ultracentrifugation. Ultracentrifugation may result in the separation of three potentially distinct phases: a pellet phase containing precipitated drug; an aqueous colloid phase containing solubilized drug; and an oily phase containing a mixture of incompletely digested lipid, any phase-separated digestion products, and incorporated drug.

The use of lipid digestion models to better understand formulation performance continues to inform the broader literature, but interpretation of data obtained across different laboratories is currently limited by variations in the experimental conditions employed such as the test pH; the volume of the test; and the concentrations of bile salt, calcium, and buffering agents employed. The current communication presents the first in a series of papers generated by a consortium of academic and industrial members [the Lipid Formulation Classification System (LFCS) Consortium] that aim to establish common and discriminating methods for the conduct of *in vitro* digestion tests.

Proposed by Pouton,[2] the LFCS categorizes lipid formulations according to formulation properties including whether or not water-soluble excipients are included, the particle size of the formulation on dispersion, and the role of lipid digestion in formulation performance *in vivo*. The initial objective of the LFCS was to generate a framework to facilitate better understanding of the performance of differing formulation types and to aid comparison of data obtained for similar "classes" of formulation.[2] In attempting to apply the LFCS, however, it has become increasingly evident that this is not possible without some level of standardization of the conditions employed to test formulations *in vitro*.[3] To address this need, the LFCS Consortium has been established to develop standardized methods for formulation assessment of LBFs using *in vitro* testing protocols. The Consortium comprises academic groups from Monash University, the University of Copenhagen, and the CNRS in Marseille and industrial collaborators organized into primary and associate Consortium members. During the first year, the primary industrial members were Capsugel, Sanofi, Gattefossé, and Merck Serono.

This first publication from the consortium establishes a panel of eight LBFs that have been chosen to span the range of the four LFCS classes and identifies the experimental variables that are critical to the *in vitro* performance of these exemplar formulations. The conditions required to facilitate the generation of consistent data across different laboratories have been evaluated and the impact of variation in sampling methodology and sample analysis across different formulation types examined. Preliminary conclusions regarding the design of testing protocols are described and form the baseline conditions for a series of subsequent investigations of the impact of method variation on *in vitro* performance.

MATERIALS AND METHODS

Materials

Details of the lipidic excipients used within the LFCS Consortium can be found in Table 1. Danazol was kindly supplied by Sterling Pharmaceuticals (Sydney, Australia) and Indis (Aartselaar, Belgium). Preliminary studies revealed that the two different batches of this drug showed comparable solubility in pH 6.5 Tris-maleate buffer (0.7–1.2 µg/mL), and the values obtained were within the range of previously reported values.[25] Sodium taurodeoxycholate >95% (NaTDC), 4-bromophenylboronic acid, and the porcine pancreatin extract [P7545, 8× United States Pharmacopeia (USP) specifications activity] were all obtained from Sigma Chemical Company (St. Louis, Missouri). Phosphatidylcholine (PC) (Lipoid E PC S, ∼99.2% pure, from egg yolk) was obtained from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). One mole per liter sodium hydroxide, which was diluted to obtain 0.2 and 0.6 M NaOH titration solutions, was purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, Massachusetts). All other chemicals and solvents were of analytical purity or high-performance liquid chromatography (HPLC) grade.

Selection of Exemplar LBFs for Investigation in the LFCS Consortium

The compositions of the eight lipid formulations investigated by the consortium are shown in Table 2. Formulations were chosen to span the four classes of the LFCS,[2] and contained either long-chain (LC) or medium-chain (MC) lipid excipients. Attempts were made to keep the lipid–surfactant ratio constant between formulations with a view to aiding interformulation comparison. For example, types II and IIIA formulations comprised an equal quantity of lipid

Table 1. Details of the Lipidic Excipients Used in the Model LBFs Investigated by the LFCS Consortium in this Study

Excipient	Source	Description of Batches
Triglyceride:		
Corn oil	Sigma, St. Louis, Missouri	Long-chain triglyceride consisting of 39.4%–62.0% linoleic acid ($C_{18:2}$), 20.0%–42.2% oleic acid ($C_{18:1}$), 8.6%–16.5% palmitic acid (C_{16}), and 1.0%–3.3% stearic acid (C_{18})
Captex 300	Abitec Corporation, Columbus, Ohio	Medium-chain triglyceride mainly consisting of 65.7% caprylic acid (C_8) and 33.7% capric acid (C_{10})
Mixed glycerides:		
Maisine^{TM} 35-1	Gattefossé, St. Priest, France	Blend of partially digested long-chain glycerides: 33.5% monoglyceride, 50.9% diglyceride, and 14.7% triglyceride, predominantly of linoleic acid (51.1%, $C_{18:2}$) and oleic acid (32.8%, $C_{18:1}$)
Capmul MCM	Abitec Corporation	Blend of partially digested medium-chain glycerides: 60.7% monoglyceride, 33.1% diglyceride, and 4.4% triglyceride, predominantly of caprylic acid (82.8%, C_8)
Surfactants:		
Tween 85	Sigma	Polyoxyethylene sorbitan trioleate (HLB 11)
Cremophor EL	BASF Corporation, Washington, New Jersey	Polyethoxylated castor oil (HLB 14–16)
Cosolvents:		
Transcutol HP	Gattefossé	Diethylene glycol monoethyl ether

and surfactant and only differed by the nature of surfactant (Table 2). Properties that varied across the formulation range included (1) the capacity to self-emulsify, (2) digestibility, (3) drug solvency in the formulation, and (4) particle size on dispersion. The disparate nature of the formulations was critical to the aims of the LFCS consortium, that is, to establish *in vitro* testing methods that are applicable across a range of lipid formulations. At this point, the Consortium has focused on liquid–lipid formulations. Solid and semisolid systems will be addressed in subsequent studies.

Determination of Danazol Equilibrium Solubility in the LBFs

The equilibrium solubility of danazol in each formulation was determined using a previously reported method.[26] In brief, danazol was added in excess to 4 mL borosilicate glass vials containing 2 g lipid formulation. Vials were allowed to equilibrate at

Table 2. Composition of the Exemplar LBFs Investigated by the LFCS Consortium in this Study and Physical Properties Following Dispersion in the Digestion Medium

Formulation Type	Composition (% w/w)	Appearance on Dispersion[a]	Particle Size[b] (nm)	Polydispersity[b]
I-MC	50.0% Captex 300 50.0% Capmul MCM	Opaque emulsion	n/a[c]	–
II-MC	32.5% Captex 300 32.5% Capmul MCM 35.0% Tween 85	Opaque emulsion	1 in 36: n/a[c] 1 in 250: 190.2 ± 2.7	0.068 ± 0.05
IIIA-MC	32.5% Captex 300 32.5% Capmul MCM 35.0% Cremophor EL	Ultrafine dispersion	1 in 36: 29.1 ± 0.6 1 in 250: 36.2 ± 0.9	0.062 ± 0.01 0.088 ± 0.02
IIIB-MC	25.0% Capmul MCM 75.0% Cremophor EL 25.0% Transcutol HP	Ultrafine dispersion	1 in 36: 21.4 ± 0.5 1 in 250: 14.0 ± 2.7	0.111 ± 0.01 0.138 ± 0.10
I-LC	50.0% corn oil 50.0% Maisine^{TM} 35-1	Coarse emulsion	n/a[c]	–
II-LC	32.5% corn oil 32.5% Maisine^{TM} 35-1 35.0% Tween 85	Coarse–opaque emulsion	n/a[c]	–
IIIA-LC	32.5% corn oil 32.5% Maisine^{TM} 35-1 35.0% Cremophor EL	Ultrafine dispersion	1 in 36: 61.8 ± 0.4 1 in 250: 58.4 ± 1.3	0.197 ± 0.01 0.149 ± 0.01
IV	50.0% Cremophor EL 50.0% Transcutol HP	Transparent solution	1 in 36: 13.1 ± 0.4 1 in 250: 9.9 ± 0.5	0.033 ± 0.01 0.111 ± 0.06

[a]Appearance following dispersion of 1 g LBF in 36 mL digestion medium (37°C).
[b]Mean particle size of the dispersed LBF following a 1 in 36 and 1 in 250 dilution in the digestion medium (37°C). Values are expressed as means ($n = 3$) ± 1 SD.
[c]Colloids formed on dispersion were too large to be measured accurately (i.e., polydispersity > 0.5).
LC, long chain; MC, medium chain.

37°C with periodic vortex mixing to ensure that undissolved drug particles were suspended in the lipid slurry. At intervals, vials were centrifuged (Eppendorf 5408R centrifuge; Eppendorf AG, Hamburg, Germany) at 4000 rpm (2800g) and 37°C for 30 min. This separated samples into a solid pellet phase and a particle-free supernatant. Accurately weighed samples were removed from the supernatant, transferred to 5 mL volumetric flasks and made up to volume with chloroform–methanol (2:1, v/v). Aliquots (50–100 μL) were diluted >10-fold with methanol and analyzed for danazol content by HPLC (see the section *HPLC Detection of Danazol* below for details). Equilibrium solubility was defined as the value attained when consecutive solubility values differed by less than 5%. For danazol, this was achieved within 24 h for most LBFs and within 72 h for all formulations.

Drug Incorporation into the LBFs

Formulations were loaded with danazol at 80% of the saturated solubility of the drug in the formulation. The required mass of danazol was weighed directly into clean screw-top glass vials, and drug-free lipid formulation was added up to the target mass loading. Vials were sealed, vortex mixed, and incubated at 37°C for at least 24 h prior to testing. The danazol content in the formulation was verified (in triplicate) on the day of testing using sampling and analysis processes as described in the section *Determination of Danazol Equilibrium Solubility in the LBFs* above.

In Vitro Evaluation of the LBFs

Digestion Experiments

Standardized equipment for performing *in vitro* digestion tests was identified based largely on earlier descriptions of the *in vitro* digestion test.[27,28] The experimental setup employed by the LFCS Consortium consisted of a pH-stat apparatus (Metrohm® AG, Herisau, Switzerland), comprising a Titrando 802 propeller stirrer/804 Ti Stand combination, a glass pH electrode (iUnitrode), and two 800 Dosino dosing units coupled to 10 mL autoburettes (Metrohm® AG). The apparatus was connected to a PC and operated using Tiamo 2.0 software (Metrohm®).

Initial Reference Conditions for In Vitro Digestion in the LFCS Consortium

The initial reference conditions for the *in vitro* digestion experiments were as follows. The digestion buffer (pH 6.5) contained 2 mM Tris-maleate, 1.4 mM CaCl$_2$·2H$_2$O, and 150 mM NaCl and was supplemented with 3 mM NaTDC and 0.75 mM PC. Concentrations of bile, phospholipid, calcium, and sodium chloride were chosen to reflect the typical concentrations present in the fasted small intestine.[29,30]

Preliminary studies (see *Supplementary Material*) were conducted to examine the importance of the source and type of bile salt utilized and compared the effect of using 3 mM of (1) NaTDC, (2) bovine bile extract, or (3) a mixture of synthetic bile salts (mimicking the typical bile salt secretion in the human gall bladder) on the rate and extent of digestion of LC lipids. These studies revealed little difference in the data obtained using the different bile salt preparations. Consequently, on the basis of cost and batch-to-batch consistency, a single commercially available source of NaTDC was considered appropriate for use during *in vitro* digestion testing. A concentration of Tris buffer (2 mM) was chosen to be sufficient to allow stabilization of pH during initial adjustment of digest pH, but sufficiently low to have a negligible effect on the downstream detection of titratable FA. Pancreatin from porcine pancreas containing pancreatic lipolytic enzymes [colipase-dependent pancreatic lipase and carboxyl ester hydrolase (CEH)] was prepared by the vortex mixing 1 g of pancreatin powder in 5 mL digestion buffer (i.e., free of bile salt and phospholipid) and approximately 17 μL of 5.0 M sodium hydroxide that adjusted the mixture pH to 6.5. The enzyme suspension was centrifuged [4000 rpm (2800g), 5°C; Eppendorf 5408R (Eppendorf AG)] for 15 min and 4 mL supernatant recovered for the digestion test. To minimize loss of enzyme activity prior to use, pancreatin extract was prepared using cold (2°C–8°C) digestion buffer and prepared fresh before each test. The batch of pancreatin used throughout this work showed a USP activity of 29.7 ± 1.9 USP units per milligram of dry extract [equivalent to ~48 tributyrin units (TBU) per milligram dry extract, and ~1000 TBU per milliliter of digest].

In the *in vitro* experiments, 1 g lipid formulation was weighed directly into a thermostat-jacketed glass reaction vessel (Metrohm® AG) and dispersed for 10 min in 36 mL digestion medium (37°C). Continuous mixing was achieved using an overhead propeller stirrer 25 mm in diameter and rotating at a speed of approximately 450 min^{-1}. Preliminary experiments confirmed that this propeller speed was sufficient to achieve homogenous mixing conditions while minimizing the risk of over-emulsifying the added lipid (data not shown). The pH during the initial dispersion phase was manually adjusted to pH 6.5 ± 0.05 using small quantities of NaOH or HCl.

Digestion was subsequently initiated via addition of 4 mL pancreatin extract. Sodium hydroxide titration solutions (titrants) of 0.2 and 0.6 M were utilized for digests containing LC and MC formulations, respectively. Titrants were automatically added (controlled via the pH-stat controller) to the reaction vessel to maintain constant pH during digestion, with the rate of titrant addition reflecting the digestibility of the lipid formulation.

Back-Titration Experiments to Estimate the Extent of Formulation Digestion

Fatty acids released during digestion of both MC and LC substrate are likely to be only partially ionized and hence partially titratable at pH 6.5.[31] To determine the total amount of FA released, back titrations were therefore performed at the end of the digestion experiments as described previously.[31,32] In these experiments, at the end of the digestion period, the pH-stat was programmed to rapidly add 1 M sodium hydroxide to the reaction vessel to increase pH to 9. The volume of sodium hydroxide added (corrected for the amount required to increase pH to 9 in the absence of lipid substrate in separate control experiments) was used to calculate the total amount of unionized FA present. The total quantity of liberated FA (i.e., the quantity of ionized FA originally titrated at pH 6.5 plus the amount of additional FA detected during the back titrations to pH 9) was subsequently compared with the total quantity of FA that was expected to be liberated if the lipid excipients were completely hydrolyzed. This provides a means of estimation of the extent of LBF digestion during the *in vitro* experiments using Eq. 1:

$$\text{Extent of digestion } (\%)$$
$$= \frac{\text{ionized fatty acid} + \text{unionized fatty acid}}{\text{theoretical maximum fatty acid in the LBF}} \times 100$$

(1)

Collection and Separation of Samples Removed During In Vitro Digestion

Digestion samples were immediately treated with a lipolysis inhibitor (5 µL/1 mL of digestion medium of 1.0 M 4-bromophenylboronic acid in methanol) to arrest digestion. Samples were then separated by centrifugation into three phases, namely a floating oily phase, a dispersed aqueous colloidal phase, and a precipitated pellet phase.

To determine the most appropriate means of separating the digestion samples, the separation efficiency of an ultracentrifuge [55,000 rpm (400,000 g), 37°C; Optima XL-200K centrifuge, SW-60 rotor; Beckman, Palo Alto, California] or a bench-top centrifuge [14,800 rpm (21,000 g), 37°C; Fresco 21 Heraeus®, Thermo Scientific, Langenselbold, Germany] was compared. The minimum sample volumes for the ultracentrifuge and bench-top centrifuge were 4 and 1 mL, respectively. The centrifugation time was constant at 30 min.

The digestion phases obtained after centrifugation were recovered according to a method described previously[28] with some modifications. The oily phase was aspirated using an adjustable pipette and transferred to a 5 mL volumetric flask that was made up to volume with chloroform–methanol (2:1, v/v). To avoid the risk of contamination of the aqueous phase with the oil, the aqueous phase was recovered directly by piercing the sample tubes with a 23 G needle–5 mL syringe assembly. Recovered aqueous phase was transferred to clean glass vials and temporarily stored at 37°C prior to dilution. To recover the pellet, tubes were cut after aspiration of the oil phase and removal of the aqueous phase to allow the pellet to be obtained free of contamination from residual amounts of oil phase that might remain around the top of the tube. The pellet was then suspended in the sectioned tube with 100 µL chloroform–methanol (2:1, v/v), transferred to 5 mL volumetric flasks, and made up to volume with methanol.

Aliquots (50–100 µL) of the recovered aqueous colloidal phase and the chloroform–methanol solutions of the oil and pellet phases were diluted further (>10-fold) with methanol, before samples were centrifuged [14,800 rpm (21,000 g), 20°C] and analyzed for danazol content by HPLC (see the section *HPLC Detection of Danazol* below for details).

Determination of Oil Droplet Size of Dispersed Formulations

The mean particle size of the dispersed formulations was determined by dynamic light scattering using a Malvern Zetasizer NanoZS (Malvern Instruments, Worcestershire, UK). Formulations were dispersed in the digestion medium (previously passed through a 0.22 µm filter to remove any dust particles) and allowed to equilibrate for 30 min. Measurements of particle diameter were performed at 37°C in triplicate.

HPLC Detection of Danazol

All HPLC analyses were conducted using a Waters Alliance 2695 Separation Module (Waters Alliance Instruments, Milford, Massachusetts) with a reverse-phase C_{18} column [150 × 15 mm², 5 µm; Waters Symmetry® (Waters Alliance Instruments)] and C_{18} security guard cartridge (4 × 2.0 mm²; Phenomenex, Torrance, California). The injection volume was 50 µL and UV detection was at 286 nm. The mobile phase consisted of methanol and water in a 75:25 (v/v) ratio and was pumped through the column at a 1 mL/min flow rate. Validation details for the danazol assays have been previously reported.[19]

RESULTS

Danazol Equilibrium Solubility in the Series of LFCS LBFs

The eight LBFs investigated were chosen to provide examples of all classes of formulations currently contained within the LFCS.[2] As a result, investigation of their solvent capacity and properties during
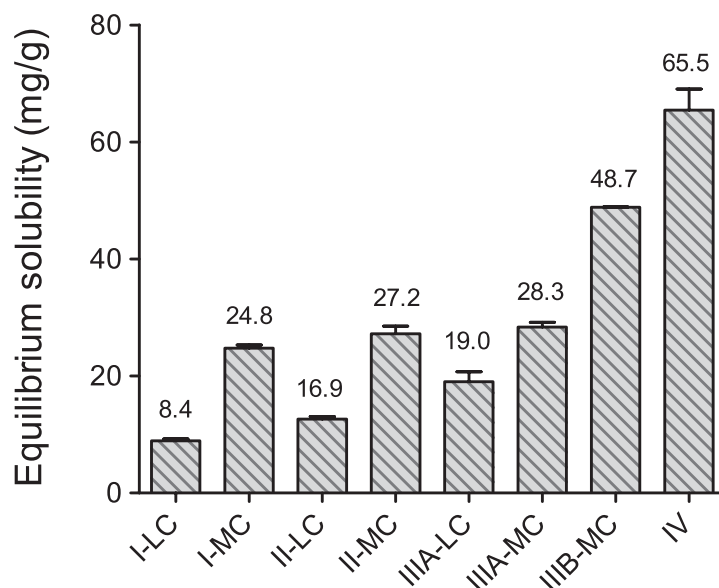
Figure 1. Danazol equilibrium solubility values in the eight investigated lipid-based formulations. Solubility determinations were determined at 37°C over 72 h. Values are expressed as means ($n = 3$) $\pm$ 1 SD. The formulation compositions can be found in Table 2. MC, medium chain; LC, long chain.

dispersion and digestion was expected to provide a broad indication of the likely performance of a range of LBFs.

The equilibrium solubility of danazol in the eight LBFs is shown in Figure 1. A trend toward increasing solubility with increasing polarity of the formulation was observed, giving rise to a wide range of solubility values. The lowest solubility was 8.4 mg/g (milligram danazol per gram of formulation) in the Type I-LC formulation, whereas the highest solubility of 65.5 mg/g occurred in the type IV formulation. Consistent with previous studies,[13,14,16,33] danazol showed greater solubility in MC lipid formulations when compared with equivalent formulations containing LC lipids. The addition of a surfactant [Tween 85 (Sigma) or Cremophor EL (BASF Corporation, Washington, New Jersey)] to LC lipid mixtures (corn oil–Maisine™ 35-1; Gattefossé, St. Priest, France) led to an increase in the amount of danazol dissolved, whereas the same change in MC formulations had only a marginal effect on equilibrium solubility. These trends reflect the smaller difference in danazol solubility between the two surfactants and MC lipid when compared with LC lipid. Danazol solubility in the cosolvent contained in the type IIIB-MC and type IV formulations (Transcutol HP; Gattefossé) was high, resulting in significant increases in solubility in these formulations.

Danazol solubility in excipient alone was utilized to explore whether it was possible to predict drug solubility in complex formulations on the basis of values obtained in the individual components. Danazol solubility values in the individual excipients are presented in Figure 2 with the results arranged in
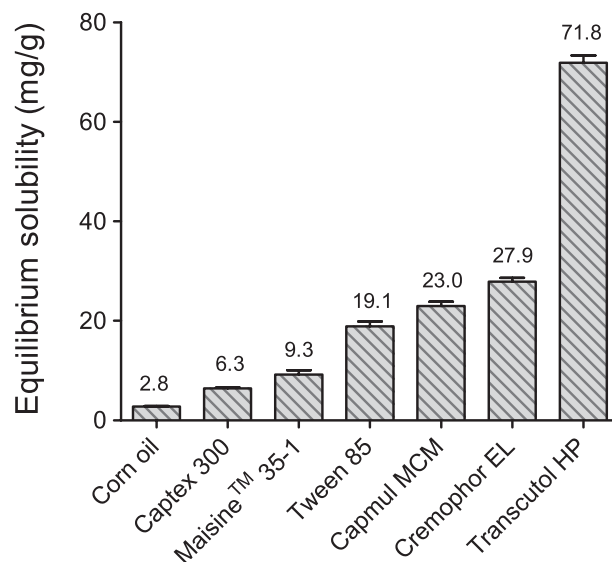

Figure 2. Danazol equilibrium solubility values in the lipid excipients utilized in the construction of the investigated lipid-based formulations. Solubility determinations were determined at 37°C over 72 h. Values are expressed as means ($n = 3$) $\pm$ 1 SD.

order of ascending solubility. In light of the trends in danazol solubility in the eight LBFs, it was unsurprising that danazol solubility was lowest in triglyceride (both MC and LC) and highest in the cosolvent Transcutol HP (Gattefossé). However, calculated (or predicted) solubilities in the eight LBFs [based on (1) danazol solubility in the individual excipients and (2) the mass ratio of excipients in the formulation] were all lower than the measured solubilities in each

formulation. For instance, danazol solubility in the individual components of the type IIIA-MC formulation [Captex 300 (Abitec Corporation, Columbus, Ohio)/Capmul MCM (Abitec Corporation)/Cremophor EL (BASF Corporation)] were 6.3, 23.0, and 27.9 mg/g respectively, providing a calculated solubility value of 19.3 mg/g. In contrast, the measured solubility in the formulation was 28.3 mg/g, and therefore, 46.6% higher than the predicted solubility value. In other cases (e.g., type I and II MC formulations), the measured solubility in LBFs was more than 50% higher than predicted values (the% difference between measured and calculated solubilities for all lipid formulations is available in the *Supplementary Material*).

Effect of Lipid Chain Length and Formulation Type on the *In Vitro* Performance of LBFs

Dispersion Properties and Titration of Liberated FA During In Vitro Digestion

The properties of the eight LBF on dispersion are summarized in Table 2. Type I lipid formulations are simple solutions of drug in oils and represent the most lipophilic of the LBFs in the LFCS.[2] In general, type I formulations, which lack true surfactants, disperse poorly. In some cases, however, the presence of more polar oils such as MC monoglycerides (which can be classified as either polar oils or very low HLB surfactants) facilitates the formation of a crude dispersion on agitation. The type I-MC formulation consisted of a mixture of triglyceride (Captex 300; Abitec Corporation) and mixed monoglycerides and diglycerides (Capmul MCM; Abitec Corporation), and provides an example of a formulation of this type. During the initial dispersion phase (predigestion), the type I-MC formulation formed a turbid and unstable opaque/white emulsion on mixing with the digestion medium. The type II-MC and IIIA-MC formulations contained surfactant [Tween 85 (Sigma) or Cremophor EL (BASF Corporation), respectively] and the IIIB-MC type also contained a cosolvent (Transcutol HP, Gattefossé), and showed evidence of spontaneous emulsion formation on addition to the digestion medium. The mean particle size of the dispersed type IIIA-MC and IIIB-MC formulations were 29.1 ± 0.6 and 21.4 ± 0.5 nm, respectively (Table 2), and therefore consistent with good self-emulsification properties and the formation of ultrafine dispersions. In contrast, the comparatively poorer dispersion properties of the type II-MC formulation precluded accurate particle size measurement at the concentrations used during *in vitro* digestion experiments. A mean particle size of 190.2 ± 2.7 nm was measured on further dilution (1 in 250). The effect of further diluting type IIIA-MC and IIIB-MC dispersions was limited (Table 2) indicating that the phases formed on dispersion were robust to increasing dilution.

For the LC formulations, the type I-LC formulation [comprising corn oil and Maisine™ 35-1 (Gattefossé)] slowly dispersed to form a coarse emulsion emphasizing the poor emulsification properties of both type I formulations, and in particular LC type I formulations. However, consistent with the MC formulations, the addition of surfactant to the type II and IIIA formulations enhanced emulsification properties significantly. An opaque/coarse emulsion was observed in the case of the type II-LC formulation, and a near translucent dispersion of 61.8 ± 0.4 nm particle size showing typical Tyndall-effect scattering was evident in the case of the type IIIA-LC formulation. The particle size of type IIIA-LC dispersions were unaffected by dilution (Table 2). The type IV formulation, which was essentially lipid free (containing only surfactant and cosolvent), dispersed to form an optically clear solution, but one containing particles (presumably micelles) of approximately 13.1 ± 0.4 nm diameter.

Titration profiles for ionized FA are shown in Figures 3a and 3b and describe the *in vitro* digestion of the MC and LC formulations, respectively. Titratable FA corresponds to FA released from the LBF (in response to digestion by pancreatic enzymes) that is ionized at experimental pH (pH 6.5). For the MC formulations, the rate of liberation of titratable FA was most rapid during the first 5 min (Fig. 3a), whereas levels of FA liberated during the remainder of the experiment were comparatively low. This was particularly true for type II and IIIA MC formulations that showed a marked decrease in FA titration after approximately 2–3 min. A more prolonged titration profile was observed for the more lipid-rich type I-MC formulation.

Despite poor dispersibility, the type I-LC formulation was rapidly digested between 0 and 5 min (Fig. 3b), although digestion reduced significantly thereafter. Notably, this profile shape was also characteristic of the digestion of the type II-LC formulation. In contrast, the type IIIA-LC formulation resulted in continuous liberation of FA over a approximately 20-min time period, and as a result, the level of titrated FA liberated from this formulation was much higher than the type I and II LC formulations despite containing similar quantities of lipid substrate.

Determination of FA Ionization and Calculation of True Extent of LBF Digestion

Quantifying digestion via titration of FA at pH 6.5 is restricted by the potential for incomplete FA ionization. This is exacerbated by the realization that the apparent pK_a of FAs in micellar solution is higher than that of a typical carboxylic acid.[34,35] Therefore, to quantify the total extent of FA digestion, complete ionization of liberated FA was forced via rapid elevation of the pH of the digest to pH 9 at the end of the experimental period level as reported previously.[31,32]
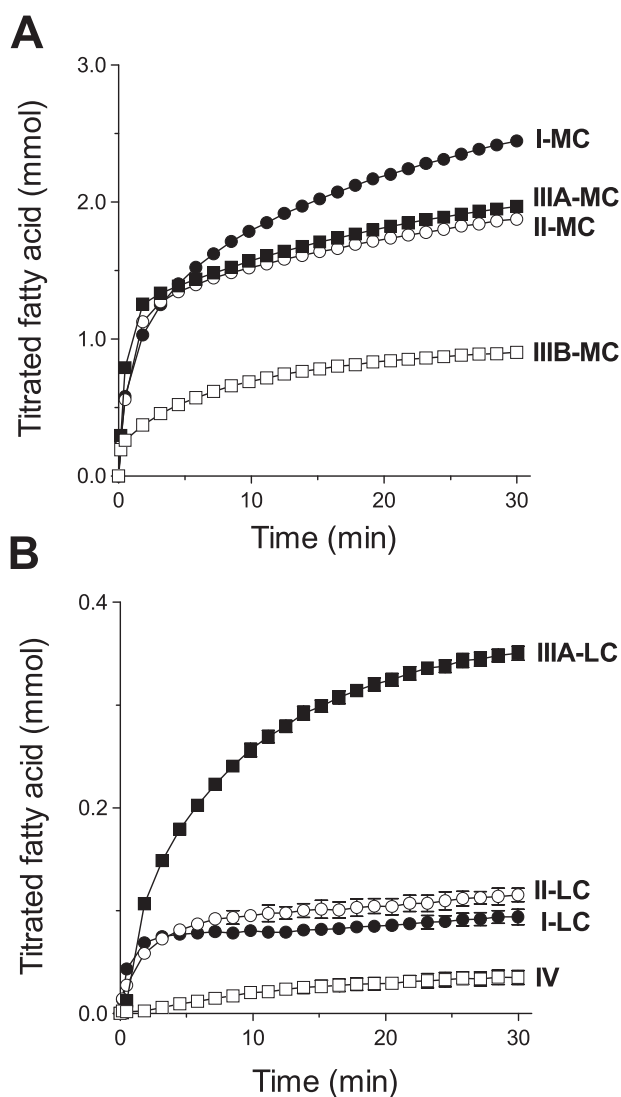
Figure 3. Apparent titration of fatty acids (FAs) released during *in vitro* digestion of (a) medium-chain lipid-based formulations (LBFs) and (b) long-chain LBFs. Digestion was initiated at $t = 0$ min on addition of pancreatin, and pH was maintained constant at pH 6.5. Titrated FA was detected by pH titration, and values are expressed as means ($n = 3$) $\pm$ 1 SD and have been corrected for the level of FA released in background digestion tests (no lipid formulation). The formulation compositions can be found in Table 2.

Comparison of the total quantity of titratable FA at pH 9 and 6.5 allows estimation of the degree of ionization at pH 6.5. Assuming constant FA dissociation throughout the *in vitro* digestion experiment, the titration data obtained by measuring ionized FA can be corrected for the degree of ionization to provide an indication of total (ionized plus unionized) FA production at pH 6.5. This approach is necessary because experiments could not be performed at pH high enough to allow complete FA ionization without risk of compromising the activity of the porcine pancreatic extract[31] or introducing the added complication

of the conduct of experiments under nonphysiological conditions. The results obtained for all eight formulations are summarized in Table 3. To further aid the description of the results, in Figure 4, the comparative levels of ionized/unionized/total FA are depicted for the digestion of a type IIIA formulation. The levels of unionized (dashed line) or total FA (dotted line) are estimated values based on the unionized-to-ionized FA ratio determined at 30 min.

Unionized–ionized FA ratios below unity indicate that the major population of liberated FA on digestion was ionized at pH 6.5. This was the case for all MC formulations (Table 3), although the unionized–ionized FA ratio was not constant across all the MC formulations. Instead, the ratio progressively decreased with decreasing LBF lipophilicity. The same trend of decreasing FA ionization with increasing LBF lipophilicity was also observed for the LC formulations. In this case, the trend was more pronounced and unionized-to-ionized ratios were much higher such that the majority of FA released during digestion of LC formulations at pH 6.5 was unionized (Table 3). The variation in ionization of MC and LC FA (during *in vitro* digestion) is illustrated in Figure 4 wherein the FA released on digestion of a type IIIA-MC formulation was predominately ionized at pH 6.5 ($\sim$75%), whereas at the same pH, the FAs released from the equivalent type IIIA-LC formulation were approximately 75% unionized. This incongruence in the degree of ionization of MC and LC FAs was also evident for type I and II formulations.

Total FA titrated over 30 min followed the rank order type I-MC > II-MC = IIIA-MC > IIIB-MC, reflecting the amount of available lipid substrate in the MC formulation (Table 2). The quantity of FA liberated from the LC formulations was lower than the equivalent quantities liberated from the corresponding MC formulations (Table 3), and is likely to be due in part to the smaller quantity (moles) of available FA in the LC formulations (that contained higher molecular weight lipids). The rank order in total FA released from LC formulations was type III > type II > type I, which is better correlated with the efficiency of formulation emulsification than the amount of available FA (cf. MC formulations).

Fatty acid liberation from the type IV formulation was very low (less than 0.1 mmol) and consistent with the lack of lipid source in this formulation other than that derived from digestion of the included surfactant (Cremophor EL; BASF Corporation).

The maximum theoretical quantity of digestible lipid (in FA equivalents) in each LBF was calculated using the composition of the lipidic excipients shown in Table 1, and assuming that on digestion, one triglyceride molecule released two FA molecules and that one molecule of diglyceride or monoglyceride (initially present in the formulation) liberated a

Table 3.   Quantification of Ionized and Unionized Fatty Acid (FA) Liberation Following 30 min Digestion of a Range of LBFs

Formulation Type	Total FA Released[a] (mmol)	Unionized–Ionized FA Ratio	Lower Theoretical Maximum FA Level (mmol)[b]	Upper Theoretical Maximum FA Level (mmol)[c]	Extent of Formulation Digested (%)[d]
I-MC	3.51	0.43	3.91	–	90
II-MC	2.54	0.36	2.54	3.11	82–100
IIIA-MC	2.66	0.35	2.54	2.96	90–105
IIIB-MC	1.12	0.23	0.96	1.57	71–117
I-LC	0.55	5.0	2.20	–	25
II-LC	0.53	3.5	1.43	2.00	27–37
IIIA-LC	1.36	2.9	1.43	2.04	67–95
IV	0.09	1.3	–	0.61	15[e]

Estimations for the extent of total digestion are also provided.

[a]Titrated FA at pH 6.5 plus FA that is unionized (and not quantified) at pH 6.5, but ionized and quantified by back titration at pH 9.

[b]Potential FA derived from complete formulation digestion (FA from surfactant not included).

[c]Potential FA derived from complete formulation digestion (FA from surfactant included).

[d]Values for the extent of digestion of type II and IIIA/B formulations are provided as a range spaning values calculated using lower and upper theoretical maximum FA levels as described above.

[e]Type IV formulations do not contain traditional lipids, hence surfactants are the only digestable components.

single FA molecule.[28,33,36] Values for total digestible lipid for each formulation are presented in Table 3. The estimated extent of formulation digestion for the evaluated formulations over 30 min was calculated using the Eq. 1.

Using this approach, type I-MC formulations were approximately 90% digested under the current experimental conditions. This value corresponds well with visual observation because only a small remaining oil droplet was observed following ultracentrifugal separation of digestion samples removed at the end of the experiment. Calculating the extent of digestion for the remaining MC formulations was complicated by the presence of surfactant that also has the potential to liberate titratable FA. Enzymatic hydrolysis of surfactant is thought to be largely mediated via CEH and pancreatic lipase-related protein 2 (PLRP 2) rather than the classical colipase-dependent pancreatic lipase.[20,31,37,38] However, the relative contribution of surfactant and lipid to the overall digestion profiles is unknown, and difficult to assess in control experiments because accurate levels of CEH in porcine pancreatin are not available. In the case of PLRP 2, the enzyme is present in human pancreatic juice[39] but has not yet been identified in porcine-derived pancreatin.[40] Estimates of the likely range of total percent digestion can therefore be made on the basis of two extremes, namely (1) no surfactant digestion and (2) complete surfactant digestion. On the basis of previous work that has shown that commonly used surfactants are digested *in vitro*,[20,31] and the visual observations made in the present study, it seems likely that the surfactants were digested to some extent, and therefore, the percent digested for the surfactant-containing LBFs will lie within the ranges suggested above. Under these circumstances, the extent of formulation digestion was 82%–100%, 90%–105%, and 71%–117% for type II-MC, IIIA-MC,

and IIIB-MC formulations, respectively (Table 3). The data therefore suggest approximately complete digestion for type II-MC and IIIA-MC. The estimated range for the type IIIB-MC formulation is wide because of the high proportion of surfactant in the formulation. Analysis of the type IV formulation, which contains Cremophor EL (BASF Corporation) as the only source of FA, revealed approximately 15% digestion. Carrying forward an assumption of a similar extent of Cremophor EL (BASF Corporation) digestion for the type IIIB-MC formulation results in an estimation of 105% for the extent of digestion of this formulation type. This corresponds well with the observed digestion profile that suggests no on-going liberation of FA for the latter period of the experiment (Fig. 3a).

Estimations of the extent of LC formulation digestion were 25% for the type I-LC systems, and (again providing a range based on zero—complete surfactant digestion) between 27%–37% and 67%–95% for the type II-LC and IIIA-LC formulations, respectively. The LC formulations were therefore only partially digested under the conditions tested.

Titration Efficiency of LBF-Derived FA

Comparison of the extent of measured digestion at pH 6.5 and back-titration data at pH 9 revealed that *in vitro* digestion of LC formulations resulted in liberation of FA that was only partially (>26%) ionized at pH 6.5. To illustrate the variation in FA ionization as a function of pH when assessing the extent of digestion, the digestion of a type IIIA-LC formulation was compared across a narrow range surrounding the target pH (pH 6.5). The data obtained are presented in Figure 5. As expected, levels of ionized FA (Fig. 5a) were highly dependent on the experimental pH, with an observable trend toward increasing titration with increasing pH. However, following back titration to
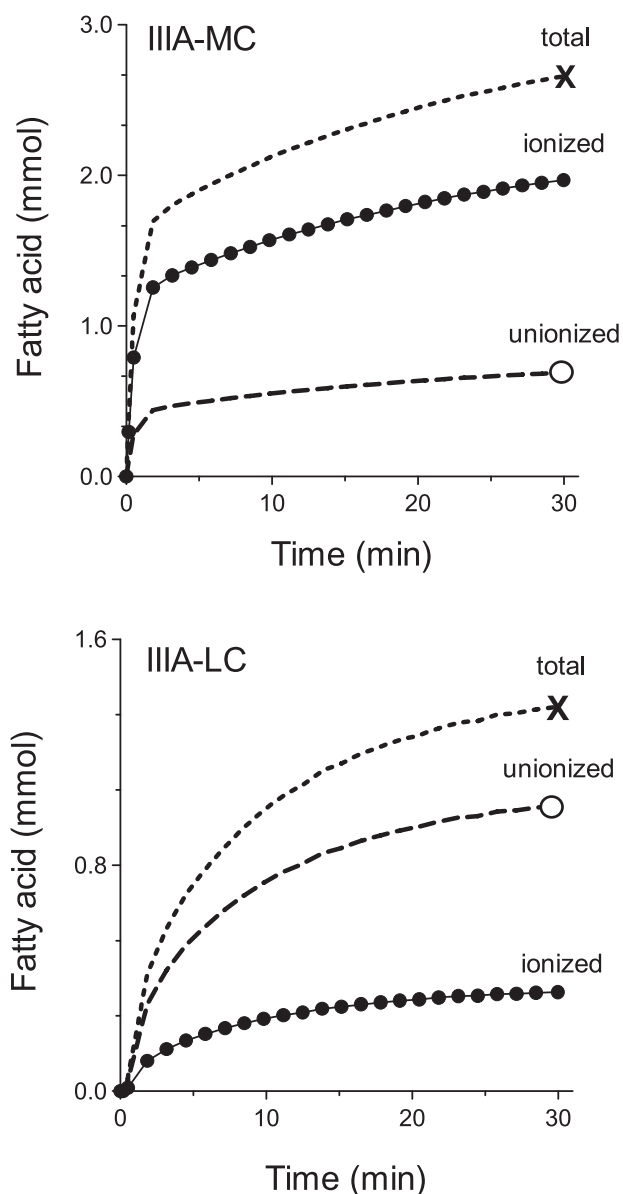
Figure 4. Levels of fatty acid (FA) released during *in vitro* digestion of type IIIA formulations containing either medium-chain or long-chain lipids. Plots for ionized FA were experimentally determined over 30 min by pH-stat titration at pH 6.5. Plots for total FAs (dotted lines) was deduced from back titration of total FAs at pH 9.0 and the unionized-to-ionized FA ratio calculated at 30 min. Plots for unionized FA (dashed lines) were estimated from the difference between total FAs and FAs, and assumes that the ratio of unionized to ionized FA was constant during the experiment.

pH 9 and subsequent total ionization of the FAs present and determination of the total quantity of FA liberated during the digestion process, it was apparent that the level of digestion was highly consistent across this small pH range (Fig. 5b).

Interlaboratory Comparison of *In Vitro* Digestion of LBFs Using Standardized Test Conditions

Equivalent *in vitro* digestion experiments were performed at Monash and Copenhagen to determine whether consistent results could be obtained using the standardized preliminary test conditions proposed by the LFCS Consortium. Experiments were performed at both sites according to the protocol described in the section *In Vitro Evaluation of the LBFs*. For consistency, the same batches of excipients were also employed (Table 1).

Comparisons of the digestion profiles obtained for MC and LC formulations are shown in Figures 6 and 7, respectively. Comparisons were made using titratable (ionized) FA at pH 6.5 (as opposed to total FA) because this was considered to be the more sensitive indicator of differences in digestion conditions. The small error (standard deviation) bars for both MC and LC formulations in Figures 6 and 7 suggest that the digestion of the LBFs was highly reproducible when tested in either laboratory. Some error in the results obtained in the digestion of type IV formulations was evident in both laboratories, but can be attributed to the very low amounts of FA detected from this formulation. The rate and extent of digestion of all MC-lipid formulations tested at Monash and Copenhagen were essentially identical as evidenced by extensive overlapping of the titration profiles in Figure 6. Digestion profiles for LC formulations were also highly consistent (Fig. 7), although some small differences in initial rate of digestion of type II-LC formulations were observed.

Evaluation of the Utility of Ultracentrifugation Versus Bench-Top Centrifugation in the Separation of *In Vitro* Digestion Samples

The fate of an incorporated drug with respect to the phases formed during dispersion and digestion of a LBF (and in particular the propensity for drug precipitation) provides an indicator of the potential utility of LBF in aiding oral absorption of poorly water-soluble drugs. Traditionally, ultracentrifugation has been employed to separate digestion samples into three discrete phases, namely (1) a poorly dispersed oily phase, (2) an aqueous colloidal phase, and (3) a pellet phase (containing any solid precipitated drug).[11,13,14,16,18,28,33,41–44] However, the use of bench-top centrifugation would significantly enhance throughput of *in vitro* digestion experiments and was therefore examined.

The distribution pattern of the model poorly soluble drug danazol across these digestion phases following 30-min digestion of the eight LBFs and subsequent separation of digestion samples by ultracentrifugation [55,000 rpm (400,000*g*)] is shown in Figure 8.
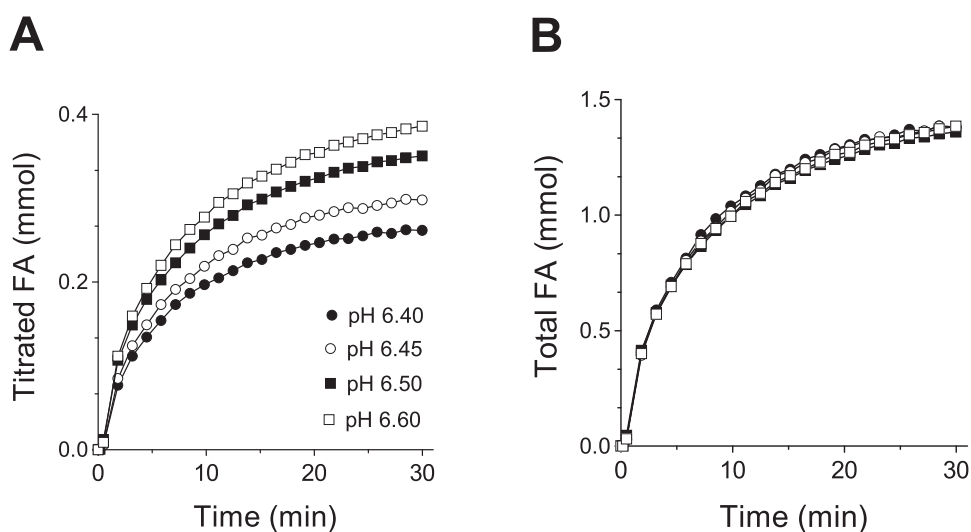
A

B

Figure 5.   The effect of a progressive change in experimental pH on the *in vitro* digestion of a type IIIA long-chain formulation. (a) The level of fatty acid (FA) titrated (ionized) and (b) the total amount of formulation digested at the pH shown in the legend.
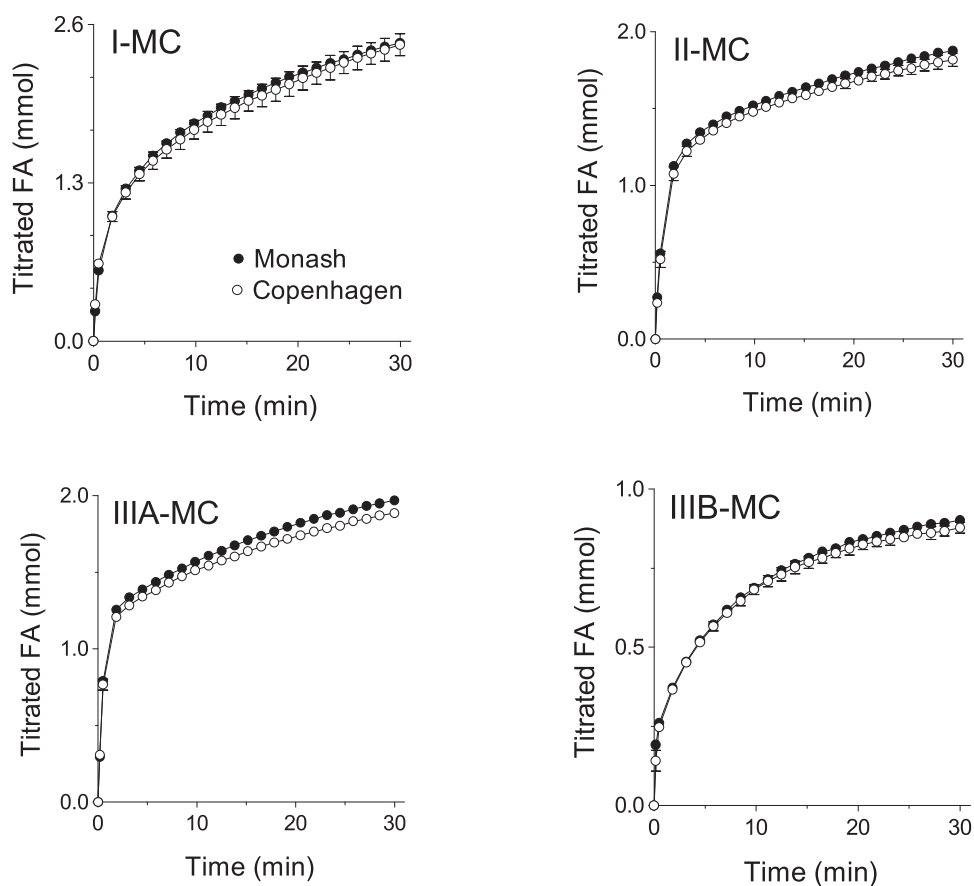

Figure 6.   Fatty acid (FA) titrated in a pH-stat apparatus during *in vitro* digestion of medium-chain lipid-based formulations tested in Monash and Copenhagen laboratories. Digestion was initiated at $t = 0$ min on addition of pancreatin, and pH was maintained constant at pH 6.5. Titrated FA values are expressed as means $(n = 3) \pm 1$ SD and have been corrected for the level of FA released in background digestion tests (no lipid formulation). The formulation compositions can be found in Table 2.

Figure 7. Fatty acid (FA) titrated in a pH-stat apparatus during the *in vitro* digestion profiles for long-chain and type IV lipid-based formulations tested in Monash and Copenhagen laboratories. Experiments were conducted at pH 6.5. Titrated FA values are expressed as means ($n = 3$) $\pm$ 1 SD and have been corrected for the level of FA released in background digestion tests (no lipid formulation). The formulation compositions can be found in Table 2.
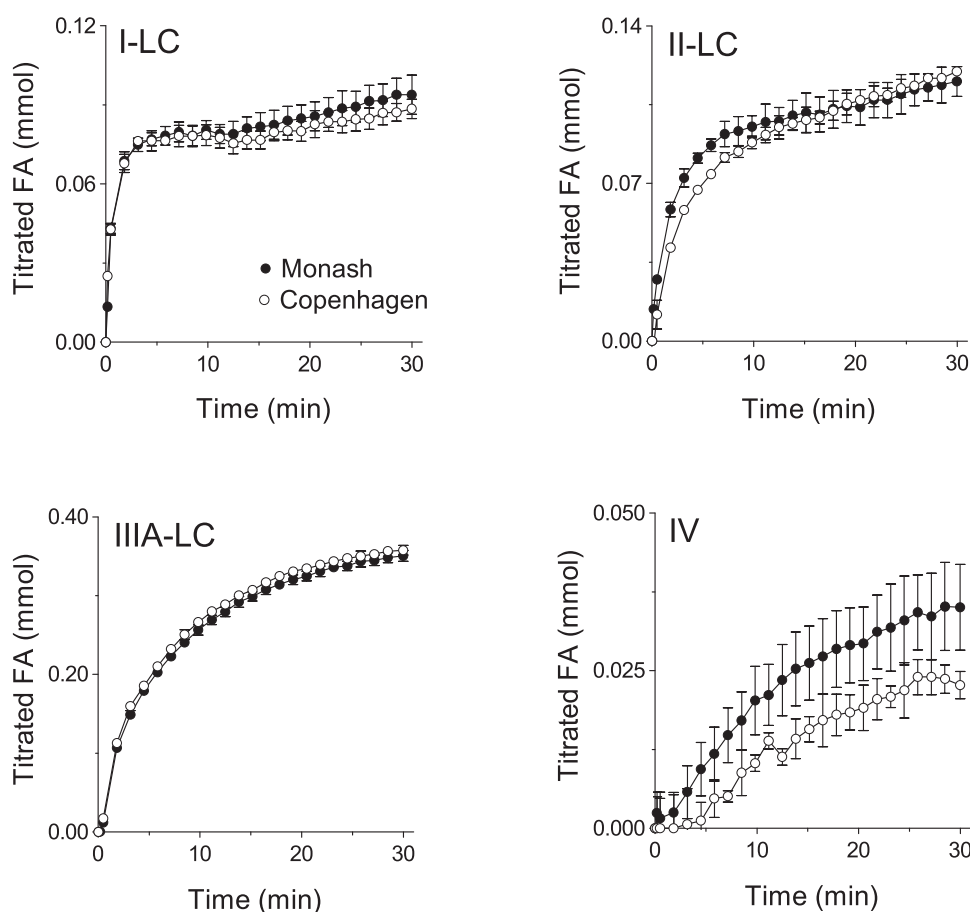
To determine whether ultracentrifugation was critical to assessing LBF performance, an additional sample was also removed at 30 min and separated at a lower speed using a bench-top centrifuge [14,800 rpm (21,000g)]. Drug distribution across the various digestion phases in bench-top centrifuge samples is also presented in Figure 8.

Following separation, samples taken from type I-LC, II-LC, IIIA-LC, and I-MC systems all contained a poorly dispersed oily phase. The effect of separation method on the distribution of danazol across the various digestion phases of these four formulations is shown in the upper panel in Figure 8. As described above, the distribution of danazol between the various digestion phases was formulation dependent; however, consistent results for each formulation were obtained following sample separation via ultracentrifugation or via bench-top centrifugation. For the type II-LC, there was some difference in the amount of danazol recovered in the pellet. Because ultracentrifuged and bench-top centrifuged samples were removed from the same digest at the same time,

this difference must reflect the separation method. In particular, the difference may be attributed to a "gel-like" phase that was present on the surface of the pellet in the samples that were ultracentrifuged, but was largely absent in samples that were separated by bench-top centrifugation. This gel phase was not evident in other formulations, and probably represents partially digested Tween 85 (Sigma) (the surfactant present in the type II formulations) because Tween 85 (Sigma) is denser than water (1.08 g/mL at room temperature). The higher quantity of drug recovered in the pellet in ultracentrifuged samples therefore suggests a greater potential to pellet the surfactant gel (and any associated drug) under the higher centrifugal force conditions in the ultracentrifuge.

For the type II-MC, IIIA-MC, IIIB-MC, and IV formulations, samples taken during the digestion phase showed no evidence of a poorly dispersed oily phase after centrifugation via either method. The distribution patterns of danazol across the aqueous phase and pellet of these formulations are presented in the lower panel in Figure 8. The majority of drug (>50%) for
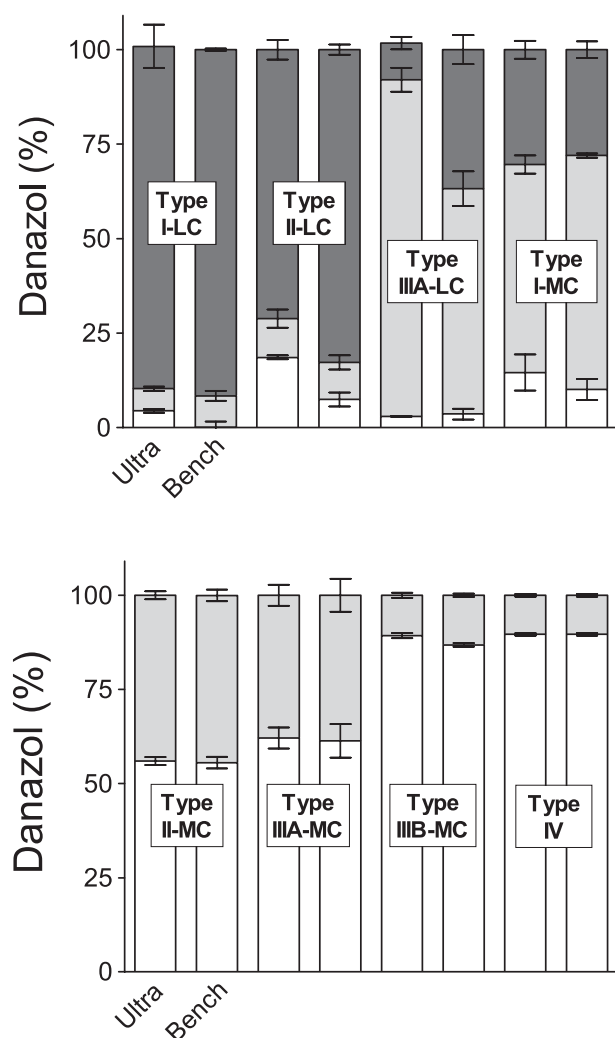
Figure 8. Comparison of danazol distribution across the various digestion phases following sample separation by ultracentrifugation or bench-top centrifugation. Dark-shaded regions show drug in a poorly dispersing oily phase, light-shaded regions show drug in a colloidal aqueous phase, and white regions show drug in the pellet phase. Values are expressed as means ($n = 3$) ± 1 SD. Formulations contained danazol at 80% of the saturated drug solubility in each formulation.

these formulation types was recovered in the pellet phase. Importantly, the proportion of drug recovered the pellet phase was highly consistent regardless of whether samples were separated using an ultracentrifuge or bench-top centrifuge, and for these formulation types, drug distribution and more specifically the level of precipitated drug was adequately captured using the bench-top centrifuge.

For the formulation types wherein a floating oil phase was present, the total drug recovery (i.e., mass balance across oil, aqueous and pellet phases) from the digestion samples separated using a bench-top centrifuge differed somewhat from the theoretical drug content. For example, danazol recovery

was 89.3 ± 6.2% for the type II-LC formulation and 126.7 ± 8.0% for the type IIIA-LC formulation. In contrast, recovery from ultracentrifuge samples varied less across the same formulation types (94.3%–102.0%). Variability in mass balance in samples taken from formulations that did not result in the production of a floating oily phase was lower, and was independent of centrifugation method (87.0%–105.2% and 91.5%–99.3% by ultracentrifugation and bench-top centrifugation, respectively). The greater variability in drug recovery using the bench-top centrifugation was therefore because of difficulty in isolating the oily phase. As described in the section *Collection and Separation of Samples Removed During In Vitro Digestion*, the floating poorly dispersed oily phase was aspirated using an adjustable pipette. In some cases, however, residual oil adheres to the inside of the sample tube and this prevents the oily phase from being completely removed using a pipette. In contrast, soft-walled polyallomer tubes were used in the ultracentrifuge separation method, and following the removal of the aqueous phase (by piercing the wall of the tube), the tubes were sectioned to allow the upper part of the tube to be rinsed with solvent therefore allowing complete removal of any residual oil adhered to the wall of the sample tube. These steps ensured the complete isolation of the three digestion phases and simultaneously minimized the risk of contaminating the aqueous phase or pellet with a potentially drug-rich oil phase. Conversely, the sample tubes used in the bench-top centrifuge were polypropylene and could not be readily punctured or sectioned. Low-volume (and puncturable) polyallomer tubes (1.5 mL) were explored as an alternative, but were not sufficiently robust to withstand centrifugation in the rotor employed and often split. Although broadly similar distribution patterns were therefore evident with both centrifugal separation techniques, the data suggest that for digests wherein an oil-rich phase is evident, the use of bench-top centrifugation is likely to lead to higher errors, and therefore, results obtained using the ultracentrifuge are likely to better reflect the true drug distribution patterns across the various digestion phases.

Danazol Solubilization Patterns Following *In Vitro* Digestion of LBFs

The distribution of danazol across the different phases of the digestion mixture following digestion of the eight LBFs is shown in Figure 8. All the LC formulations and the type I-MC formulation showed evidence of a floating oily phase after separation, consistent with incomplete lipid digestion (Table 3). For the type I and II LC formulations—formulations that showed the lowest extent of digestion, the majority of danazol (>65%–70%) was recovered from the oil phase (i.e., the dark-shaded region in Fig. 8).

In contrast, type IIIA-LC and I-MC systems under the same conditions were more completely digested, and significantly greater quantities of drug were recovered in the solubilized aqueous phase. Corresponding aqueous phases appeared "hazy" for the type I-MC formulation and "milky-turbid" in the case of the type IIIA-LC formulation, suggesting the presence of high concentrations of digested lipids. The solubilized concentrations of danazol in the aqueous colloidal phase for the type IIIA-LC (359.3 ± 8.2 µg/mL) and type I-MC (233.2 ± 31.5 µg/mL) formulations were high, and constituted over 50% of the incorporated dose. The level of precipitated drug recovered in the pellet across the partially digested formulations (i.e., those that showed evidence of an oil phase) was low (<20%).

In contrast, most of the MC formulations (types II, IIIA/B MC) and the IV formulations showed no evidence of an oil phase following 30-min digestion. This observation was consistent with complete digestion of type II, IIIA/B MC formulations (Table 3) and the low level (or absence) of lipid in the type IIIB-MC and IV formulations, respectively. However, although digestion was complete, drug solubilization was poor for the type IIIB-MC, and IV formulations and moderate for the type II-MC and IIIA-MC formulations, and drug precipitation was significant. The level of drug precipitate (as a % of the initial drug load) was approximately 56% and approximately 62% for type II-MC and IIIA-MC formulations, respectively, and more than 90% for the less lipophilic type IIIB-MC and IV formulations. To determine whether this loss of solubilization capacity (as evidenced by drug precipitation) was simply because of dilution effects, samples were removed from the dispersion phase at 10 min (i.e., immediately prior to commencement of digestion) and analyzed for levels of dissolved drug. For the type II-MC and IIIA-MC formulations, there was no evidence of drug precipitation during the initial dispersion phase. In contrast for the type IIIB-MC and IV formulations, more than 70% of danazol had precipitated during the dispersion phase.

DISCUSSION

Several recent studies suggest that changes to the solubilization properties of LBF as they are processed in the GI tract are key determinant of *in vivo* performance.[12,19,20,45] The major changes imparted by GI processing are formulation dilution, formulation digestion, and the interaction of formulation components and digestion products with biliary secretions—all of which are expected to impact on patterns of drug solubilization.[5] Recent studies have suggested that *in vitro* lipid digestion models may be employed to mimic the GI processing of LBF under physiologically relevant conditions, and an increasing number of studies have shown that at least rank-order correlation between patterns of solubilization obtained during *in vitro* digestion and *in vivo* exposure are possible for some compounds.[12,17,18,20,42,45]

Wider application of these test methods, however, has been limited by the lack of a standardized approach and considerable variation is apparent in the values used for several critical test parameters (e.g., test volume, pH, bile salt concentration, buffer, etc.) across different groups. One of the key objectives of the LFCS Consortium has been to establish standardized methods for formulation assessment of LBFs using simple *in vitro* testing protocols. The present submission represents the first in a series of publications to address this goal. Here, the *in vitro* digestion behavior of a standardized series of eight LBFs, each containing danazol as a model poorly water-soluble drug, is described. The formulations have been selected to capture the likely patterns of drug solubilization across a wide range of LBF classes with differing dispersion/digestion properties. A standardized set of digestion conditions have been proposed and utilized, and the impact of alterations in the centrifugation method employed to facilitate separation of samples after digestion has been explored. The importance of pH and the inclusion of a back-titration step (i.e., complete titration of free FA via ionization at elevated pH) on the quantification of digestion across the range of formulations have also been investigated.

Danazol Solubility in Type I, II, IIIA/B, and IV Lipid Formulations

The equilibrium solubility of the model drug danazol in the investigated LBFs (Fig. 1) progressively increased with decreasing lipid content in the formulations, and consistent with previous work,[13,14,16,46] was higher when the included lipid was based on MC FA rather than LC FA. The highest solubility was obtained in the formulations containing Transcutol HP (Gattefossé) as a cosolvent. This is consistent with studies that have reported solubility advantages for Transcutol HP (Gattefossé) when compared with other cosolvents (e.g., ethanol, propylene glycol, polyethylene glycol).[47–49] Danazol solubility in the formulations could not be predicted accurately on the basis of solubility in the individual excipients and measured solubilities were consistently higher than predicted values. Because drug loading capacity is a primary design criteria for many LBFs,[50–52] and the potential to reach a load target is often estimated from drug solubility data in individual excipients, the current data suggest that calculated solubility estimates based on data obtained in individual excipients are conservative, and that measured solubilities may be considerably higher.

Comparative *In Vitro* Digestibility of Type I, II, IIIA/B, and IV Lipid Formulations

Quantification of *in vitro* lipid digestion (as % digested) has been traditionally calculated using the moles of FA detected relative to the moles of FA equivalents in the formulation.[20,28,33,36,53,54] This approach, however, is limited by incomplete FA ionization at GI pH (Table 3 and Fig. 4). Evaluation on the basis of detected (ionized) FA therefore leads to a significant underestimation of the extent of digestion and is complicated by the formulation dependence of FA ionization, and in particular the much lower ionization of LC (when compared with MC) FAs.[34,35,55]

To limit the effect of reduced FA ionization at lower pH, lipid digestion experiments may be conducted at pH toward the upper end of the likely intestinal pH range (e.g., pH 7.5). In the current studies, however, pH was instead maintained at levels more representative of upper small intestinal pH[56,57] (pH 6.5) and unionized FA quantified by back titration. During back titration, the pH of the digest is rapidly increased to pH 9, creating conditions favoring complete ionization. Comparison of the extent of titration before and after pH shift subsequently provides a measure of differential ionization that can be used to correct digestion calculations.[31,32]

For the LC lipid formulations, levels of unionized FA were highly significant at pH 6.5 and exceeded that of ionized FA (Table 3). FA ionization was also lower during the digestion of the more lipophilic type I and II systems, consistent with less effective FA solubilization, and more constrained access to ionization sites when emulsified in larger colloidal droplets. Determination of total (ionized and unionized) FA using back titration therefore allowed much better estimation of formulation digestion. Using these methods, the extent of digestion of the type I-LC formulation was 25% and slightly higher for the type II-LC formulation [27%–37% depending on estimates of the expected maximum extent of FA liberation from Tween 85 (Sigma) surfactant] (Table 3). Digestion profiles are shown in Figure 3 and suggest that ongoing digestion of type I-LC and II-LC formulations was not limited by the duration of the experiment, and that continuing digestion was more likely limited by the solubilization capacity of the digestion media for the LC digestion products generated.[28,58,59] In the absence of the absorption sink that is present *in vivo*, the *in vitro* digestion of LC lipids is limited by the build up of poorly water-soluble digestion products on the droplet surface, effectively "poisoning" further enzyme binding and digestion.[7,59,60] As a result of incomplete digestion, samples from type I-LC and II-LC digestion tests contained significant quantities of a poorly dispersed oily phase, which likely comprised undigested formulation and some phase-separated digestion products.[28] In contrast, the type IIIA-LC formulation showed greater evidence of prolonged and more efficient digestion (between 67% and 95%). The greater level of digestion may be attributed to (1) the enhanced dispersion properties of the type IIIA-LC system (the average oil droplet particle size was 61.8 ± 0.4 nm following dispersion in the digestion medium) leading to higher levels of available lipid surface area for enzyme binding,[61,62] (2) improved solubilization of lipid digestion products by Cremophor EL (BASF Corporation) (contained in the type IIIA-LC formulation) compared with Tween 85 (Sigma) (in the type II-LC formulation), or (3) differences in enzyme inhibition mediated by the two surfactants.[37] Major differences in direct effects of Cremophor EL (BASF Corporation) and Tween 85 (Sigma) on enzyme activity were considered unlikely, however, because little difference in digestibility of the equivalent type II-MC and IIIA-MC formulations was apparent (Fig. 3a).

For the MC lipid formulations, the rate and extent of digestion of MC formulations was high and far less dependent on formulation–dispersion properties because the addition of surfactant had less effect on digestion when compared with addition of the same surfactant to the equivalent LC formulations and digestion of II-MC and IIIA-MC formulations was consistent despite differences in particle size (Table 2). For the MC systems, the greater intrinsic solubility of MC digestion products (when compared with the LC equivalents) is likely to have promoted the trafficking of digestion products away from the droplet surface, reducing the need for surfactant-mediated solubilization to maintain digestion. The presence of increased solubilization power therefore had little impact on digestion and the quantity of liberated FA was instead more highly correlated with the quantity of lipid in the formulation. The data indicate that the digestibility of a LBF is often independent of dispersion properties and instead is better correlated with lipid chain length, and more specifically, the fate of the lipid digestion products. Where digestion products readily enter the aqueous colloidal phase (e.g., in the case of MC formulations), digestion is rapid, complete, and formulation independent, whereas for lipids with less water-soluble digestion products, digestion is slower, incomplete, and significantly enhanced by the addition of excipients that promote solubilization.

Although pancreatic lipase is essential to the digestion of triglycerides and displays high specific activities toward both triglycerides and diglycerides, human pancreatic lipase has no or weak activity on monoglycerides.[63,64] This has been suggested to reflect the greater affinity of pancreatic lipase for substrates forming an oil-in-water interface. The more polar monoglycerides have the propensity to form

micellar structures and remain dispersed in solution, whereas the pancreatic lipase binds at the oil–water interface.[31,38] In contrast, CEH and PLRP2 have a reverse affinity for lipidic substrates and show superior activity toward monoglycerides[63,64] and digestible surfactants such as Labrasol and Gelucire[31,38] (Gattefosse). In the current studies, differing enzymes are therefore likely to have been involved to varying degrees in the digestion of type I–IV formulations. Nonetheless, the porcine pancreatic extract utilized here contains both the classical colipase-dependent pancreatic lipase (PPL) and CEH, and is therefore expected to provide representative digestive activity against a range of lipidic and surfactant substrates.

Danazol Solubilization on *In Vitro* Digestion of Type I, II, IIIA/B, and IV Lipid Formulations

The solubilization behavior of danazol, a model lipophilic drug ($\log P = 4.5$), after dispersion and digestion of the series of LFCS formulations was highly dependent on formulation type. Notably, however, solubilization patterns were largely independent of simple descriptors of formulation performance such as digestibility or degree of initial dispersion. For the most hydrophilic type IV and IIIB-MC formulations, wherein initial dispersion and subsequent digestion were rapid, drug precipitation was evident even on dispersion and was almost complete by the end of the 30-min digestion period. The loss of solubilization capacity resulting from surfactant and cosolvent dilution, and the limited quantity (or absence) of lipid digestion products to assist ongoing solubilization, therefore reduced formulation performance. For the type II-MC and IIIA-MC formulations, solubilization properties were somewhat improved, although precipitation remained significant. These formulations were rapidly and completely digested; however, the aqueous phase generated retained limited solubilization capacity, reflecting the lower ability of MC digestion products to swell micellar structures (compared with LC equivalents) and improve the solubilization capacity of endogenous mixed micelles.[65]

For the LC formulation, the patterns of *in vitro* solubilization were complicated by incomplete formulation digestion. For the poorly dispersed and most lipophilic type I-LC and II-LC formulations, an oil phase persisted after digestion, resulting in drug retention within the incompletely digested and dispersed oil phase. This is in line with previous studies,[14,17,45] and reflects a combination of incomplete digestion under *in vitro* conditions (Table 3) and relatively poor emulsification properties. In comparison, the type IIIA-LC formulation dispersed rapidly and effectively to form submicron-sized emulsion droplets and was very effectively digested under the current conditions. Unlike the well dispersed MC formulations, however, digestion of the type IIIA-LC formulation did not result in a decrease in solubilization properties and precipitation was negligible.

In contrast to the other MC formulations and the type I-LC formulation, the type I-MC formulation resulted in an incompletely dispersed "oil" phase after digestion; however, digestion was largely complete and the "oil" phase therefore contained phase-separated digestion products rather than incompletely digested formulation. The oil phase was also apparent only after centrifugation and was readily redispersed, suggesting the presence of larger emulsified lipid droplets or vesicles that "creamed" on centrifugation rather than a phase-separated oil phase. Unlike the other MC formulations drug precipitation from the type I-MC formulation was limited. The large quantity of MC lipid present in the type I formulation, coupled with near-complete digestion, therefore appears to have resulted in the generation of an aqueous phase rich in lipid digestion products that maintain drug solubilization, but that are also of sufficient large particle size to cream or float on centrifugation, providing the visual impression of phase separation into an oil phase (suggesting incomplete digestion).

Although the current study is the first to specifically compare *in vitro* digestion performance across the complete range of LFCS formulation types, the data obtained are consistent with much of the available literature. Thus, Mohsin et al.[46] showed using fenofibrate as a model drug that type IIIB-MC and IV systems were prone to drug precipitation following simple dilution, and that type II-MC and IIIA-MC systems were more able to resist precipitation on dispersion. Cuine et al.[19] showed that danazol solubilization following digestion of a type IIIA-LC formulation, very similar to that employed here [30% soybean oil, 30% Maisine[TM] 35-1 (Gattefossé), and 40% Cremophor EL (BASF Corporation)] but utilizing slightly different experimental conditions, was able to maintain drug solubilization after digestion, much more effectively than a lipid-free type IV formulation [65% Cremophor EL (BASF Corporation) and 35% ethanol]. This marked difference in *in vitro* performance was later correlated with bioavailability after formulation administration to (fasted) beagle dogs, where danazol exposure was approximately 3.2-fold higher in the case of the type IIIA-LC formulation. Differences between type IIIA MC and LC formulations were also examined by Porter et al.[16] and consistent with the data reported here, the MC formulations resulted in significantly greater precipitation on *in vitro* digestion when compared with equivalent type IIIA-LC formulation and this again translated to differences in *in vivo* exposure.

The impact of drug precipitation during dispersion and digestion of lipid formulations on drug absorption may also be dependent on the physical form of the precipitate. In the event that the precipitate is amorphous,[11,66] rapid redissolution may be sufficient to replenish the decrease in solubilized drug concentrations caused by precipitation. In the case of danazol, previous work has shown that precipitates analyzed following *in vitro* digestion LBFs contain crystalline drug.[67,68] Where possible, however, the physical form of any precipitate formed during lipid digestion experiments might usefully be checked.

Evaluation of Ultracentrifugation Versus Bench-Top Centrifugation in the Separation of *In Vitro* Digests

Considering both efficiency and convenience, the most suitable method for separating digestion samples prior to analysis for drug distribution across the various digestion phases was dependent on the degree of formulation dispersion and digestibility. Poor dispersion and incomplete digestion of the more lipophilic formulations [i.e., type I (both MC and LC), II-LC and IIIA-LC systems] resulted in the retention of a poorly dispersed oil phase after digestion, and ultracentrifugation was required to most effectively separate the oil phase from the aqueous and pellet phases without compromising mass balance. However, for the less lipophilic formulations that lacked the oil phase, bench-top centrifugation was sufficient to allow phase separation of solid precipitated drug from the aqueous phase (Fig. 8). The use of bench-top centrifugation is more convenient in terms of availability and cost and also permitted the use of lower sample volumes. The bench-top method was therefore preferred for sample separation when no oil phase was present and provides an advantage where multiple samples are envisaged to provide a kinetic pattern of drug solubilization–precipitation on digestion. Given the increasing propensity toward the use of self-emulsifying LBF that disperse to form nanosized emulsion droplets or micellar solutions (i.e., type IIIA/B and IV formulations), that do not generate oil phases on digestion, bench-top centrifugation is likely to be an acceptable (and therefore preferred on the basis of ease of use) method of sample separation for many prototype formulations. Where the precipitate consists of a fine dispersion of low-density amorphous nanoparticles, a bench-top centrifuge may not separate all of the solid material from the aqueous phase[69,70] and the use of an ultracentrifuge in these situations may be more appropriate.

Cross-Laboratory Evaluation of the Robustness of *In Vitro* Digestion Data

Equivalent *in vitro* digestion tests were performed on all eight LBFs in laboratories at Monash and Copenhagen according to the initial test conditions proposed by the LFCS Consortium. These experiments were undertaken to determine whether consistent results could be obtained in different laboratories using the same protocol, and therefore to probe the robustness of the evaluation methodology. Using the standard protocol, consistent patterns of FA titration were obtained in both locations (Figs. 6 and 7), suggesting that the assay as described was robust. It was evident, however, that variability in the *in vitro* data was more common for the LC formulations when compared with equivalent MC formulations. This likely reflects (1) the higher pK_a of LC FA at pH 6.5,[34] and therefore, greater sensitivity to fluctuations in pH around the set point, and (2) the relatively poorer dispersion properties of the LC formulations, and therefore, greater sensitivity to stirring conditions (see *Supplementary Materials*). Nonetheless, the consistent digestion data obtained here for even the most poorly dispersed formulations (i.e., type I) across both laboratories confirm the suitability of the experimental setup, including the use of an overhead propeller stirrer for the conduct of *in vitro* digestion profiling of candidate LBF.

CONCLUSIONS

Standardized baseline conditions for the evaluation of LBF using *in vitro* lipid digestion tests have been proposed and a broad range of LBF types examined. The data obtained under a consistent set of *in vitro* digestion conditions provide an opportunity to directly compare LBF performance and permit initial identification of key experimental variables. Experiments were conducted at pH 6.5, using 2 mM Tris-maleate buffer containing 150 mM NaCl, 1.4 mM $CaCl_2$, 3 mM NaTDC, and 0.75 mM PC. To better estimate the extent of LBF digestion, levels of unionized FA were determined by back titration. Consistent digestion data for the investigated LBFs were obtained at two sites (Monash and Copenhagen), providing some confidence in the robustness of the methods employed, and evaluation of separation techniques indicated that under many conditions, bench-top centrifugation may be an easier and more high throughput methods of sample separation. Using these conditions, good differentiation of formulation performance was possible. Digestion of type II-MC and IIIA-MC formulations resulted in rapid loss of drug solubilization capacity, whereas the more hydrophilic type IIIB-MC and IV formulations exhibited a similar loss of solubilization during the initial dispersion phase. In contrast, levels of drug precipitate were considerably lower in the case of LBFs containing LC lipids and the lipophilic type I-MC formulation.

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