Interactions of Artefenomel (OZ439) with Milk during Digestion: Insights into Digestion-Driven Solubilization and Polymorphic Transformations

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Supporting Information

ABSTRACT: Milk has been used as a vehicle for the delivery of antimalarial drugs during clinical trials to test for a food effect and artefenomel (OZ439) showed enhanced oral bioavailability with milk. However, the nature of the interaction between milk and OZ439 in the gastrointestinal tract remains poorly understood. To understand the role of milk digestion on the solubilization of OZ439 and polymorphism, we conducted real-time monitoring of crystalline drug in suspension during in vitro intestinal lipolysis of milk containing OZ439 using synchrotron X-ray scattering. OZ439 formed an unstable solid-state intermediate free base form (OZ439-FB form 1) at intestinal pH and was partially solubilized by milk fat globules prior to lipolysis. Dissolution of the free base form 1 and recrystallization of OZ439 in a more stable polymorphic form (OZ439-FB form 2) occurred during in vitro lipolysis in milk. Simply stirring the milk/drug suspension in the absence of lipase or addition of lipase to OZ439 in a lipid-free buffer did not induce this polymorphic transformation. The formation of OZ439-FB form 2 was therefore accelerated by the solubilization of OZ439-FB form 1 during the digestion of milk. Our findings confirmed that although crystalline precipitates of OZ439-FB form 2 could still be detected after in vitro digestion, milk-based lipid formulations provided a significant reduction in crystalline OZ439 compared to lipid-free formulations, which we attribute to the formation of colloidal structures by the digested milk lipids. Milk may therefore be particularly suited as a form of lipid-based formulation (LBF) for coadministration with OZ439, from which both an enhancement in OZ439 oral bioavailability and the delivery of essential nutrients should result.

KEYWORDS: milk, lipid-based formulation, drug solubilization, polymorphism, X-ray scattering, in vitro digestion, antimalarial, artefenomel, weakly basic drug, precipitation

INTRODUCTION

Malaria is a mosquito-borne parasitic disease that poses a global concern; about half a million children under the age of 5 die from malaria each year. The emergence of parasite resistance to current artemisinin-based therapies has led to the need for new antimalarial drugs. OZ439 is a synthetic trioxolane drug (Figure 1) that is highly potent against artemisinin-resistant strains of Plasmodium falciparum and can potentially be used as a single-dose cure in combination with a partner drug.

OZ439 is an amphiphilic poorly water-soluble drug that can self-assemble into colloidal structures in aqueous solution. Although the mesylate salt of OZ439 has an apparent high solubility in water, the free base forms of the drug are poorly soluble in fluids representative of those in the gastrointestinal (GI) tract. Consequently, solubilization of drug in the aqueous environment is anticipated to be a limitation to absorption through the intestinal membrane.

Figure 1. Chemical structure of OZ439-free base.

GI environment is anticipated to be a limitation to absorption through the intestinal membrane.
Lipid-based formulations (LBFs) such as triglycerides, mixed glycerides, and self-micro- or nanoemulsifying drug delivery systems (SMEDDS/SNEDDS) have been widely used to improve the oral bioavailability of poorly soluble lipophilic drugs by enhancing drug solubilization in the GI tract, often due to enhanced solubility in the fatty acids and monoglycerides generated upon digestion of the lipid components.\textsuperscript{11−13} The digestion process is commonly assessed by in vitro lipolysis experiments followed by separation of the colloidal phases\textsuperscript{14,15} and more recently has been adapted to the study of the fate of the drug during digestion.\textsuperscript{16}

Milk can be considered in some respects to be the ultimate lipid formulation: it serves a range of functions, including supporting the solubilization and delivery of poorly soluble vitamins and nutrients in the critical early stages of life. Milk fat is composed of 98% triglycerides emulsified as fat globules,\textsuperscript{17} and the underlying concept for milk to serve as a lipid formulation to improve the oral bioavailability of poorly water-soluble drugs is therefore similar to other digestible lipid-based formulation systems.\textsuperscript{16,18,19} The potential use of milk and milk components as formulation excipients targeted for pediatric populations has been reported in the literature,\textsuperscript{20−22} although the impact of milk digestion and consequent lipid digestion products on drug solubilization has not been studied.

The dispersion of antimalarial active pharmaceutical ingredients (APIs) in milk-based LBFs could therefore serve as suitable means for oral drug delivery with pediatric acceptability. Milk has been used to coadminister OZ439 in laboratory studies and clinical trials to enhance drug absorption.\textsuperscript{6,25,26} An increased level of OZ439 in blood was observed when full cream cow’s milk was coadministered with the drug when compared with fasted volunteers.\textsuperscript{25,27} This suggested the capability of milk fat globules (MFG) and or the products of milk digestion to maintain and present OZ439 in a solubilized or absorbable form to enable intestinal absorption, although the mechanistic processes remained unknown. Hence, this study aims to provide the physico-chemical understanding of the role of milk as a LBF for delivery of OZ439. Drug crystallinity during dispersion of drug in milk and during digestion of the formulation in vitro was monitored in real-time using synchrotron small-angle X-ray scattering. High performance liquid chromatography (HPLC) was used in tandem to quantify the phase distribution of drug during the dispersion and digestion of milk containing OZ439.

## MATERIALS AND METHODS

**Materials.** OZ439 was provided as the mesylate salt by the Medicines for Malaria Venture. Full fat bovine milk (3.8 wt % fat) was either supplied by the Australian Synchrotron or purchased from Coles supermarkets (Brunswick or Mt Waverly, Victoria, Australia). Trizma maleate (reagent grade), trifluoroacetic acid (≥99% purity), 4-bromophenylboronic acid (4-BPBA, > 95% purity), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Missouri). Calcium chloride dihydrate (>99% purity) and sodium hydroxide pellets (min 97% purity) were purchased from Ajax Finechem (Seven Hills, New South Wales, Australia). Hydrochloric acid (aqueous solution) was purchased from LabServ (Ireland). Sodium chloride (>99% purity) was purchased from Chem Supply (Gillman, South Australia, Australia). Sodium azide (299% purity) was purchased from Fluka (Sigma-Aldrich, St. Louis, Missouri), acetonitrile (liquid chromatography grade) was purchased from Merck (Darmstadt, Germany), and phospholipid (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, DOPC) was purchased from Sapphire Bioscience (New South Wales, Australia). USP grade pancreatin extract was purchased from Southern Biologicals (Nunawading, Victoria, Australia). Unless otherwise stated, all chemicals were used without further purification and water was sourced from a Millipore Milli-Q purification system.

**Preparation of OZ439-HCl and OZ439-FB Powders.** The HCl salt of OZ439 was prepared by dissolving 99 mg of OZ439-mesylate in 2.5 mL of water, followed by the addition of 0.25 mL of 1 M HCl solution. Samples were vortexed until thoroughly mixed and then passed through a vacuum filter unit with a 0.45 μm nylon membrane. OZ439-FB (form 1) was prepared by addition of 1 M NaOH solution to a solution of OZ439-mesylate (99 mg) in water (2.5 mL) to raise the pH to approximately 8.0. The sample was vortexed until thoroughly mixed and vacuum filtered as described above. The resultant powders were analyzed using SAXS within 1 h of preparation.

**Preparation of OZ439 in Milk Formulations and Their In Vitro Lipolysis.** OZ439-mesylate salt (99.0 mg, equivalent to 82.2 mg of OZ439-FB) was added to 2.75 mL of water containing 0.25 mL of 1 M HCl solution. The drug sample was vortexed until thoroughly mixed prior to the addition of 17.5 mL of milk. This dosage was selected based on a clinically effective dose of 800 mg of OZ439 free base in 200 mL of fluid, i.e., 4 mg of OZ439-FB/mL.\textsuperscript{28,29} The resulting drug/milk mixture that contained 123.6 mg of OZ439-FB equivalent/gram of milk fat (or 4 mg of OZ439-FB/mL) was incubated in a thermostated (37 °C) glass vessel under constant magnetic stirring. The pH of the sample in the digestion vessel was monitored at 6.5 during digestion using either 2 or 1 M NaOH solution titrated in by a pH stat control module (Metrohm AG, Herisau, Switzerland). 2 M NaOH was used to minimize the effects of titrant dilution during SAXS and HPLC runs, while 1 M NaOH was used during titration and back-titration experiments to determine the extent of lipolysis, as described forthwith. Tris-maleate buffer (50 mM pH 6.5) was used for the digestions and was prepared by dissolving 11.86 g of Trizma-maleate, 0.74 g of CaCl₂·2H₂O (5 mM), and 8.77 g of NaCl (150 mM) in water to a total volume of 1000 mL. Calcium chloride was added to remove the free fatty acids that could inhibit lipolysis from the lipolysis medium and to more closely resemble in vivo conditions where the free fatty acids are absorbed.\textsuperscript{14}

The amount of titrated (ionized) fatty acids during the digestion of milk and OZ439/milk suspensions was calculated from the volume of NaOH required to maintain the pH of the samples at 6.5, with background correction from a digestion of Tris-maleate buffer with OZ439 without lipid present. To determine the total amount of fatty acids released (ionized and unionized). Back-titration experiments were performed after 1 h of lipolysis that increased the pH to 9.0. The extent of digestion was calculated based on eq 1, and the theoretical amount of fatty acids (FA) released (2 mmol) was estimated using the total FA content in bovine milk reported in the literature,\textsuperscript{30} assuming that 2 mol of free FA were liberated from 1 mol of triglyceride.
Isolation of Drug Precipitates for Characterization of the Solid-State Structures. Precipitates containing OZ439 were isolated from digested milk samples to identify the solid-state forms of OZ439. First, 15 mL of a sample was collected from the digestion vessel after 1 h in vitro lipolysis of 247.2 mg of OZ439-FB equivalent/gram of milk fat and centrifuged for 15 min at 7378 g (25 °C). The upper layers of the centrifuged samples that contained both the lipid phase and the aqueous supernatant phase were discarded. The pellets were rinsed by adding 10 mL of water prior to recentrifugation under the same conditions as above. The aqueous layer was again removed and the pellets were analyzed using SAXS.

Synchrotron Small-Angle X-ray Scattering: Flow through Digestion Measurements. The small and wide-angle X-ray scattering (SAXS/WAXS) beamline at the Australian Synchrotron (ANSTO, Clayton, Victoria) was used to monitor real-time liquid crystalline structure formation in milk, the solid state crystalline form of OZ439 (inferred from the presence of crystalline drug peaks), and polymorphic transformations of OZ439. The instrumental setup of the digestion apparatus interfaced to the SAXS/WAXS beamline has been described previously. The digestion medium containing the formulation was readjusted to pH 6.5 and aspirated from the digestion vessel (thermostated to 37 °C) through a peristaltic pump operating at approximately 10 mL/min. An X-ray beam with a wavelength \( \lambda \) of 0.954 Å (13 keV) was used throughout this study. The liquid crystalline structures in milk were detected using a sample-to-detector distance of approximately 1.6 m (covering a \( q \) range of 0.01 < \( q \) < 0.67 Å⁻¹), while the solid crystalline drugs were detected with a sample-to-detector distance of approximately 0.6 m (0.04 < \( q \) < 2.00 Å⁻¹). \( q \) is the length of the scattering vector defined by \( (4\pi/\lambda)\sin(2\theta/2) \), where 2\( \theta \) is the scattering angle. 2D SAXS patterns were recorded using a Pilatus 1 M detector with a 5 s acquisition time and a delay of 15 s between measurements (one measurement every 20 s). The raw data were reduced to 1D scattering functions \( I(q) \) by radial integration using the in-house-developed software ScatterBrain.

Synchrotron Small-Angle X-ray Scattering: Static Capillary Measurements. SAXS patterns of the OZ439-mesylate, the OZ439-HCl, and the OZ439 free base powders were recorded using a Pilatus 1 M detector with a 5 s acquisition time and a delay of 15 s between measurements (one measurement every 20 s). The same setup was used to analyze the precipitates of OZ439 after 1 h digestion in milk and tris buffer.
Distribution and Quantification of OZ439 in the Digested Milk Phases. Quantification of the amount of drug solubilized after dispersion and digestion of OZ439 in milk and tris-maleate buffer (i.e., dynamic solubilization) were carried out using HPLC. Digestion experiments were carried out using methods described in the in vitro lipolysis section. To quantify the amount of drug dissolved during the digestion of milk and the amount of undissolved drug remaining in the system, aliquots (300 μL) were taken at various time points throughout the digestion experiments (0, 2, 5, 10, 30, and 60 min). These aliquots were pipetted into 2 mL glass vials containing 30 μL of lipase inhibitor solution (0.05 M 4-BPBA in methanol).31 Subsequently, an aliquot (200 μL) was then transferred from each glass vial to a polycarbonate centrifuge tube (7 mm × 20 mm, Beckman Coulter) and centrifuged for 40 min at 329177 g using an Optima MAX-TL ultracentrifuge (Beckman Coulter, Indiana, U.S.A.). After the digested samples were centrifuged, the resultant samples consisted of three phases (except in the case of buffer-only samples). The three phases were the upper lipid layer and the middle aqueous layer that consist of digested milk products and colloidal phases, and the pellet phase, which contained precipitated drug. The aqueous colloidal phase was first removed with a 1 mL syringe and 25 gauge needle and transferred to a separate 2 mL glass vial. The remaining lipid layer that settled at the top of the centrifuge tube was then also transferred to a separate 2 mL glass vial. A 25 gauge needle was used for this purpose, and the lipid layer was collected into the vial. The needle was subsequently rinsed with DMSO, and 800 μL of DMSO was added to the separated aqueous colloidal phase and lipid layer phase. These samples were then diluted in 5:95 v/v mobile phase B:mobile phase A (mobile phase A was water with 0.1 vol % TFA, and mobile phase B was acetonitrile with 0.085 vol % TFA) before the drug was quantified using HPLC. The pellet phase was also dissolved separately in DMSO and diluted in mobile phase before analysis by HPLC.

The HPLC system included a Shimadzu CBM-20A system controller, an LC-20AD solvent delivery module, an SIL-20A auto sampler, and a CTO-20A column oven set at 35 °C, coupled to an SPD-20A UV detector (Shimadzu Corporation, Kyoto, Japan). A reverse-phase C18 column was used (4.6 × 75 mm2, 3.5 μm; Waters Symmetry, MA, USA), and the UV detector was set to 260 nm for the detection of OZ439 using a binary gradient of mobile phase A and B. The buffer gradient consisted of 5–95% B for 6 min, 95–5% B for 0.2 min, and 5% B for 5.8 min at 0.5 mL/min flow rate. The injection volume was 20 μL. A standard curve was prepared from a stock solution of OZ439 prepared in DMSO at a concentration of 5 mg/mL. This stock solution was diluted in a mixture of DMSO and digested milk, which represented the sample matrix (1:4 v/v of digested milk to DMSO), to provide standards at different concentrations ranging from 1 to 40 μg/mL. Diazepam was used as an internal standard in all the HPLC measurements. The retention time of OZ439 was 7 min, while that for diazepam was 5 min. The limit of detection for OZ439 was 0.05 μg/mL and the limit of quantitation was 0.10 μg/mL, with the respective signal-to-noise ratio of 5 and 10.

RESULTS AND DISCUSSION

Effects of OZ439 on the Extent of Digestion and Lipid Self-Assembled Structures in Milk. Our group has previously shown that liquid crystalline nanostructures are formed during the digestion of bovine milk due to the self-assembly of amphiphilic digestion products that consist of fatty acids, monoglycerides, and diglycerides.32,33 As shown in the X-ray scattering pattern of milk in Figure 2a of ref 32, an inverse bicontinuous cubic phase Im3m, an inverse hexagonal phase H2, and a lamellar phase Lα coexist upon complete digestion of full cream milk. However, these structures were altered when OZ439 was present in the digestion system at different concentrations (Figure 2a). Major changes in the self-assembled structures in milk occurred at >50 mg of OZ439-mesylate, i.e., 62.4 mg of OZ439-FB equivalent/gram of milk fat. Above this concentration, the Im3m phase did not form during the 1 h digestion period. Only the Lα phase related to the formation of the calcium soaps of fatty acid digestion products and an H2 phase with the prominent (100) peak at q = 0.12 Å−1 were seen. The time-dependent colloidal structure formation during the digestion of milk in the presence of OZ439 is shown in Figure 2b. A shift of the scattering peaks of the H2 phase toward higher q values with increasing concentration of drug is illustrated in Figure 2c, signifying a contraction of the lattice spacing, i.e., closer packing of adjacent cylindrical aqueous channels between lipid domains.

The finding that OZ439 interferes with the self-assembly of lipids during the digestion of milk was anticipated. The presence of drug molecules in liquid crystalline systems does not necessarily impact on the self-assembly of lipids,34,35 but OZ439 is amphiphilic and was therefore expected to participate in altering the global curvature of the amphiphilic digestion components, in this case driving the self-assembly toward a less swollen inverse hexagonal phase. This drive toward more negative overall curvature indicates that OZ439 is acting as a more hydrophobic component than the overall influence on packing of the lipid digestion components alone.

The total amount of liberated fatty acids after 60 min digestion of milk and milk containing 99 mg of OZ439 (2.35 ± 0.13 vs 2.29 ± 0.20 mmol, respectively) were similar, indicating that although the surface-active OZ439 molecules altered the lipid packing on digestion, the overall digestibility of milk was not significantly affected by the presence of OZ439. Changes in the structures were therefore not attributable to the different extents of digestion. The influence of loading of amphiphilic drug on the release of fatty acids from LBFs has been reported previously with variable findings.36–38 For example, Williams et al. reported no significant effects of varying the concentration of danazol on the digestibility of medium and long chain lipids,38 while Thomas et al. observed a decrease in the titrated fatty acids as a function of simvastatin concentration during digestion in medium chain-SNEDDS but not long chain-SNEDDS.37 Contrary to Williams et al.,38 studies by Arnold et al. suggested that the presence of a variety of amphiphilic drugs (including danazol) in LBFs reduced the extent of lipolysis of medium chain triglycerides.39 While the basis of the discrepancies between these observations were not initially clear due to the complexity of the mechanisms involved, it was generally recognized that poorly water-soluble drugs may change the surface compositions of the lipid droplets available for digestion.36,37 This could arise from interactions between the drugs and the lipid droplets, surface localization, or partial solubilization of the drugs.36,37 In addition to the effects of drugs on the removal of lipolytic products from the oil/water interface through changes in surface characteristics, interactions between the drugs and the lipid digestion products such as ion-pairing may also occur, which could potentially affect the formation of liquid crystalline structures as was seen in the case of OZ439 in milk.
Solid-State Properties of OZ439 Prior to Exposure to Milk. Transit of a drug through the GI tract following oral administration may result in dissolution, salt formation, and/or solid state transformations of the drug due to variations in the intraluminal pH across the different segments. The pH-induced changes in the solid-state properties of OZ439 have
Solid-State Disposition of OZ439 in Milk and Buffer Pre- and Postdigestion. Milk fats are dispersed as globules that are composed principally of triglycerides, which are anticipated to be capable of solubilizing the antimalarial drugs. Solubilization of poorly water-soluble drugs and the ability of LBFs to maintain drug solubility in solution during GI transit have been reported as key to improved oral bioavailability. Solubilization of drugs in LBF during in vitro digestion has recently been used to predict the performance of the lipid excipients following oral administration by assessing the fraction of precipitated and dissolved drugs. Samples were removed at time intervals, and the lipolysis reactions were inhibited prior to ultracentrifugation of the inhibited digests. This typically led to separation into three layers: an upper lipid layer containing the digestion products and associated drugs, the aqueous supernatant, and a lower pellet layer containing undissolved drug. The distribution of the drugs in the separated phases were then analyzed and quantified using HPLC. However, recent studies have highlighted the importance of in situ monitoring of drug solubilization using a synchrotron small-angle X-ray scattering and Raman spectroscopy to account for the dynamic processes of the lipid digestion. These techniques therefore provide critical information regarding the solubilization of OZ439 in the rapidly digesting milk, analogous to studies on colloidal phase transitions. In addition, elucidation of the solution-mediated or digestion-mediated polymorphic transformations of drugs that typically occurred within short time frames could also be achieved.

Parts a and b of Figure 4 summarize the SAXS profiles of milk in the high-q range during the course of 25 min dispersion (stirring drug in the milk) and digestion in the presence of OZ439. The proportional changes in the amount of OZ439-FB form 1 and form 2 detected were determined by measuring the area of the characteristic peaks at q = 1.31 (OZ439-FB form 1) and 1.27 Å⁻¹ (OZ439-FB form 2) shown in Figure 4c. Formation of OZ439-FB form 1 was observed immediately after the addition of milk to OZ439-HCl solution and pH adjustment to 6.5. With continuous stirring at 37 °C for 25 min at pH 6.5, no further changes in diffraction from the drug were evident. Interestingly, a decrease in the peak area of OZ439-FB form 1 was seen upon dispersion in milk, which could be related to the solubilization of OZ439-FB form 1 into the undigested milk fat globules (Figure 4c).

Injection of pancreatic lipase to initiate the digestion of milk containing OZ439 resulted in a decrease in the area of the characteristic peak in OZ439-FB form 1, which disappeared completely after approximately 15 min digestion. Concurrent with the disappearance of the characteristic peaks of OZ439-FB form 1, new peaks appeared at q values of 1.27 and 1.35 Å⁻¹ approximately 3 min into the digestion. The positions of these new peaks point to the formation of the thermodynamically stable form of the free base (OZ439-FB form 2) as was discussed previously. A progressive decrease in the peak area of OZ439-FB form 2 was subsequently observed from about 8 min of digestion until the conclusion of the measurement. This decrease was not due to dilution from the addition of the NaOH titrant (titrated volume of NaOH was about 3% of the total sample volume after 60 min digestion) but was attributed to gradual but incomplete solubilization of the crystalline FB form 2 into the digested milk products. Dissolution of crystalline drug precipitates (collected after digestion) has also been reported by Stillhart et al. and Sassene et al., but more research is needed to understand the influences of self-assembled colloidal structures formed by the lipid digestion products on the dissolution of the crystalline drugs.

Simple stirring OZ439 in milk without digestion for an extended period of time did not induce the formation of OZ439-FB form 2 (Figure 4a), indicating that digestion provided the required conditions for the polymorphic transformation from OZ439-FB1 to OZ439-FB2. This transformation was not influenced by the initial form of the drug (both the HCl and the mesylate salts of the OZ439 converted to the FB form 2 upon digestion), similar to observations made by Porter et al. with halofantrine. Clinical exposure of these drugs would therefore be largely determined by the final form of the drugs postdigestion and their dissolution into the digests.

Precipitation of OZ439 by crystallization of the FB form 2, which was not completely solubilized during digestion, was confirmed by the solid-state measurements of the pellet after 60 min of lipid digestion. It has been previously suggested that weakly basic drugs can readily form amorphous noncrystalline precipitates during digestion in LBFs, depending on the pK_a of the drugs and the pH of the digestion medium. Ionized drugs have been shown to precipitate in the amorphous form due to charge interactions with liberated fatty acids, while neutral drugs are generally precipitated as crystalline solids. OZ439 has a pK_a of approximately 6.5–6.7 (extracted from the literature and from Scifinder based on ACD/Laboratories Software V11.02) and would therefore exist both in the unionized and ionized states (in equal concentrations) under the digestion conditions at pH 6.5. As a consequence, it was anticipated that OZ439 would be precipitated in a mixture of crystalline and amorphous solids, but solid-state measurements of the precipitated OZ439 revealed a predominantly crystalline solid (Figure 4d). However, the slight increase in the background intensities observed between q values of 1.3 and 1.7 Å⁻¹ in the collected pellets compared to as-prepared OZ439-FB form 2 powder may indicate the presence of some amorphous precipitates. Precipitation of predominantly crystalline solids with some degree of amorphous-salt after lipolysis of cinarizine at pH similar to its pK_a has also been previously reported.

Finally, a “sham” digestion of OZ439 in tris buffer medium was carried out as a control to determine the behavior of drug in the absence of digesting milk lipids. No decrease in the peak area of OZ439-FB form 1 was observed after pancreatin addition, indicating that no solubilization occurred (see Figure S1 in the Supporting Information). No polymorphic transformations were also observed. Schematic representation of the dynamic processes that occur during the digestion of milk containing...
Figure 5. Schematic representation of the time-course formation of liquid crystalline structures and the corresponding solid-state form of OZ439 during digestion in buffer and milk.

Figure 6. Partitioning of OZ439 in the pellet, aqueous, and the lipid layers after ultracentrifugation of OZ439 in (a) milk and (b) tris buffer, before (0 min) and after digestion for 2, 5, 10, 30, and 60 min. The digestion reactions were terminated using 0.05 M 4-BPBA in methanol. The extent of digestion of milk in the presence of 99 mg OZ439-mesylate is also shown in (a). (c) Comparison showing the effects of inhibitor on the partitioning of OZ439 in the digested milk phases at the 60 min time-point (validating its use for the shorter time points to suspend lipolysis before ultracentrifugation). (d) Solubilized OZ439 in the lipid and aqueous layers during 60 min digestion in milligram per gram of milk fat. The theoretical maximum value for the amount of OZ439 that can be solubilized per g of milk fat is indicated by the dashed line. Data are mean ± sd, n = 3 separate digestions.
OZ439 and the control experiment in which drug was dispersed in tris buffer and the solid state interrogated after addition of lipase is shown in Figure 5.

Quantification of the Dynamic Solubilization of OZ439 in Milk and Buffer during in Vitro Digestion Using HPLC. The partitioning of OZ439 into the colloidal lipid phases during the course of digestion in milk and lipid-free buffer was quantified using HPLC after ultracentrifugation. The process of ultracentrifugation of the digested milk samples resulted in the formation of three phases (i.e., the upper lipid layer, the aqueous layer, and the pellet layer), while only two phases were seen in tris buffer due to the absence of lipids. Separation of the digested milk-only formulation (no OZ439) resulted in only an upper lipid layer enriched in digestion products and an aqueous layer with no observable precipitates that were more dense than the aqueous supernatant.

Figure 6a shows that OZ439 was largely present in an undissolved state during the initial dispersion prior to the addition of lipase at the 0 min time point. As the digestion of milk was initiated, OZ439 was solubilized into the digested milk lipids, which was evident from the increase of OZ439 content in the lipid and aqueous layers and the reduction in the amount of OZ439 in the pellet phase. These observations were supported by visual observation of the separated phases after ultracentrifugation in Figure S2 in the Supporting Information. The solubilization of OZ439 in the lipid and aqueous phases was quantified in milligrams per gram of milk fat (Figure 6d). The amount of OZ439 that resided in the separated phases remained relatively constant after about 10 min of digestion, which coincided with the formation of the final liquid crystalline structure (the H2 phase) of the digested milk containing OZ439, which occurred at approximately 9 min into each digestion.

In contrast to the phase distribution during digestion of milk, there was little to no solubilization of OZ439 when tris buffer alone was used as the digestion medium (Figure 6b). OZ439 remained in the pellet phase at all sampling points throughout the 60 min lipid-free digestion. The formation of digestion products during lipid hydrolysis was therefore essential to the solubilization of OZ439 in milk, where the drug may be partitioned into the various colloidal structures. Previous studies have shown that the aqueous supernatant layer of the digested milk medium consisted of mainly unilamellar vesicles with multilamellar fragments, while the H2 phase was predominantly located in the upper lipid layer. It was therefore speculated that OZ439 was preferentially localized within the H2 phase instead of the vesicular phase, which may also be linked to the greater oil–water surface area provided by the H2 phase for the amphiphilic OZ439 molecules.

Although inhibition of the lipase activity can be achieved using 4-BPBA to halt the digestion of triglycerides in order to obtain time-dependent drug solubilization profiles, it was not known if the presence of 4-BPBA (prepared in methanol) would alter the distribution of OZ439. To investigate this, comparisons were made between the inhibited and uninhibited samples collected at the 60 min end point. It was assumed that little or no further lipolysis could occur following the 40 min ultracentrifugation at 37 °C, as the digestion had reached completion (Figure 6a). As was shown in Figure 6c, no significant differences in the distributions of OZ439 between samples with and without added 4-BPBA were observed. This observation indicates minimal interference of methanol on the partitioning of OZ439 in the digested milk phases.

CONCLUSIONS

Assessing the solubilization behavior of drugs during digestion is important to understand their fate after oral administration. In this study, in situ monitoring of diffraction peaks and thereby solubilization of OZ439 in milk as the formulation vehicle (inferred through the loss of Bragg peak intensity) during the course of digestion was investigated by using synchrotron X-ray scattering. The following key conclusions can be drawn from the observations. OZ439 transforms from the mesylate salt form to the hydrochloride salt form at the low pH of the gastric phase, which then changes to the free base form when the pH of the environment increases to 6.5, mimicking the small intestine. This free base form (FB form 1) is an unstable intermediate and can undergo polymorphic transformation to FB form 2 during digestion but not during dispersion in milk. Digestion of OZ439 in lipid-free formulation (buffer) also does not cause the polymorphic transformation, suggesting that the formation of OZ439 free base form 2 in digesting milk is triggered by solubilization of FB form 1. This highlights the potential complexity in the solid-state behavior of poorly water-soluble drugs and that consideration of the solid state of the drug and the colloidal state of the lipids at the end point of digestion may be critical to understanding consequent patterns of absorption. In addition, the presence of lipase and the process of digestion are essential for the solubilization of OZ439 under intestinal conditions based on the in vitro findings. Inclusion of a digestion step to the drug solubilization experiments in milk-based oral formulations is therefore strongly recommended. The influence of fat content on the solubilization of OZ439 also warrants further investigation, and by introducing milk powders, modulation of the ratios of milk fat to the solubilization of a fixed dose of OZ439 may become possible.

ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b00541.

X-ray scattering patterns of OZ439 during dispersion and digestion in tris buffer and the changes in the area under a Bragg peak of OZ439-FB form 1 during digestion; visual observations of the separated phases in the undigested (0 min) and digested (2–60 min) OZ439-containing milk samples after ultracentrifugation.

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ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b00541.

X-ray scattering patterns of OZ439 during dispersion and digestion in tris buffer and the changes in the area under a Bragg peak of OZ439-FB form 1 during digestion; visual observations of the separated phases in the undigested (0 min) and digested (2–60 min) OZ439-containing milk samples after ultracentrifugation.

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**ABBREVIATIONS USED**

FB, free base; LBF, lipid-based formulation; GI, gastrointestinal; SMEDDS, self-microemulsifying drug delivery systems; SNEDDS, self-nanoemulsifying drug delivery systems; API, active pharmaceutical ingredient; MFG, milk fat globules; HPLC, high performance liquid chromatography; SAXS, small-angle X-ray scattering; 4-BPBA, 4-bromophenylboronic acid; FA, fatty acid.

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