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Evaluating human milk as a drug delivery vehicle for clofazimine to premature infants

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ABSTRACT

Human milk is proposed as a drug delivery vehicle suitable for use in neonatal patients. Clofazimine, traditionally used for the treatment of leprosy and tuberculosis, is emerging as a treatment for cryptosporidiosis in infants, however its poor aqueous solubility has led to its commercial formulation as a waxy lipid formulation in a capsule, a format that is not suitable for infants. In this study, the evaluation of pasteurised human milk for the delivery of clofazimine was investigated using an *in vitro* lipolysis model to simulate gastric and intestinal digestion. The total lipid composition of the human milk was characterised alongside the liberated fatty acid species following digestion for comparison to alternative lipid-based delivery systems. Small-angle X-ray scattering was used to measure the presence of crystalline clofazimine during digestion and hence the extent of drug solubilisation. High-performance liquid chromatography was used to quantify the mass of clofazimine solubilised per gram of human milk fat (drug-to-fat ratio) in digested and undigested human milk. The digestion process was essential for the solubilisation of clofazimine, with digested human milk solubilising a sufficient dose of clofazimine for treatment of a premature infant. Human milk solubilised the clofazimine to a greater extent than bovine milk and infant formula during digestion, most likely as a result of differing lipid composition and increased long-chain fatty acid concentrations. These findings show that human milk enhances the solubility of clofazimine as a model drug and may be a suitable drug delivery vehicle for infant populations requiring therapeutic treatment.

1. Introduction

Following birth, many neonates who require pharmacological treatments will be admitted to the neonatal intensive care unit. This is especially the case for premature infants who are more likely to develop complications such as respiratory distress syndrome or necrotizing enterocolitis (NEC) as a result of their immature physiological development [1].

Drug delivery to neonates presents a challenge due to the lack of age-appropriate formulations, which is especially problematic for coadministration of different compounds. The oral delivery of medicines is preferred, with the European Medicines Agency suggesting the use of oral liquid preparations (solutions, suspensions and droplets) as appropriate drug delivery forms following birth [1]. Although oral delivery is a less invasive delivery option than intravenous administration, the lack of appropriate neonatal formulations often results in high occurrences of off-label oral drug use, with up to half the medicines used in the United States not providing adequate labelling for use in children [2]. Off-label use in infants poses a large safety risk as neonates and adults exhibit vastly different stages of development and physiology, resulting in differences in safety, dosing and efficacy requirements [3]. Additionally, the low volumes of fluids (10–20 mL/h) that can be safely tolerated orally by the neonates is an additional factor that must be considered when delivering therapeutics to this vulnerable population [4].

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Consequently, parenteral intravenous administration is often used despite a lack of accurate dosing information and limited pharmacokinetic and pharmacodynamic data in infants. Administration *via* the parenteral route in neonates exhibits many challenges, including being a more invasive and demanding process, requiring a trained health professional and the increased risk of infection and pain at the administration site [5].

Orally delivered lipid-based formulations often facilitate the improved gastrointestinal solubility of poorly water-soluble drugs by the generation of lipophilic colloidal domains [6], however, commercial lipid-based formulations often contain excipients such as surfactants and solvents that are unacceptable for use in neonates. In recent years, the use of milk-related systems such as infant formula, has been of interest as low-cost drug delivery vehicles for paediatric populations [7–12]. Specifically, the co-administration of drug dispersed in reconstituted powdered milk or infant formula provides benefits from a drug stability perspective and amenable to use in harsh climatic conditions compared to liquid formulations. The delivery mechanism relies on digestion of the lipids in the gastrointestinal tract to form fatty acids and monoglycerides that act to provide a dissolution sink for the drug, maximising its solubility and providing a means to enhance bioavailability.

Human milk is recommended as the ideal source of nutrition for infants in the first six months of life [13]. Additionally, the use of an exclusively human milk diet has been shown to potentially decrease the risk of NEC when compared to a diet containing bovine milk-based products such as infant formula and breast milk fortifiers [14–16]. Therefore, from a safety perspective, its use is envisaged to be favoured over infant formula as a drug delivery excipient. Although the use of human milk has been proposed previously as a means of dissolving solid dose forms to enable delivery during breast feeding [17–19], the solubilisation of drugs in human milk during digestion has not been assessed.

To investigate the suitability of human milk as a drug delivery vehicle for neonates, the solubilisation of clofazimine, a highly lipophilic poorly water-soluble drug (log $P = 7.1$) [20] was selected. As a biopharmaceutical class (BSC) II drug, the dissolution of clofazimine can often be the rate limiting step for oral absorption in the gastrointestinal tract. Clofazimine is currently used in an off-label compounded suspension form in children for tuberculosis and also is emerging as a treatment for crytosporidiosis, a conditon that can result in severe diarrhoea and even death in children [21,22]. Clofazimine has been previously shown to be solubilised during the digestion of milk-based formulations *in vitro* [9]. Dosing of clofazimine following a high-fat meal has also been shown to improve bioavailability compared to fasted conditions suggesting administration within a suspension of human milk may show similar enhancements [23].

In this study, as illustrated schematically in Fig. 1, the solubility of clofazimine in human milk and the solubilisation behaviour of clofazimine in digesting human milk were measured under gastric and intestinal *in vitro* conditions. Synchrotron small-angle X-ray scattering (SAXS) was used to monitor the presence of crystalline drug *in situ* during digestion and used as an indirect measurement of the solubilisation of clofazimine in the digesting milk. The lipid and micronutrient content of the human milk used in the solubilisation studies were characterised using gas chromatography with flame ionisation detector (GC-FID) and inductively coupled plasma-optical emission spectroscopy (ICP-OES), respectively, as these components are known to vary across postpartum stage, duration of feeding, nutritional status and maternal diet [24,25]. The influence of the lipid composition on the solubilisation of clofazimine was compared to the solubilisation behaviour of clofazimine in alternative milk-based formulations used in neonatal populations, namely infant formula and bovine milk [9].

2. Material and methods

2.1. Materials

Pasteurised donor human milk samples ('human milk') were donated by Australian Red Cross Lifeblood (Alexandria, NSW, Australia) with ethics approval from the Mercy Health Human Ethics Research Committee (2017–035) and recognition from Monash University and Lifeblood. Pasteurised milk at Lifeblood was pooled from a single donor in 3 to 5 L batches, aliquoted into individual bottles, and Holder pasteurised (62.5 °C for 30 min).

Calcium chloride dihydrate (> 99% purity), salicylic acid (> 99% purity), sodium hydroxide pellets (> 97% purity) and nitric acid (70% purity) were purchased from Ajax Finechem (Sydney, Australia). Hydrochloric acid (36%) was obtained from LabServ (Ireland). Sodium chloride (> 99.7% purity) was purchased from Chem Supply (Gillman, Australia). Chloroform (HPLC grade), methanol (HPLC grade), glacial

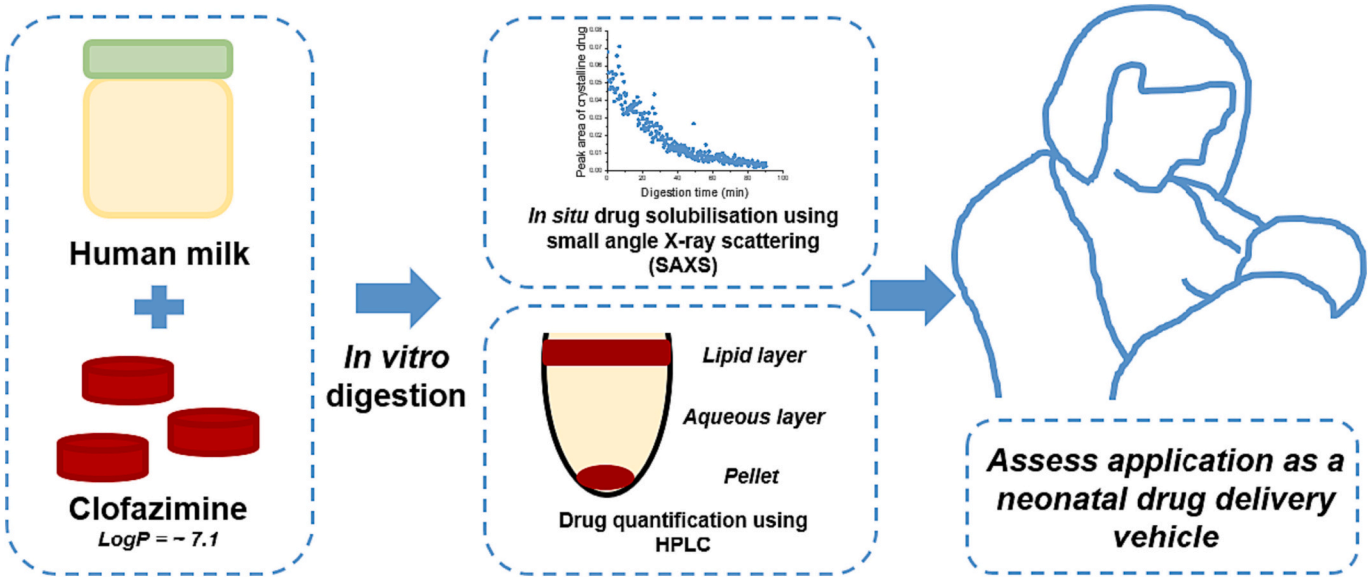

Fig. 1. Schematic representation of the key components of this study. The poorly water-soluble drug, clofazimine, was combined with human milk in a suspension as a potential neonatal drug delivery vehicle. The solubilisation behaviour of clofazimine during *in vitro* digestion was assessed using small angle X-ray scattering and HPLC.

acetic acid, 2-propanol (>99.9%, HPLC grade), *n*-hexane (≥96.0%, GC grade), sodium azide (> 99% purity) and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine, > 99%) was purchased from Cayman Chemical Company (Michigan, United States). Pepsin (porcine gastric mucosa), fungal gastric lipase (*candida rugosa*, type VII, ≥700 unit/mg solid), Trizma® maleate (reagent grade), sodium hydroxide (≥99% purity), sodium taurodeoxycholate hydrate (>95% purity), methanolic HCl (3 M), methyl *tert* butyl ether (≥99.8%, HPLC grade) and 4-bromophenylboronic acid (4-BPBA, >95% purity) were purchased from Sigma-Aldrich (Missouri, United States). USP grade pancreatin extract was obtained from Southern Biologicals (Nunawading, Victoria, Australia). Clofazimine (≥98% purity) was purchased from Sigma-Aldrich (Missouri, United states). C11:1 FAME methyl undecenate (C11:1, >99% purity) and C13:0 TAG glyceryl tridecanoate (C13:0, >99% purity) were purchased from Nu-Chek Prep (Minnesota, United States). A fatty acid methyl ester (FAME) standard mixture containing the following methyl esters was also purchased from Nu-Chek Prep (Minnesota, United States): methyl butyrate (C4:0), methyl pentanoate (C5:0), methyl hexanoate (C6:0), methyl heptanoate (C7:0), methyl octanoate (C8:0), methyl nonanoate (C9:0), methyl decanoate (C10:0), methyl undecanoate (C11:0), methyl laurate (C12:0), methyl tridecanoate (C13:0), methyl myristate (C14:0), methyl pentadecanoate (C15:0), methyl palmitate (C16:0), methyl heptadecanoate (C17:0), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2), and methyl α-linolenate (C18:3).

Unless otherwise stated, all chemicals were used as received without further purification and water was sourced from Millipore Milli-Q water purification systems at the point of use.

2.2. Quantification of the total fat content of human milk

The total fat content of the human milk was determined using a previously established gravimetric method [26]. A sample of human milk (1.61 mL) was combined with a 32.2 mL mix of chloroform/methanol (2:1, *v/v*) and vortexed before centrifuging at 1503 *g* for 10 min at room temperature. The clear homogenate was transferred to a separating funnel, combined with water (6.29 mL) and allowed to stand until phase separation occurred. The lower layer containing the lipid components was collected into a round bottom flask. The remaining aqueous phase was extracted with the chloroform/methanol mix (1:1 *v/v* aqueous to organic solvents) to collect any remaining lipids. The resulting organic layers were subsequently collected in the same round bottom flask. The collected organics were evaporated to dryness using a rotary evaporator (400 mbar, 100 rpm, 45 °C, where the pressure was continuously dropped to 0 mbar to remove any remaining solvents). The total lipid content was then determined by weighing the difference between the round bottom flask containing the dried lipid layer and the empty flask.

2.3. Characterisation of the micronutrient composition in human milk

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to determine the concentration of trace elements (potassium, sodium, calcium, phosphorous, magnesium and iron) within the human milk samples. These experiments were carried out at The University of Melbourne in collaboration with the Melbourne TrACEES Platform.

Sample preparation of the human milk was carried out with a Milestone Ethos Easy microwave digestion system. Human milk samples were thawed to room temperature and mixed by vortex. Approximately 0.5 g of thawed human milk was combined with 5 mL of nitric acid and acid-digested at 240 °C for 30 min following a 25-min temperature ramp from 30 °C. The digested samples were diluted to 50 mL with water and the concentrations of trace micronutrients were analysed using an inductively coupled plasma optical emission spectrometer (PerkinElmer Optima 4300DV). The sample introduction system consisted of a concentric nebuliser, a baffled cyclonic spray chamber, and a 2.0 mm alumina injector.

2.4. In vitro lipolysis of human milk and preparation of clofazimine in human milk formulation

For each digestion, human milk (18 mL) with or without a dose of clofazimine powder was combined and vortexed well prior to transfer into a thermostatted glass vessel (temperature is maintained at 37 °C) connected to a pH stat auto titrator (Metrohm® AG) with constant magnetic stirring. Prior to gastric digestion, the pH of the sample was adjusted to a value of 4.500 ± 0.003 using HCl or NaOH (0.2–5.0 M). A 2 mL suspension of gastric lipase (1.389 mg/mL) and pepsin (0.694 mg/mL) was injected to initiate gastric lipolysis. Following a 60-min period of gastric lipolysis the pH of the sample was manually adjusted to 6.500 ± 0.003 using NaOH (0.2–5.0 M) to mimic intestinal conditions and bile salts micelles included. Bile salt micelles were prepared with DOPC and sodium taurodeoxycholate using a previously reported method [27] and incorporated to provide a final concentration of 0.98 mM and 4.7 mM, respectively. A 2 mL suspension of freeze-dried pancreatic lipase reconstituted in tris buffer (about 700 tributyrin unit enzyme activity) was injected into the vessel to initiate intestinal lipolysis for a period of 90 min. The tris maleate digestion buffer was prepared with 50 mM Trizma® maleate, 5 mM $CaCl_2.2H_20$, 150 mM NaCl and adjusted to pH 6.5 using NaOH and HCl solutions.

2.5. Characterisation of free fatty acid composition in pasteurised human milk

2.5.1. Preparation of digested human milk samples

Gas chromatography with flame ionisation detector (GC-FID) was used to quantify the fatty acid composition of human milk as well as total amount of free fatty acids released after digestion. Digestion of the human milk was performed as described in section 2.4. Digested human milk samples (1 mL) were collected following the completion of gastric and intestinal digestion and placed into Eppendorf® tubes (1.5 mL) containing 10 μL orlistat (2 mg/mL in methanol). Samples were stored at −20 °C until use. Following quantification, the extent of digestion of the human milk was calculated using the total amount of fatty acids (mol) released at individual time points during the *in vitro* digestion (0, 30, 60 and 150 min). The theoretical amount of fatty acids was determined based on the assumption that one mol of triglycerides released two mols of fatty acids. The total fat content of the milk was 3.65 g/100 mL and the average molecular weight of human milk triglycerides was taken to be 764 g/mol.

2.5.2. Extraction and derivatisation of FA species

Samples (250 μL) of the digested human milk were thawed, combined with 6 mL of hexane: methyl *tert* butyl ether: acetic acid (100:3:0.3 *v/v/v*) and centrifuged at 1555 *g* for 5 min to extract the total lipid species. The upper layer (5.5 mL) of the centrifuged sample was passed through an aminopropyl column (ISOLUTE ® NH_2 500 mg/6 mL) that has been preconditioned with acetone: water (7:1 *v/v*) followed by hexane. A 4.5 mL volume of hexane:chloroform:ethyl acetate (100:5:5 *v/v/v*), chloroform:isopropanol (2:1 *v/v*) and chloroform:methanol: acetic acid (100:2:2 *v/v/v*) were passed through the column to elute the triacylglycerols (TAG; also known as triglycerols), mono- and diacylglycerols (MAG/DAG; also known as monoglycerides and diglycerides respectively) and free fatty acids (FFA) species, respectively. The eluted excess solvent was evaporated using a Buchi Rotavapor® (Model R-205) and the resultant lipids were left to dry overnight under vacuum (Labtech LVO-2030).

Derivatisation of fatty acids into fatty acid methyl esters (FAME) was performed using an acid-catalysed method previously reported by Cruz-Hernandez et al. [28]. To glass vials containing the dried lipid samples, 3 mL of methanol/HCl (3 N) and hexane containing 240 μL TAG (13:0; 3

mg/mL) and 170 μL FAME (11:1; 5 mg/mL) internal standards was added. The vials were firmly capped and vortexed before being heated at 100 °C for 60 min with occasional mixing. After heating, samples were left to cool to room temperature and 2 mL of water was added followed by vortexing and 5 min of centrifuging at 1110 *g*. The upper hexane layer was collected and transferred to 2 mL glass vials for GC analysis.

2.5.3. Chromatographic conditions for FA analysis

The analysis of the FAME species was performed on an Agilent 7820A gas chromatograph, equipped with a fused silica capillary (SUPELCOWAX™10), column length of 30 m, internal diameter of 0.32 mm and 0.5 μm film thickness (Supelco, Inc., Bellefonte, PA). Samples (0.5 μL,1:10 split ratio) were injected into the GC-FID under programmed conditions. The initial oven temperature was set to 45 °C, then increased to 220 °C at a rate of 15 °C/min, once this temperature was reached it was then increased to the maximum temperature of 250 °C at a rate of 8 °C/min and held for 1 min. The temperature of the injector and detector ports were held constant at 250 °C. The carrier gas was helium (1.288 mL/min, constant flow) and the FID was kept at 300 °C. The concentration of individual FAME species was automatically determined using the instrument software in relation to the FAME standards.

2.6. Synchrotron small angle X-ray scattering coupled with in vitro digestion

A pH stat digestion apparatus was coupled to the SAXS/WAXS beamline at the Australian Synchrotron, as previously described [29]. A peristaltic pump (Masterflex™ C/L Variable-Speed Tubing Pump) was used to continuously circulate the digestion media at a constant flow rate (about 10 mL/min) *via* silicone tubing (2 mm ID, 4 mm OD) through a 1.5 mm diameter quartz capillary mounted in the X-ray beam (wavelength = 0.954 Å, photon energy = 13 keV). A Pilatus 2 M detector was used to acquire two-dimensional SAXS images with a 5 s acquisition time and a 15 s delay with a sample-to-detector distance of approximately 700 mm (covering a q range of $0.04 < q < 2.00$ Å^{-1}), where $q = (4\pi/\lambda)\sin\theta$, with λ being the photon wavelength and 2θ the scattering angle. Raw data was analysed using the in-house software package, Scatterbrain version 2.71. The peak area of diffraction peaks of interests was determined using Origin software 2021b (version 9.85) using a linear baseline. No background subtraction was performed.

2.7. Solubility of clofazimine in digested and undigested human milk

Excess clofazimine was weighed into glass vials containing undigested or digested (section 2.4) human milk. Sodium azide (0.39 g/L) was included as a preservative and digestion was inhibited using orlistat (1:100 *v/v*, 2 mg/mL in methanol). The glass vials were kept on a shaker in an incubator at 37 °C for 24 h. Samples (200 μL) were removed at specified time points (0, 4, 8 and 24 h) and ultracentrifuged at 434900 *g* for 60 min at 37 °C (Optima MAX-TL ultracentrifuge, Beckman Coulter, IN, US). The resulting upper lipid layer and the aqueous supernatant containing solubilised clofazimine were transferred into separate 1.5 mL Eppendorf® tubes using a 1 mL syringe and 25-guage needle. Samples were stored at −20 °C prior to drug quantification (section 2.8).

2.8. Quantification of clofazimine in digested and undigested human milk using HPLC

Frozen samples of clofazimine in human milk were thawed to room temperature and prepared for quantification using a previously established HPLC method [9]. To Eppendorf® tubes containing the upper lipid layer, 200 μL of methanol was added and the samples vortexed for five minutes to extract the clofazimine. Following extraction, 800 μL of methanol containing a salicylic acid internal standard (40 μg/mL) was added to all samples and the samples centrifuged (Sigma unrefrigerated ultracentrifuge 1–14) at 16160 *g* for 7 min to sediment any undissolved components. The supernatant was collected and diluted 1 in 20 *v/v* in mobile phase (26:74 *v/v* 0.25 M sodium acetate trihydrate, pH 4.5/methanol) prior to measurement on the HPLC. The separation of clofazimine was performed using a reversed-phase C8 column (Phenomenex®, 150 mm × 5 μm × 100 Å, 4.6 mm) on a Shimadzu Nexera instrument equipped with model LC-30 CE pumps, SIL-30 AC autosampler, CTO-20 AC oven, SPD-M30A DAD detector and LabSolutions software. The oven temperature was set to 35 °C and a UV detection wavelength of 286 nm was used. The injection volume of the samples was 10 μL and the flow rate was 1 mL/min, which gave a retention time of 4.5 min for clofazimine and 1.7 min for the internal standard. A standard curve was prepared from 0.01 to 40.0 μg/mL of clofazimine made up in methanol and spiked with human milk. Following quantification, GraphPad Prism software (version 9.0.1) was used to perform statistical analysis and a two-way ANOVA conducted to determine the significance ($P < 0.05$) of any differences in the mass of solubilised clofazimine in the human milk.

3. Results and discussion

3.1. Characterisation of total fat content in human milk samples

Total lipid content has been shown to be the most variable macronutrient in human milk and can vary greatly depending on a number of maternal factors [24,30,31]. These factors may include gestational age, time post-partum as well as the diet of the mother. As the total fat content and lipid composition of milk-based formulations have been shown to influence the extent of solubilisation of co-administered drugs [11,32], it was necessary to characterise these components in the human milk used within this study. Mature human milk (> 30 days post-partum) typically contains about 3–5 g fat/100 mL milk [33], which was comparable with the human milk samples used in this study as shown in Fig. S1. The total fat content from four different batches was found to range from 3.76 to 5.63 g/100 mL, with all batches excluding batch B matching previously reported values. As the error in measurement of the fat content for batch B was low, it is indicating a high fat level specific to that batch, highlighting the need for this characterisation rather than an assumption of a typical average fat content.

It is worth noting that the milk samples used for fat content determination were also sonicated prior to gravimetric analysis to ensure sample homogeneity following the freezing and thawing processes, which had previously resulted in phase separation.

3.2. Characterisation of the micronutrient composition in human milk

Compositions of the micronutrients (potassium, sodium, calcium, phosphorous, magnesium and iron) in the three human milk samples were quantified using ICP-OES and the results summarised in Table S1. When considering the application of human milk as an oral drug delivery vehicle, the micronutrient content must be characterised to ensure safe trace element levels. As the micronutrient content of human milk can vary greatly depending on time post-partum and maternal diet [34–37], a large range between donors was expected. Potassium was determined to be the most abundant micronutrient with a concentration of 402 ± 26.0 mg/L, followed by calcium (247 ± 34.7 mg/L), phosphorous (149 ± 13.5 mg/L), sodium (69.3 ± 32.4 mg/L), magnesium (34.7 ± 5.21 mg/L) and iron (0.792 ± 0.0465 mg/L) (Table S1). Notably, although the concentrations of zinc and copper were investigated, their respective concentrations were below the limit of detection. As zinc and copper are two of least abundant micronutrients in human milk, a more sensitive methodology such as inductively coupled plasma mass spectrometry may need to be utilised for future analysis.

From the quantified micronutrient levels, both sodium (69.3 ± 32.4 mg/L) and iron (0.792 ± 0.0465 mg/L) were outside the scope of values previously reported in the literature [34–37]. The concentration of

sodium in the human milk was approximately half of the expected range, whereas the concentration of iron exceeded the previously reported values of 0.300–0.700 mg/L. A prior study has found that lower sodium contents in human milk can be associated with increased daily feedings [38]. Other maternal factors, including time post-partum and method of milk expression, have also been shown to influence the sodium content of the milk. A decline in sodium content has been observed to correlate with time post-partum whereas lower sodium levels were found in milk expressed *via* a pump compared to manually, most likely as a result of intercellular leakage and/or cellular damage from mechanical stress during expression [39]. Although the iron content of human milk has not been found to be influenced by maternal factors or dietary uptake [40], a recent study has found that maternal iron levels during pregnancy may influence the quantity of iron present during lactation following birth [41].

3.3. Characterisation of the fatty acids and monoglycerides formed during the digestion of the human milk samples

Prior to digestion, approximately 98 wt% of all lipid species found within human milk are triglycerides and account for half of the energy content of human milk. These triglycerides comprise a glycerol backbone joined to three fatty acid molecules in *sn*-1, *sn*-2 and *sn*-3 stereospecific positions respectively. Through the digestion process, human gastric lipase selectively hydrolyses and releases fatty acids from the *sn*-3 position of triglycerides to liberate a free fatty acid and diglyceride. Following transition to the small intestines, pancreatic lipase acts to further hydrolyse these molecules into mono-glycerides and free fatty acids through further action on the *sn*-1 and *sn*-3 position [42]. Identifying the distribution of fatty acids and mono-glycerides following digestion is an important consideration as it is known that the solubilisation capacity of a drug can be affected by the lipid species present in the milk-based formulation [9,32].

Table 1 shows the fatty acid distribution of the human milk samples quantified using GC-FID after digestion. Following simulated gastric and intestinal digestion, the most abundant fatty acids were oleic (C18:1) and linoleic acid (C18:2), followed by palmitic acid (C16:0) (Table 1). The prevalence of these fatty acids and relative abundance is comparable to values previously seen in the literature [43,44]. Overall, the abundance of the oleic and palmitic acid may be slightly lower than expected, but still within range of previously reported values when accounting for the large variability seen in the lipid composition of human milk. In particular the percentage of the free oleic acid was lower than expected, with an average of 23.1%mol in the human milk compared to 41.9%mol reported in the literature. Comparatively, the molar percentage of oleic acid in the monoglyceride component of the human milk was 16.8%mol, slightly higher than the 11.5%mol reported in the literature. Although care was taken to simulate the digestion process of infants as closely as possible during the *in vitro* digestion process, fungal gastric lipase was used in place of recombinant human gastric lipase. The fungal gastric lipase used in these studies was not selective for the *sn*-1 or *sn*-3 position in comparison to human gastric lipase and may have resulted in hydrolysis of both positions during simulated gastric digestion, potentially deviating the results from normal physiological results [45]. Additionally, as longer chain fatty acids in human milk have been found to degrade over time, there is potential that some degradation may have occurred during storage time [46]. For further application as a drug delivery vehicle *in vivo*, stricter control over storage time and conditions would need to be employed compared to in a research setting.

3.4. Monitoring of crystalline clofazimine in human milk during dispersion and digestion

The decreasing presence of crystalline clofazimine in digesting human milk was observed and used as an indication of drug solubilisation. A characteristic peak of the form I clofazimine polymorph, the original material used in the study, was initially tracked across a dispersion stage followed by a gastric and intestinal digestion stage (Fig. 2 a, b, c). Furthermore, to understand the capacity of human milk to solubilise clofazimine, increasing quantities of clofazimine (100, 150, 200 mg) were dispersed in human milk of uniform fat content. Bile salt micelles representing the fasted state of an infant were also included to create the suspension. Solubilisation of the crystalline clofazimine was determined by quantifying the area of the characteristic diffraction peak for crystalline clofazimine during dispersion, gastric and intestinal digestion of human milk using synchrotron X-ray scattering. This method has previously been used to monitor the *in-situ* solubilisation of other poorly water-soluble drugs in digesting lipid-formulations [8–11,32,49,50]. The characteristic diffraction peak for clofazimine at $q = 0.66$ Å^{-1} was monitored throughout dispersion and digestion and integrated to determine the peak area. A decrease in peak area was used to indicate a decrease in crystalline drug (Fig. 2d).

Prior to the initiation of digestion, the solubilisation behaviour of clofazimine in the undigested human milk was monitored throughout a dispersion stage. Small decreases of approximately 5% in the peak area of the crystalline clofazimine were observed across all doses, however these were quite inconsistent and appeared to plateau the longer the dispersion time (Fig. S2). The reduction in crystalline clofazimine in the undigested human milk may have been a result of the clofazimine exhibiting a finite solubility in the undigested lipid component of the human milk. Measurement of the particle size distribution of the clofazimine active pharmaceutical ingredient (API), human milk and human milk suspension containing clofazimine API and bile salt micelles showed an averaged $D_{4,3}$ of 59.5, 20.2 and 9.25 μm, respectively, indicating some interaction between the clofazimine API and human milk could be occurring (Fig. S3). A simulated digestion of clofazimine in a lipid-free environment (tris maleate buffer) showed an even smaller

Table 1
Fatty acid composition (%mol) of human milk following gastric and intestinal digestion. Samples of digested milk were collected from three separate batches (batch A, batch B, batch F) and all results are represented as mean ($n = 3$) ± standard deviation. The fatty acid composition of human milk [47] and bovine milk [48] reported in the literature have been included for comparison.

FA	Free fatty acids (*sn*-1 and *sn*-3)			Fatty acids in monoglycerides (*sn*-2)		
	Human milk (current study)	Human milk (literature)	Bovine milk	Human milk (current study)	Human milk (literature)	Bovine milk
C6:0	3.0 ± 0.6	–	8.5	5.1 ± 1.9	–	0.1
C8:0	1.3 ± 0.3	–	2.6	2.2 ± 0.8	–	2.9
C10:0	0.9 ± 0.2	1.4 ± 1.1	11.8	1.6 ± 0.6	0.6 ± 0.7	7.8
C12:0	3.0 ± 1.3	8.5 ± 4.2	4.2	1.6 ± 0.4	7.8 ± 4.3	8.1
C14:0	3.4 ± 1.1	7.4 ± 3.1	10.1	3.7 ± 1.6	12.5 ± 3.6	23.9
C16:0	12.5 ± 3.3	12.6 ± 5.2	27.1	15.9 ± 7.4	51.2 ± 1.5	36.7
C18:0	8.1 ± 1.5	10.6 ± 1.7	12.5	11.1 ± 4.4	1.5 ± 0.5	3
C18:1	23.1 ± 8.7	41.9 ± 6.4	20.1	16.8 ± 8.8	11.5 ± 5.2	7.2
C18:2	12.7 ± 7.7	16.4 ± 2.8	1.6	1.4 ± 2.1	8.3 ± 1.0	0.6
C18:3	0.8 ± 0.2	1.2 ± 0.8	1.4	1.3 ± 0.5	0.8 ± 0.8	0.3

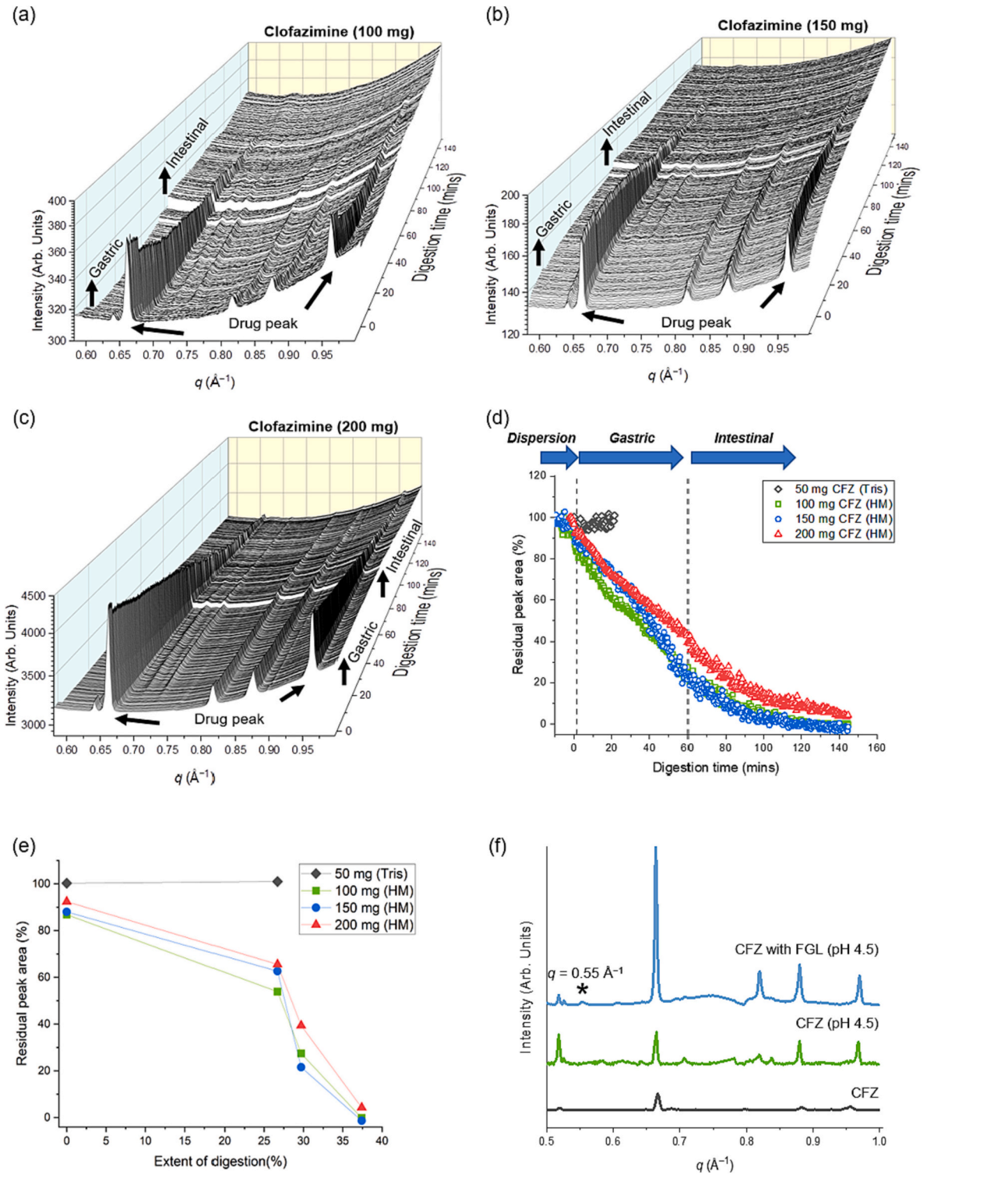

Fig. 2. Panels (a), (b) and (c) X-ray scattering patterns of clofazimine during *in vitro* digestion of human milk between $q = 0.58$ and 1.00 $Å^{-1}$ at a dose of 100 mg, 150 mg and 200 mg respectively. Panel (d) Residual integrated peak area for clofazimine (CFZ) at $q = 0.66$ $Å^{-1}$ in human milk at a dose of 100 mg (green square), 150 mg (blue square) and 200 mg (red triangle) derived from panels (a), (b) and (c). Panel (e) illustrates the residual crystalline drug ((determined from panel (a – c)) as a function of the total extent of digestion determined by the fatty acid content using GC-FID. Panel (f) shows the X-ray scattering patterns of clofazimine after 60 min of incubation in human milk, human milk at gastric pH (pH 4.5) and human milk (pH 4.5) in the presence of fungal gastric lipase (FGL). The asterisk at $q = 0.55$ $Å^{-1}$ denotes a peak from a potential different polymorphic form of clofazimine in the presence of FGL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduction in the total peak area during dispersion, indicating that the lipophilic clofazimine did display a higher innate solubility in the triglycerides of the human milk. Comparison of the solubilisation behaviour of clofazimine in the tris buffer showed there was no decrease in the intensity of the diffraction peak during dispersion or following initiation of gastric digestion conditions (Fig. 2d). Subsequently, the measurement was ceased following 20 min of no decreases in peak intensity. Following initiation of gastric digestion in the human milk samples, a more pronounced decrease in the intensity of the crystalline clofazimine peak was observed (Fig. 2d). After 60 min of gastric digestion conditions, approximately 75% of the 100 mg and 150 mg doses of clofazimine was solubilised, whilst approximately 55% of the largest 200 mg dose was solubilised. Following transition to intestinal conditions, all three doses of clofazimine were solubilised at the completion of the 80-min digestion time. The human milk used during these *in vitro* digestions had a fat content of 3.65 g/100 mL and therefore contained sufficient lipids to solubilise all doses of the drug. Accounting for a digestion volume of 23 mL and dilutions from injections of the lipase suspensions, it was concluded that the ratio of solubilised clofazimine to human milk fat (drug-to-fat ratio) was 152 mg/g, 228 mg/g and 304 mg/g respectively for the increasing doses of clofazimine.

The kinetics of the digestion process was estimated by obtaining samples of human milk throughout the digestion process (0, 30, 60 and 140 min) and determining the free fatty acid content with GC-FID. The extent of digestion was determined by comparing the theoretical mass of free fatty acids released at that time point to the total expected mass of fatty acids calculated from the average molecular weight of the human milk prior to digestion. Following gastric and intestinal digestion, it was determined that approximately 40% of the total fatty acids in the human milk sample were liberated in the *in vitro* digestion apparatus. During *in vivo* digestion the extent of digestion would be expected to reach 100% as liberated digestion products would be removed and absorbed from the site of digestion. As mentioned previously, the concentration of nutritionally relevant salts in the human milk was an important consideration when assessing optimal digestion of the human milk following oral administration. Cations such as calcium and magnesium have been shown to interact with free fatty acids and form insoluble soaps as part of the digestion process; as calcium is the most abundant cation within human milk, the formation of calcium soaps is most common. [42]. Throughout the digestion process, lipase adsorbs to the oil-water interface of milk fat globules and hydrolyses the triglycerides to form free fatty acids. As longer-chain fatty acids are more hydrophobic and exhibit poorer aqueous solubility, they are more likely to accumulate at the oil-water interface [51]. Hence, adequate calcium content in human milk is beneficial for the complexation and removal of the fatty acid digestion products from the oil-water interface, thereby facilitating increased lipase activity at the oil-water interface. The scattering profiles generated during digestion of the human milk with clofazimine displayed lamellar structures associated with the presence of calcium soaps (Fig. S4) [52]. Although calcium soaps were observed, the natural calcium levels in the human milk did not appear to be sufficient to drive digestion to completion in the *in vitro* system. A previous study by Binte Abu Bakar et al., studying the *in vitro* digestion of pasteurised human milk observed a similar extent of digestion, 42.1 ± 8.1%, with native calcium levels as well as an increased extent of digestion in a simulated higher calcium concentration environment [27].

Following determination of the extent of digestion, the time-dependent solubilisation data (Fig. 2d) was converted to solubilisation as a function of lipolysis extent (Fig. 2e). As seen in Fig. 2e there was a clear decrease in the amount of crystalline clofazimine with increased fatty acid production, indicating the importance of lipid digestion and liberation of digestion products (FFA and MAG/DAGs) on the extent of solubilisation of clofazimine in human milk. As the extent of digestion of the human milk vehicle progressed above 25% the rate of drug solubilisation and subsequent decrease in crystalline peak area increased rapidly. Following this point, the trend between decreasing peak area and extent of digestion had an approximately linear correlation, which supports previous data [11]. This correlation is most likely a result of increased interaction between the negatively charged fatty acid digestion products and weakly basic clofazimine drug. Clofazimine contains an ionisable amine group (pKa 8.51) that exhibits an extent of ionisation under both gastric and intestinal pH conditions [20]. The free fatty acids liberated during digestion exhibit pKa values of 5–9, depending on their chain length [53]. Although free fatty acids would be produced under gastric conditions, it would be expected that following transition to intestinal pH more ionised free fatty acids would be present and hence greater interaction between the protonated clofazimine and ionised fatty acids. Ion pairing behaviour between weakly basic drugs and digesting lipid formulations has previously been investigated using alternative model drugs such as cinnarizine [54–56]. It is hypothesised that through increased interaction of the weakly basic drugs with the fatty acid digestion products to form lipophilic ion pairs, the drug molecules may be more effectively solubilised in the subsequent colloidal structures formed in the digested human milk [57].

It should also be noted that from the third set of data points to the last data sets in Fig. 2b, intestinal digestion was initiated through remote injection of pancreatic lipase. As intestinal digestion was carried out at pH 6.5, there is a possibility there was combined action of both the pancreatic and fungal gastric lipase in the lipolysis process of the digesting human milk. Lipases derived from fungal sources have been shown to exhibit similar digestive activity to human gastric lipase across pH ranges of 2–8, with optimum activity over a pH-range of 4–6 [45].

As discussed, care was taken to simulate the gastrointestinal conditions of a premature infant as closely as possible where appropriate. Studies by Kamstrup et al. and de Oliveira et al., have investigated the design of relevant and comprehensive *in vitro* digestion models for neonatal and premature infants that are comparable to the *in vitro* model used within this study (Table S2) [58,59]. Gastrointestinal tract (GI) conditions vary greatly between premature infants, neonates and adults, with the GI tract of premature infants largely characterised by higher gastric pH, lower enzymatic activity, rapid gastric emptying and low electrolyte levels [60,61]. As such, attention was paid when replicating these conditions. *In vitro* models incorporating a drug absorption step have yet to be fully established and of the various methods attempted such as contact with cell lines [62] there is as yet no consensus on a preferred approach. Furthermore, as clofazimine is a BSC class II/IV drug, the absorption process following dissolution would not be expected to be the rate limiting step following administration *in vivo*.

As multiple polymorphs of clofazimine exist that may change throughout dispersion and digestion, the characteristic peaks of the clofazimine API powder were initially characterised and compared to the peaks observed during dispersion and digestion in the human milk (Table S3). The initial material used in the study was a form I polymorph of the clofazimine API with observed characteristic peaks at $q = 0.66$ and 0.96 $Å^{-1}$ [63]. One additional peak ($q = 0.55$ $Å^{-1}$) corresponding to the form II polymorph appeared only under gastric conditions following the injection of fungal gastric lipase (Fig. 2f). As this peak was not present following initial adjustment to gastric conditions (pH 4.5) it is unlikely that a more acidic environment and increased ionisation of the clofazimine molecule was responsible for this transformation. However, after the initiation of intestinal digestion, the peak at $q = 0.55$ $Å^{-1}$ decreased in size, suggesting that solubilisation was occurring.

3.5. Determination of equilibrium solubility of clofazimine in digested and undigested human milk using HPLC

The enhancement of drug solubility upon digestion, *i.e.* between undigested milk to digested milk is considered the driving force for dynamic solubilisation during digestion [11]. Therefore, determining the difference in solubility before and after digestion in human milk was important in understanding the overall results in this work. The

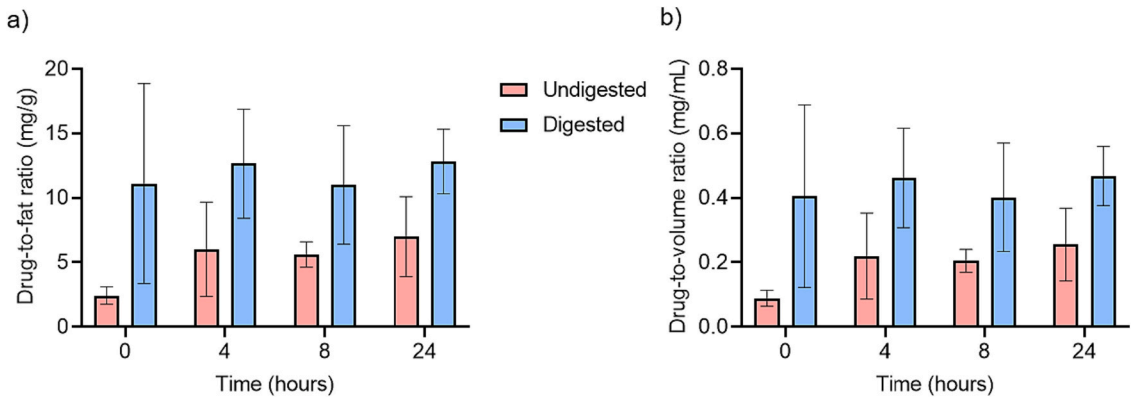

Fig. 3. Comparison between the amount of clofazimine solubilised in undigested or digested human milk after 0, 4, 8, and 24 h. The equilibrium solubility of clofazimine is expressed as (a) ratio of solubilised drug to gram of fat in the human milk and (b) ratio of drug that would be solubilised per mL of human milk. Data is presented as mean ($n = 3$) ± standard deviation.

solubility of clofazimine in pre-digested and undigested milk upon incubation for 24 h was quantified and the final solubility value expressed as a ratio of solubilised clofazimine equivalent to fat (mg/g) (Fig. 3a). The use of a drug-to-fat ratio (mg/g) in this context allowed for clearer comparison between samples of differing fat contents or volumes of human milk would be required. The results confirmed that clofazimine dissolved at a higher drug-to-fat ratio in pre-digested human milk than undigested human milk, 11.2 mg/g and 4.3 mg/g fat respectively. (Fig. 3a). The increase in the amount of solubilised clofazimine in the digested human milk sample was confirmed to be significantly higher ($P \leq 0.001$) following statistical analysis. This demonstrated there was over a three-fold increase in the extent of solubilisation of clofazimine in the digested human milk, confirming that the weakly basic drug was more soluble in the fatty acid and monoglyceride-rich digestion products than triglycerides in the undigested milk. Overall, the equilibrium solubility of clofazimine in the digested human milk was reached rapidly, with no large increase in measured drug-to-fat ratio from the initial time point to the four-hour timepoint. This result suggests that the maximum drug-to-fat ratio of 11.2 mg/g fat could be achieved within an appropriate physiological time period where transition of drug dosage forms throughout the small intestine can average three hours [64]. For this *in vitro* solubility study, there were significant difficulties sampling the clofazimine human milk suspension. As an excess of clofazimine was included for the equilibrium solubility study, the excess hydrophobic and unsolubilised clofazimine appeared to begin to form larger aggregates that prevented homogenous sampling (Fig. S5). This sampling difficulty is reflected in the results where some uncertainty is seen across the samples. For *in vivo* studies and further application this inhomogeneity issue would not be expected as more physiologically appropriate doses would be chosen and solubilised clofazimine would be absorbed from the site of delivery.

Further contextualisation of the equilibrium solubility values involved expression as a ratio of drug that could be solubilised in one millilitre of human milk with an equivalent fat content. The quantitative drug-to-volume of human milk ratio results shows that an appropriate dose of clofazimine could be delivered in human milk to premature infants. The equilibrium drug-to-volume ratio for clofazimine was approximately 0.19 mg/mL and 0.43 mg/mL in undigested and digested human milk (Fig. 3b). Hence, for an infant with a weight of 2.5 kg and a required dose of 1 mg/kg/day [65] (2.5 mg total dose), a full dose could be administered within approximately 6 mL of human milk, allowing for variability in the fat content of the human milk sample. This small volume is ideal for premature infants who often display lower milk intake on a weight scaled basis and may require additional fluid intake from other medical therapies, restricting their overall daily fluid intake [66].

It should be noted that there was a marked difference between the drug-to-fat ratio determined from the dynamic *in vitro* digestions and the equilibrium solubility study, 304 mg/g and 11.2 mg/g respectively. For the *in vitro* digestion studies, clofazimine was introduced to the human milk under gastric conditions prior to transition into intestinal conditions. For the equilibrium studies, the solubility of clofazimine was determined in human milk at pH 6.5 following digestion under gastric and intestinal conditions. It is possible that during the dynamic studies the suspension became supersaturated under gastric conditions. A previous study by Zhang et al. investigating the design of clofazimine nanoparticles observed similar solubilisation behaviour, with increased (> 90 times) dissolution and supersaturation levels seen across gastric and intestinal fluid conditions compared to equilibrium solubility measurements [67].

3.6. Comparison between solubilisation behaviour of clofazimine in human milk relative to alternative lipid-based formulations

In this study, the use of human milk as a novel milk-based formulation was investigated. Previous literature has assessed the solubilisation behaviour of a range of model poorly water-soluble drugs in alternative milk-based formulations and hence may be used for comparison. As mentioned, human milk may display advantages as a drug delivery vehicle for premature infants over alternative milk-based formulations.

In a previous study published by our group [9] the solubilisation behaviour of a 50 mg dose of clofazimine was assessed in bovine milk, infant formula, a long chain triglyceride (LCT) emulsion and a medium chain triglyceride (MCT) emulsion. The brand of infant formula was not disclosed, however it was reported to be a commercial, low lactose brand. The MCT emulsion consisted of triglycerides of a caprylic/capric acid mix and the LCT emulsion contained high amounts of *sn*-2 palmitate. By tracking a representative diffraction peak ($q = 0.96$ Å^{-1}), the solubilisation behaviour of clofazimine in each vehicle could be monitored. The clofazimine API was found to be completely solubilised in the LCT and MCT emulsions, but exhibited incomplete solubilisation in bovine milk and infant formula following 30 min of digestion (Fig. S6 a and b). As a uniform fat content (3.8%) was used across all the lipid-based formulations it was hypothesised that differences in the lipid composition, namely the chain length and saturation of the fatty acid species resulting from the digestion process, may have influenced the solubilisation behaviour of clofazimine. Milk-based vehicles with varying lipid compositions have shown different self-assembly lipid behaviour during digestion, with human milk, infant formula and bovine milk displaying varied liquid crystalline structures [68]. Furthermore, the *in vivo* absorption of a model poorly water-soluble drug, cinnarizine, has

been shown to be dependent on the liquid crystalline structures formed by the lipid-based delivery vehicle [69].

The MCT emulsion had the greatest solubilisation capacity for clofazimine, followed by LCT, infant formula and bovine milk. Although not disclosed in the study, infant formula typically contains a higher quantity of medium-chain lipids compared to bovine milk. Human milk used within this study displayed a higher abundance of long chain free fatty acids than bovine milk, in particular oleic acid (23.1% to 7.2%) and linoleic acid (12.7% to 0.6%) (Table 1). Compared to the previous study, human milk displayed similar clofazimine solubilisation behaviour to the MCT and LCT. Although *in vitro* digestions of clofazimine at higher doses were not performed in the MCT and LCT mixes, the equilibrium solubility of clofazimine was found to be comparable in human milk. The MCT mix with corresponding fatty acids demonstrated a higher equilibrium solubility than the LCT mix, with drug-to-fat ratios of 12.1 $\pm$ 0.5 and 6.8 $\pm$ 0.5 mg drug/g lipid, respectively. As discussed above, the equilibrium solubility of clofazimine in digested human milk was 11.2 mg drug/g lipid (Fig. 3a). This indicates that although human milk did not display a higher solubilisation capacity than the MCT mix, it would perform comparably as a drug delivery vehicle. This result has positive implications for the use of human milk as a delivery vehicle in low resource or economy settings where human milk may be more readily available than commercial lipid formulations. Access to affordable medicines in low- and middle-income settings has been acknowledged by the World Health Organisation as a healthcare challenge [70]. In order to address the needs of these countries, medicines should be readily available, accessible and affordable [71]. Commonly used excipients in lipid-based formulations, such as synthetic surfactants, fatty acid derivatives and triglycerides are often costly as a result of processing and may require infrastructure that is not readily available in low- and middle- income countries [72]. Although regulatory challenges exist for human milk as a drug delivery vehicle, as a ready source of healthy fat as an excipient, intended to be used locally rather than as a marketed product, human milk may be advantageous as a low-cost delivery vehicle for clofazimine and other drugs.

4. Conclusion

This work has shown the potential for application of human milk for drug delivery to premature infant patients. The digestion process and liberation of free fatty acids was essential for the solubilisation of the poorly water-soluble drug clofazimine. This importance was further confirmed by equilibrium studies that showed a marked increase in the solubility clofazimine in digested human milk. Initial analysis of the lipid composition of the human milk samples showed a higher abundance of medium to long chain fatty acids, in particular oleic, linoleic and palmitic acid, following digestion. The unique composition of fatty acids in the human milk proved to be more effective at solubilising clofazimine compared to the alternative lipid-based vehicles of infant formula and bovine milk [9]. Further compositional analysis of the lipids and micronutrients in the human milk showed some variability between donors, highlighting the potential regulatory hurdles to the use of whole milk, however further work must be completed to assess how large of a role this variability would play in drug solubilisation during digestion.

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CRediT authorship contribution statement

Ellie Ponsonby-Thomas: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Malinda Salim:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Laura D. Klein:** Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. **Andrew J. Clulow:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Susi Seibt:** Methodology, Data curation. **Ben J. Boyd:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2023.08.037.

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