European Journal of Pharmaceutical Sciences 194 (2024) 106681

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Developing an *in vitro* lipolysis model for real-time analysis of drug concentrations during digestion of lipid-based formulations

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ARTICLE INFO

Keywords:
Lipid-based formulations
In vitro lipolysis
Fenofibrate
Lipid digestion

ABSTRACT

Understanding the effect of digestion on oral lipid-based drug formulations is a critical step in assessing the impact of the digestive process in the intestine on intraluminal drug concentrations. The classical pH-stat *in vitro* lipolysis technique has traditionally been applied, however, there is a need to explore the establishment of higher throughput small-scale methods. This study explores the use of alternative lipases with the aim of selecting digestion conditions that permit in-line UV detection for the determination of real-time drug concentrations. A range of immobilised and pre-dissolved lipases were assessed for digestion of lipid-based formulations and compared to digestion with the classical source of lipase, porcine pancreatin. Palatase® 20000 L, a purified liquid lipase, displayed comparable digestion kinetics to porcine pancreatin and drug concentration determined during digestion of a fenofibrate lipid-based formulation were similar between methods. In-line UV analysis using the MicroDISS Profiler™ demonstrated that drug concentration could be monitored during one hour of dispersion and three hours of digestion for both a medium- and long-chain lipid-based formulations with corresponding results to that obtained from the classical lipolysis method. This method offers opportunities exploring the real-time dynamic drug concentration during dispersion and digestion of lipid-based formulations in a small-scale setup avoiding artifacts as a result of extensive sample preparation.

1. Introduction

The delivery of poorly water-soluble drugs in lipid-based formulations (LBFs) is well-established for enhancing oral bioavailability, but a key challenge facing formulators is to identify as early in the development cycle whether a drug displays properties that would be suited to an LBF. Multiple biorelevant *in vitro* lipolysis models have been developed with varying complexity and number of gastro-intestinal compartments investigated (Fernandez et al., 2009; Minekus et al., 2014; Sek et al., 2002; Zangenberg et al., 2001). These models are designed to more closely simulate the *in vivo* conditions, with varying degrees of complexity, which make them more suitable for formulation characterisation of late-stage LBFs such as clinical or commercial lead formulations. There is a need to advance the *in vitro* lipolysis method to support early-stage screening and development of LBFs, and to advance the use of in-line analytic tools that allow following dynamic real-time concentrations (Kuentz, 2019).

The most commonly utilised *in vitro* lipolysis method to test LBFs is in a pH-stat method. In this method the pH of digestion media is controlled throughout the experiment by the addition of sodium hydroxide to counteract the free fatty acids which are formed due to digestion of LBFs. This method employs relatively high quantities of both media and drug compound involving extensive sample handling and analysis, which limits its application in a high-throughput industrial screening setting (Williams et al., 2012). In early drug development, there are typically only small quantities of drug candidates available, therefore screening methods can operate with limited drug quantities. The sampling must be completed by off-line analytics where samples are removed and

List of abbreviations: FFA, free fatty acid; HPLC, high-performance liquid chromatography; LFCS, lipid formulation consortium system; SAXR, small-angle X-ray; UV, ultra violet.

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https://doi.org/10.1016/j.ejps.2023.106681
Received 22 September 2023; Received in revised form 22 November 2023; Accepted 19 December 2023
Available online 20 December 2023

analysed with high-performance liquid chromatography (HPLC) (Kuentz, 2019). In addition, real-time in-line analytic tools to detect the dynamic drug concentration in micellar systems are valuable to avoid artifacts as result of the extensive sample preparation with phase separation and analysis (Kuentz, 2019). The *in vitro* lipolysis model has been optimised by different research groups to develop methods that comply with the request for higher throughput, smaller quantities, and real-time analytics. Mosgaard et al. (2015, 2017) developed an *in vitro* lipolysis model and media for a 96-well plate format. While this method advanced the opportunity for high throughput screening, this model uses off-line analytics with HPLC. Khan et al. (2022) developed a small-volume lipolysis method using real-time analysis with small-angle X-ray (SAXS) to follow digestion products and drug solubility during digestion; however, this sophisticated method has a low throughput and a need for a high capital investment. This is similar to the practical limitations of raman spectroscopy for in-line analytics during *in vitro* formulation digestion (Stillhart et al., 2013).

Porcine pancreatin is typically used as the source of lipase in lipolysis models to digest lipids, including the model described by the lipid formulation consortium system (LFCS) (Williams et al., 2012). Porcine pancreatin is an extract from pig intestine and contains several digestive enzymes such as amylase, trypsin, lipase, ribonuclease, and protease (MERCK, 2023). The crude extract is believed to contribute to high turbidity and high light scattering when dispersed in water, making it unsuitable for in-line ultra-violet (UV) detection (Phan et al., 2015). Lipases are enzymes that can catalyse the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, free fatty acids (FFAs), and glycerol, and can be found in most organisms from the microbial, plant, and animal kingdoms (Aloulou et al., 2006). More recently, purified liquid lipases have become commercially available, as an aqueous solution with a stabiliser to prevent enzyme denaturation, and a preservative to inhibit microbial growth. In addition, immobilised lipases are also commercially available. This is where the lipase enzymes are immobilised in their activated form on a carrier, with the advantage of making the enzyme more stable and facilitating the ability to withdraw samples from media without the need to add lipolysis inhibitors to halt the digestion (Facin et al., 2019; Phan et al., 2015). In previous *in vitro* permeation studies, the non-specific immobilised lipase, Novozym® 435, has been used as an alternative to crude porcine pancreatin, as it has been reported to be compatible with the Caco-2 cell monolayer used for permeation studies (Keemink et al., 2019; Keemink and Bergström, 2018). Novozym® 435 has been reported to form a medium that causes less scattering and turbidity which makes it compatible with UV detection and SAXS and to digest the formulations and excipients tested. However, in general, a smaller extent of digestion and a prolonged digestion time was reported when compared to porcine pancreatin (Alvebratt et al., 2020; Keemink et al., 2019; Keemink and Bergström, 2018; Phan et al., 2015).

The aim of this work was to develop an *in vitro* lipolysis model for real-time analysis of drug concentration during the dispersion and digestion of LBFs. Firstly, a range of purified liquid and immobilised lipases were explored with the goal of establishing conditions that facilitate in-line UV detection of drug concentration under digestion conditions. Secondly, a range of experimental conditions were explored with these different lipases to explore impact of calcium, lipid load, and drug concentration on the kinetics of lipolysis. Finally, a feasibility study was conducted using the MicroDISS Profiler™ for detection of drug concentration upon dispersion and digestion of LBF. The MicroDISS Profiler™ has previously been used for determining in-line real-time drug concentrations in LBFs (Bennett-Lenane et al., 2021; Keemink et al., 2019). It offers opportunity to determine in-line drug concentrations with fiber optic UV spectroscopy with eight probes simultaneously and are temperature and stirring controlled (Avdeef and Tsinman, 2008).

Table 1
Composition of the LBFs investigated.

Formulation	Excipients
Medium-chain LBF	40 % Miglyol 812 40 % Tween 85 20 % Cremophor RH 40
Long-chain LBF	40 % Olive oil 40 % Tween 85 20 % Cremophor RH 40
Surfactant-only	67 % Tween 85 33 % Cremophor RH 40
LFCS IIIA medium-chain	32.5 % Captex 300 32.5 % Capmul MCM 35 % Tween 85
LFCS IIIA long-chain	32.5 % Olive oil 32.5 % Maisine CC 35 % Tween 85
LFCS surfactant-only	50 % Cremophor EL 50 % Transcutol HP

2. Materials and methods

2.1. Materials

The lipases, Novozym® 435, Lipozyme® RM, Lipozyme® CALB L, NovoCor® AD L, Lipozyme® TL 100 L, and Palatase® 20000 L were purchased from Strem Chemicals Ltd (Suffolk, UK), and the immobilised lipase from thermomyces lanuginous was purchased from Sigma-Aldrich (Schnelldorf, Germany) (referred to as Sigma beads). Porcine pancreatin, corn oil, Tween 85, and olive oil were purchased from Sigma-Aldrich (Gillingham, UK). Capmul MCM and Captex 300 were kindly donated by ABITEC Corp (Wisconsin, USA), Cremophor EL and Cremophor RH 40 (Ludwigshafen, Germany) were donated by BASF, and the Transcutol HP and Maisine CC were donated from Gattefossé (Saint-Priest, France). Miglyol 812 was purchased from IOI GmbH (Witten, Germany), and fenofibrate from Kemprotec Ltd. (Carnforth, UK). The FaSSIF/FeSSIF/FeSSGF powder was purchased from Biorelevant.com (London, UK). All other chemicals were purchased from Sigma-Aldrich (Gillingham, UK). Water used for the experiments was obtained from a MilliQ water system.

2.2. Methods

2.2.1. Formulations

A medium-chain LBF, long-chain LBF, and surfactant-only formulation that previously have been investigated *in vitro* and *in vivo* (Bennett-Lenane et al., 2021; Griffin et al., 2014), and three formulations tested and described, the class IIIA medium-chain LBF (LFCS IIIA medium-chain), class IIIA long-chain (LFCS IIIA long-chain), and class IV (LFCS surfactant-only) (Williams et al., 2012) were investigated in this study. The compositions of all formulations can be found in Table 1.

The formulations were prepared by weighing in exact amounts of the excipients into glass vials and incubating them at 37 °C and 300 rpm stirring overnight.

The medium-chain LBF, long-chain LBF, and surfactant-only were also investigated with fenofibrate dissolved in the formulation. The formulations were prepared as previously described by Griffin et al. (2014) and included 80 mg/g fenofibrate that was added to the lipid blend. The formulation was mixed at 50 °C and 300 rpm for approximately 30 min followed by 37 °C overnight to allow the fenofibrate to dissolve.

2.2.2. pH-stat method

The *in vitro* lipolysis experiments were performed using a pH-stat apparatus (Metrohm AG, Herisau, Switzerland) comprising an 836 Titando, 804 Ti Stand, pH electrode (Metrohm), and two 800 Dosino 20 mL dosing units. The system was operated by the Tiamo 2.5 software

Table 2
Overview of the lipases with name, origin, immobilised/liquid, selectivity, and concentration used in digestion experiments. The LU/g reflect the amount of enzyme which generates 1 μmol of butyric acid from glycerol tributyrate per minute, whereas PLU/g reflects the amount of enzyme which generates 1 μmol of propyl laurate per minute.

Lipase	Origin	Immobilised/liquid	Selectivity	Concentration	Refs.
Porcine pancreatin	Porcine	Liquid	Sn-1,3	8 x USP	
Novozym® 435	C. Antarctica B	Immobilised	Non	125 PLU/mL	(Strem Chemicals, 2022a)
Sigma beads	T. Lanuginosus	Immobilised	Sn-1,3	125 LU/mL	
Lipozyme® RM	R. Miehei	Immobilised	Sn-1,3	6.25 LU/mL	(Strem Chemicals, 2022b)
Lipozyme® CALB L	C. Antarctica B	Liquid	Non	125 LU/mL	(Strem Chemicals, 2022c)
Palatase® 20000 L	R. Miehei	Liquid	Sn-1,3	125 LU/mL	(Strem Chemicals, 2022d)
Lipozyme® TL 100 L	T. Lanuginosus	Liquid	Sn-1,3	125 LU/mL	(Strem Chemicals, 2022e)
NovoCor® AD L	C. Antarctica A	Liquid	Non	125 LU/mL	(Strem Chemicals, 2022f)

(Metrohm).

The compositions of the media were: (i) 2 mM tris maleate and 150 mM NaCl, and (ii) 2 mM tris maleate, 150 mM NaCl, and 1.06 mM $CaCl_2$. The pH of both buffers was adjusted to pH 7.5 as previously described by O'Dwyer et al. (2020) and Koehl et al. (2019). The studies were conducted at pH 7.5 to be in line with previous studies (O'Dwyer et al., 2020; Koehl et al. 2019) and to limit the effect of fatty acid ionisation that has been reported at lower pH (Williams et al., 2012). pH 7.5 was also considered the optimal pH across all lipases as per manufacture recommendations. The buffers were supplemented with FaSSIF powder a day prior to the experiment resulting in a composition of the final digestion media of 3 mM taurocholate and 0.75 mM phospholipids.

A range of immobilised and liquid lipases was investigated and an overview of the lipases including origin, immobilised/liquid, selectivity, and concentration used in digestion experiments are found in Table 2. The lipase concentrations selected were based on previous work with the Novozym® 435 (Keemink and Bergström, 2018). The immobilised and liquid lipases are ready to use. The porcine pancreatin extract (8 x USP) was reconstituted immediately prior to use by adding 5 mL of the aqueous buffer to 1 g of porcine pancreatin, vortexed thoroughly, and centrifuged at 2,900 g for 15 min at 4 °C (Centrifuge 5702 R, Eppendorf, Germany).

The LBF was introduced to the digestion buffer in a ratio of 1:40 (v/v) (high lipid load) or 1:200 (v/v) (low lipid load) and stirred for 15 min to disperse the formulation. Afterward, the pH was automatically adjusted to pH 7.5, and the digestion was initiated by adding the lipase. The volume of the digestion buffer was corresponding to a final volume of 40 mL after the lipase was added. The pH was maintained at pH 7.5 throughout the experiment by automatic titration with 0.6 M NaOH for the medium-chain formulations and 0.2 M NaOH for the long-chain formulations and the surfactant-only formulations. The amount of NaOH dispersed was recorded by the system and used to assess the rate and extent of digestion. After 60 min of digestion, the pH was back titrated to pH 9 to determine the release of non-ionised FFA. Post-experiment, a picture of the media was taken to assess visual turbidity.

Blank titrations with the biorelevant media alone were performed to account for the background digestion. The released mmol of FFA was subtracted from the mmol FFA released with formulations. The % digested was calculated as previously described by Koehl et al. (2020).

$$Theoretical\ FFA\ [mmol] = \frac{SV\ [mg]}{56.1056\ \left[\frac{g}{mol}\right]} \quad (1)$$

where theoretical FFA is the amount of FFA that theoretically can be released from the excipient in mmol, SV is the saponification value in mg KOH per gram of the excipient from the certificate of analysis. The 56.1056 g/mol is the molecular weight of KOH.

The extent digested was then calculated by the released FFA in mmol in the digestibility experiment including the amount detected by back titration to pH 9 divided by the theoretical amount of FFA (Eq. (2).

$$\%digested = \frac{released\ FFA\ [mmol]}{theoretical\ FFA\ [mmol]} \cdot 100\% \quad (2)$$

Experiments, where the formulation contained fenofibrate, were conducted with high lipid load and otherwise as described above. Samples of 1 mL were withdrawn 5, 10, 20, 40, and 60 min after the addition of lipase. Each sample was immediately treated with 1 M 4-bromophenylboronic acid in methanol (5 μL/mL) and mixed well to stop further digestion. The samples were kept at 37 °C until centrifugation. The samples were centrifuged at 37 °C and 21,000 g for 30 min (Mikro 200 R, Hettich, Germany). The supernatant was withdrawn and diluted in 80 % acetonitrile and 20 % 25 mM sodium acetate (pH 5.0). In samples digested with porcine pancreatin undissolved particles occurred post-dilution due to the porcine pancreatin. These samples were additionally centrifuged for 5 min at 6,000 g (Mikro 120, Hettich, Germany) to remove the undissolved particles, and the supernatant was collected for analysis as described below.

2.2.3. Quantification of fenofibrate

The concentration of fenofibrate in the samples was determined by reverse-phase-HPLC on an Agilent 1200 series system using a previously described method (Bennett-Lenane et al., 2021). In short, 50 μL samples were injected into an Agilent reverse C18 column (150 mm x 4.6 × 5 μm) at 25 °C; UV detection at 286 nm, mobile phase 80 % acetonitrile and 20 % 25 mM sodium acetate buffer (pH 5.0).

2.2.4. Data analysis

The data analysis was conducted in Excel. A one-way ANOVA was performed on the lipolysis data with a Bonferroni post hoc test to compare the performance of the different lipases under the different conditions. T-tests were performed for each lipase between lipolysis results with calcium vs. without calcium and the high vs. low lipid load. A significance level of $p < 0.05$ was used.

2.2.5. MicroDISS Profiler™

The experimental protocol was adopted from Bennett-Lenane et al. (2021). In short, fenofibrate solubility ($n = 3$) was determined in the MicroDISS Profiler™ (Pion Inc., USA). The instrument settings were a temperature of 37 °C and a stirring rate of 250 rpm. The path length of the *in situ* UV probe was 2 mm. Standard spectra were collected for each probe in 1:1 water and acetonitrile. The concentration range was 0 μg/mL–567.20 μg/mL, and a linear relationship ($r^2 > 0.99$) was established between fenofibrate concentration and absorbance. The experimental run was performed in four vials. The vials contained 15 mL FaSSIF media prepared from 29 mM phosphate buffer (pH 7.5), the formulation in the ratio of 1:200 (v/v) (medium-chain or long-chain LBF), and a cross-stirring bar. After 15–30 min of dispersion, fenofibrate was added to three of the vials. Approximately double the amount of fenofibrate previously reported to be soluble in the LBF dispersed in FaSSIF was added (Bennett-Lenane et al., 2021). Excess of the drug was added to account for the potential supersaturation upon digestion. After one hour of dispersion, Palatase® 20000 L was added corresponding to

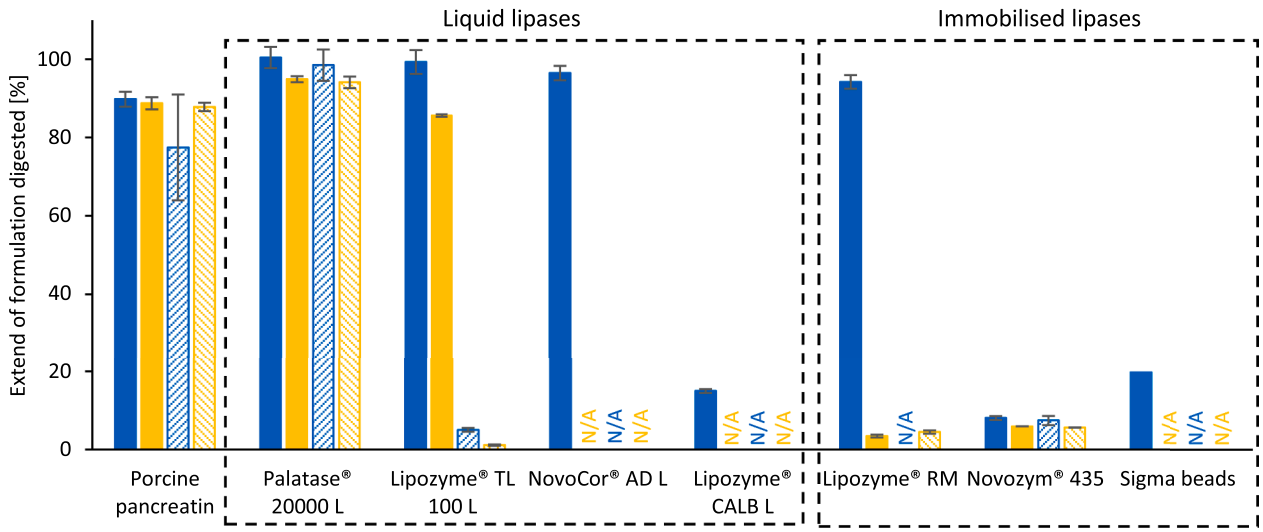

Fig. 1. Extent of digestion from the medium-chain LBF (40 % Miglyol 812, 40 % Tween 85, and 20 % Cremophor RH40) in percent from experiments performed in the pH-stat setup with experimental parameters: low lipid load with calcium (blue), high lipid load with calcium (orange), low lipid load without calcium (scattered blue), and high lipid load without calcium (scattered orange). The data shown is $n = 3 \pm$ SD. Experiments that have not been conducted are marked with "N/A".

125 PLU/mL. The digestion was running for three hours. An additional channel was used as blank to consolidate for potential issues with changes to the background of FaSSIF UV absorbance over time and upon digestion. The *in situ* UV scans were done every 30 s. Concentrations were determined by the area-under-the-curve in second derivative spectra (314–318 nm). The AuPRO software (version 7, Pion Inc, MA, USA) was used to interpret the data. The pH of the media was measured at the end of the experiment.

3. Results

3.1. Exploration of immobilised and liquid lipase as an alternative to crude porcine pancreatin extract

The extent of digestion of the medium-chain LBF was investigated across a range of different lipases using the classical lipolysis setup (Williams et al., 2012). The range of lipases included immobilised lipases (Novozym® 435, Lipozyme® RM, and Sigma beads), liquid lipases (Lipozyme® CALB L, Lipozyme® TL 100 L, Palatase® 20000 L, and NovoCor® AD L) and porcine pancreatin which was considered the reference conditions (Williams et al., 2012). The extent of digestion, corrected for the background digestion was initially investigated under conditions considered to be the most favorable for digestion. These conditions were a low lipid load and calcium present in the digest media, which has previously shown to be required (Alvarez and Stella, 1989; Devraj et al., 2013; Torcello-Gómez et al., 2018). Subsequently, if the lipases showed comparable digestion to porcine pancreatin, other conditions which include higher lipid load and digest media without calcium was used to investigate the robustness of the method. Palatase® 20000 L, Lipozyme® RM, NovoCor® AD L and Lipozyme® TL 100 L demonstrated comparable extent of digestion to porcine pancreatin under the initial digestion conditions. In contrast, Sigma beads and Lipozyme® CALB L displayed relatively poor extents of digestion and hence these enzymes were not progressed for further testing. Novozym® 435 also exhibited a poor level of digestion; since it has been previously employed in lipolysis setups, it was selected for further testing (Keemink et al., 2019; Keemink and Bergström, 2018). Although NovoCor® AD L showed the same extent of digestion as porcine pancreatin, it was not progressed because the media post-digestion appeared cloudy/turbid (see supplementary material, Fig. S1), hence it was anticipated that it was not compatible with UV detection. Palatase® 20000 L, Lipozyme® TL 100 L, Lipozyme® RM, and Novozym® 435 were subsequently evaluated under the different digestion conditions.

Generally, a correlation between the specificity of the lipases and the extent of digestion was observed. The lipases with sn-1,3-specificity, Lipozyme® RM, Palatase® 20000 L, and Lipozyme® TL 100 L, digested to a comparable extent as porcine pancreatin. The exceptions were the Sigma beads, which showed significantly lower digestion than the other sn-1-3 specific lipases, and NovoCor® AD L which showed significantly higher digestion than the other non-specific lipases. The sn-1,3-specific lipases are known to have a preference for the fatty acids in positions 1 and 3 on the glycerol backbone, whereas the non-specific lipases have no preference for the fatty acid position on the glycerol backbone (Soumanou et al., 2013).

The rate of digestion (as indicated by FFA release as a function of time) for the range of lipases is presented in Fig. 2. The amount of FFA released was generally higher for digestion with porcine pancreatin compared to the other lipases which would reflect a higher extent of digestion, however, these figures did not account for background digestion. Since porcine pancreatin shows high background digestion, the higher release of FFA was not necessarily equivalent to a higher extent of digestion; however, this was captured with digestion of the blank FaSSIF media to adjust for this background digestion. Palatase® 20000 L showed the digestion profile most similar to porcine pancreatin in all experimental conditions (see blue curves in Fig. 2 A1, B1, C1, D1). First, a steep incline in FFA release was observed in the first 10 min, followed by a plateau indicating that full digestion was reached. In general, the other lipases explored were less comparable to porcine pancreatin and reached a plateau within the first 20 min. While Lipozyme® TL 100 L and Lipozyme® RM showed comparable digestion kinetic to porcine pancreatin under the low lipid load conditions the digestion kinetic deviated considerably under high lipid load conditions. Lipozyme® TL 100 L showed a delay in release of FFA to around 30 min thereafter there was an indication of a plateau within 10 min (see yellow curve in Fig. 2 A2). Whereas Lipozyme® RM showed a significantly lower digestion profile caused by the low lipase activity (see blue curve in Fig. 2 B2). NovoCor® AD L showed a relatively slow and consistent increase in released FFA throughout the experiment indicating that digestion was ongoing when the experiment ended (see green curve in Fig. 2 A1).

Guided by the observations for the medium-chain formulation digestibility, the digestion of a selected range of lipases (Novozym® 435, Lipozyme® RM, Palatase® 20000 L, and Lipozyme® TL 100 L) was subsequently investigated for the digestion of the long-chain LBF. The

trend in the extent of digestion of the long-chain LBF broadly followed what was observed for the medium-chain LBF. The sn-1,3-specific lipases digested to the same extent as porcine pancreatin, whereas the non-specific lipases showed a significantly lower extent of digestion (Fig. 3). The rate of digestion also followed the same trend as seen for the medium-chain LBF (see supplementary material, Fig. S2).

3.2. Influence of calcium

Traditionally, calcium is used in the digestion media to remove FFAs from the oil-water interface, however, studies have shown that the concentration of calcium influences the solubilisation of drugs, disturbs the colloidal equilibrium and hence the solubilisation capacity of the system (Alvarez and Stella, 1989; Devraj et al., 2013; Torcello-Gómez et al., 2018). The influence of calcium in the biorelevant media was investigated in this study by having two arms of the study, one arm having the calcium concentration according to the standard lipolysis method and another arm without calcium in the biorelevant media. The results showed no significant difference between the extent of digestion when performed with or without calcium in the biorelevant media (Figs. 1 and 3). The only exception was Lipozyme® TL 100 L, where significantly lower digestion was observed in the experiments without calcium (Figs. 1 and 3). This most likely indicates that Lipozyme® TL 100 L was dependent on the calcium effect to remove fatty acids to retain a suitable enzymatic activity, whereas the other lipases did not appear to be as heavily influenced by calcium. However, calcium had a visual influence on the degree of turbidity of the media post-digestion for Palatase® 20000 L. In the experiments with calcium the media developed into cloudy turbid media after digestion, whereas the medium was clearer in the experiments without calcium (see supplementary material, Fig. S1) due to low presence of precipitated fatty acids.

3.3. Influence of lipid load

Two different lipid loads were investigated using the standard lipid load of 1:40 lipid to biorelevant media which in this study is referred to as the high lipid load (Williams et al., 2012), and a low lipid load with a ratio of 1:200 lipid to biorelevant media as have been used in previous studies (Bennett-Lenane et al., 2021). Generally, no difference in the range of digestion was seen between the experiments with high and low lipid loads for the medium-chain LBF (Fig. 1) which indicates that an excess of lipase was present at the low lipid load experiment, and therefore the lipase ability to digest was the limiting factor, rather than the lipase concentration used. Lipozyme® RM was an exception, it displayed a significantly lower extent of digestion in experiments with high lipid load. This was most likely caused by the lipase activity of Lipozyme® RM, as from an experimental point of view it was not possible to run the experiments with the same lipase concentration of 125 PLU/mL as in the other experiments because the increased number of beads in the vessel would disrupt the stirring (Keemink et al., 2019; Keemink and Bergström, 2018). Therefore, a lower activity of 6.25 LU/mL was necessary in the experiments with Lipozyme® RM.

In the case of long-chain LBF, there was a clear impact of lipid load on the extent of digestion (Fig. 3) where a lower percentage of digestion was observed under high lipid load conditions (Fig. 3). This may have reflected that lipases have a lower activity for long-chain triglycerides compared to medium-chain and short-chain triglycerides, as shown in previous studies (Han et al., 2009; Sek et al., 2002; Williams et al., 2012).

3.4. Post-digestion medium

In the classical lipolysis setup with porcine pancreatin extract, the digestion medium develops a turbid/cloudy appearance that is reflective of extensive light scattering in the post-digestive media. This highly turbid medium limits the application of in-line UV detection of drug concentration. To allow visual comparison of the extent of turbidity developed during digestion, images of the media were taken in the pH-stat setup after the digestion experiments (see supplementary material, Fig. S1). The key observation from these experiments was that Palatase® 20000 L without calcium in the digest media resulted in a transparent clear media, with less scattering when compared to porcine pancreatin, which was considered favourable in terms of in-line UV detection (see supplementary material, Fig. S1).

3.5. Assessing suitability of Palatase® 20000 L for digestion of a range of lipid formulations

The digestion with Palatase® 20000 L was further assessed using three different LBFs to establish the ability of the lipase to digest across different formulations. The digestion experiments were conducted with the LFCS formulations IIIA medium-chain, IIIA long-chain, and LFCS surfactant-only (Williams et al., 2012). Palatase® 20000 L displayed similar extents and rates of digestion for the LFCS IIIA medium-chain and long-chain formulations as porcine pancreatin (Fig. 4). In contrast, the digestion of the LFCS surfactant-only showed a higher extent of digestion with Palatase® 20000 L compared to porcine pancreatin (Fig. 4B). Interestingly, the same trend for higher digestion of surfactant-only formulations was observed during the digestion of a formulation consisting of Cremophor RH40:Tween 85 (see supplementary material, Fig. S3).

3.6. Drug distribution in post-digested LBF

Given the general comparability in digestion kinetics observed between porcine pancreatin and Palatase® 20000 L, a comparison of drug concentration observed during digestion was subsequently evaluated. Formulations loaded with 80 mg/g fenofibrate were prepared and investigated by digestion in the pH-stat setup. Fenofibrate concentration in the aqueous digest media after 60 min of digestion were determined (as presented in Fig. 5). The medium-chain and long-chain formulation showed no significant differences between the concentration of fenofibrate in the aqueous phase after one hour of digestion with Palatase® 20000 L and porcine pancreatin. Whereas a significant difference was found for the surfactant-only formulation ($p = 0.019$), which most likely caused by the difference in digestion characteristics between Palatase® 20000 L and porcine pancreatin as described above (see Fig. 4B and supplementary materials, Fig. S3).

3.7. Real-time in-line detection of drug concentration upon digestion

The MicroDISS Profiler™ was employed to assess real-time drug concentration of fenofibrate during dispersion and digestion of LBFs. Palatase® 20000 L showed that determination of fenofibrate concentration was possible with the in-line UV probes throughout one hour of dispersion followed by three hours of digestion (Fig. 6). The experimental setup was built on a previous setup which monitored the drug concentration upon dispersion of LBFs in FaSSIF (Bennett-Lenane et al., 2021). FaSSIF was prepared from phosphate buffer because previous studies have shown it is compatible with UV-detection, contrary to tris-maleate buffer which was not feasible to use for determination of drug concentration because it is a chromophore and absorbs light. The experiment was initiated by adding excess of fenofibrate to a blank LBF dispersion in FaSSIF, as depicted in Fig. 6 the in-line analysis showed gradual dissolution of drug during the one hour of dispersion. Digestion was initiated after one hour by addition of Palatase® 20000 L. UV detection was maintained throughout the three hours of digestion where dynamic drug concentration was determined and showed that drug concentration gradually declined indicating a digestion-induced reduced solubilisation capacity as digestion proceeded. A plateau in drug concentration appeared after 130 min for the medium-chain LBF and 160 min for the long-chain LBF which allows an estimate of the

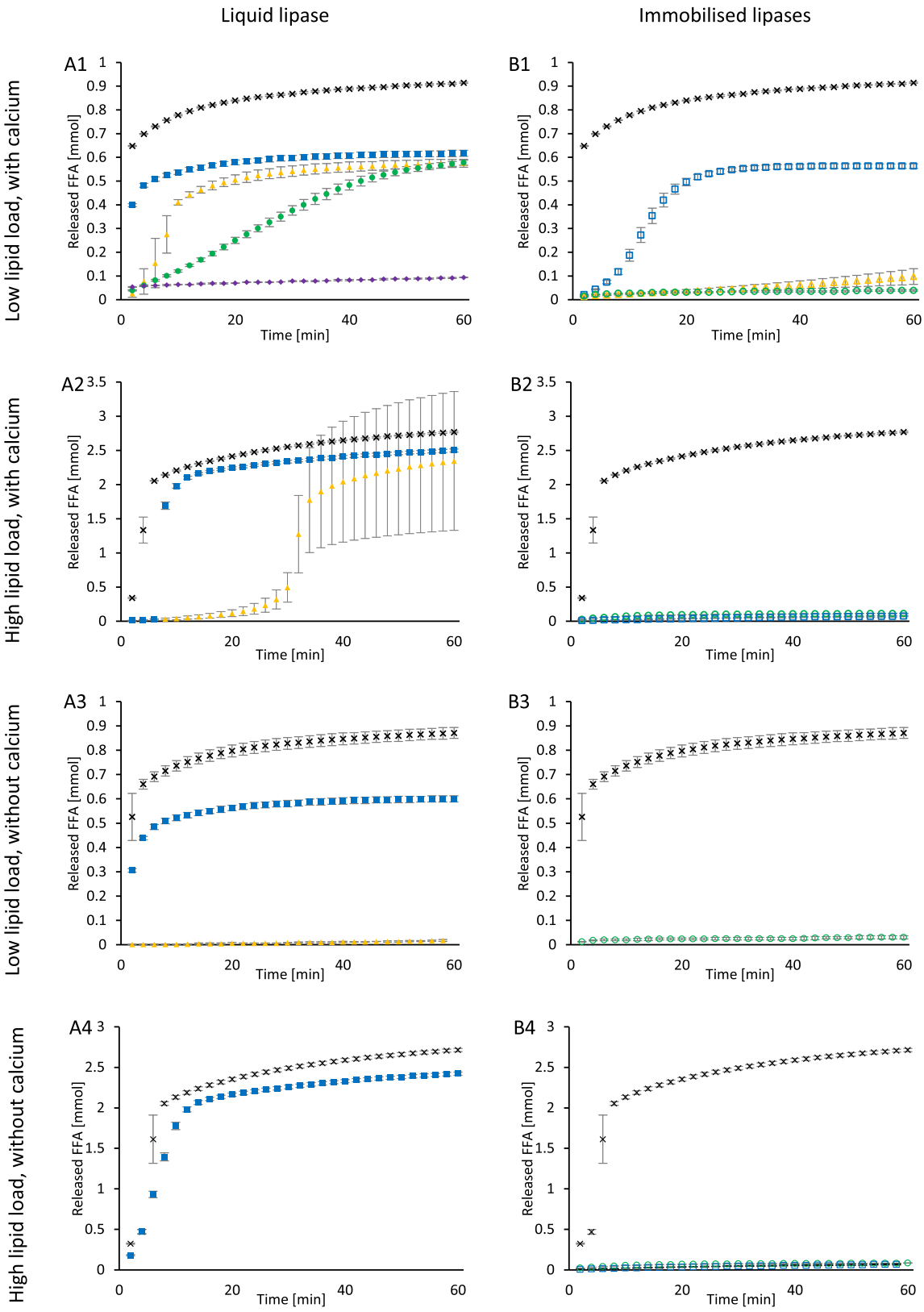

Fig. 2. Digestion profiles from pH-stat experiments showing the release of FFA versus time for the range of lipases investigated. The digestion profiles are for the medium-chain LBF (40 % Miglyol 812, 40 % Tween 85, and 20 % Cremophor RH40). Porcine pancreatin (×) is in all graphs as a reference. The A figures are liquid lipases: Palatase® 20000 L (■), Lipozyme® TL 100 L (▲), NovoCor® AD L (●), and Lipozyme® CALB L (♦), and the B figures are the immobilised lipases: Lipozyme® RM (□), Sigma beads (△), and Novozym® 435 (○). Experiments with low lipid load (1:200) are shown in 1 and 3, and high lipid load (1:40) are shown in 2 and 4. Experiments with calcium are shown in 1 and 2, and experiments without calcium are shown in 3 and 4. Every 24th data point is shown in the graph (i.e., a data point every 2 min) to improve graph legibility. The data shown is $n = 3 \pm$ SD.

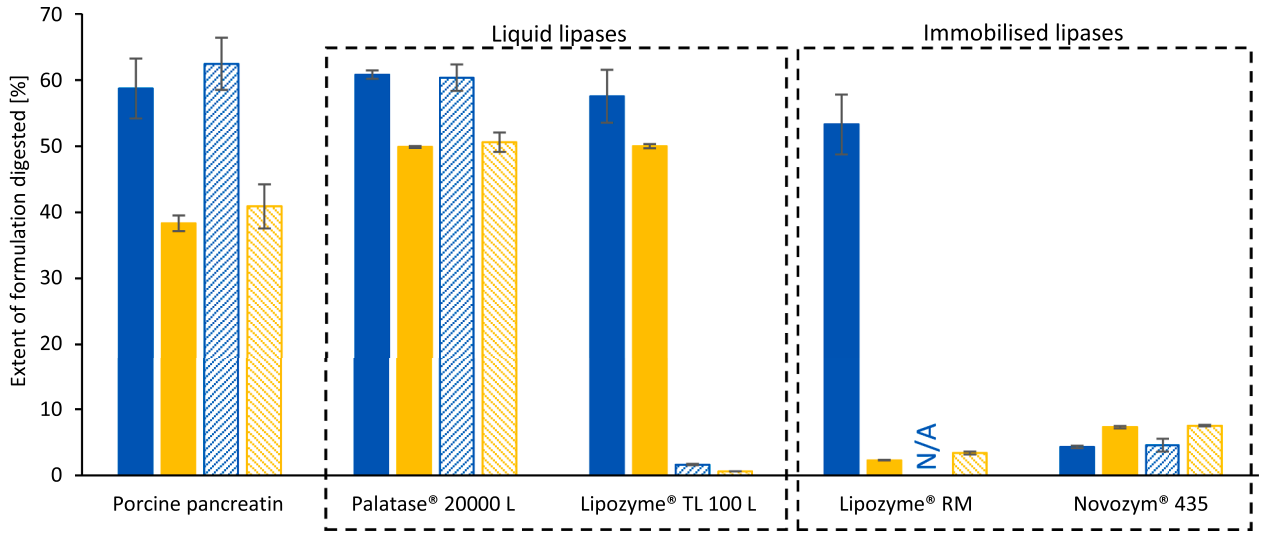

Fig. 3. Extent of digestion from the long-chain LBF (40 % Olive oil, 40 % Tween 85, and 20 % Cremophor RH40) in percent from experiments performed in the pH-stat setup with experimental parameters: low lipid load with calcium (blue), high lipid load with calcium (orange), low lipid load without calcium (scattered blue), and high lipid load without calcium (scattered orange). The data shown is $n = 3 \pm$ SD. Experiments that have not been conducted are marked with "N/A".

solubilisation capacity of the post-digestive media.

Given that there was no continual adjustment of pH in the MicroDISS setup, the pH was determined at the start and end of the experiment. A pH drop from the initial pH of 7.5 to 6.560 ± 0.002 and 7.006 ± 0.004 was seen for medium-chain LBF and long-chain LBF, respectively.

4. Discussion

With an increasing focus on early-stage drug development to inform formulation selection for the clinical and/or commercial formulation, it is imperative to develop small scale, biorelevant, *in vitro* methods for screening the impact of digestion on prototype formulations. The advantages of real-time analytics to inform about formulation performance range from a finer resolution of kinetics to avoidance of issues caused by analytical sampling (Kuentz, 2019). While previous studies have advanced the use of immobilised lipases (Keemink et al., 2019; Keemink and Bergström, 2018; Phan et al., 2015), this is to the best of our knowledge the first study that explores these types of lipases more systematically and explores the use of in-line UV detection method to access drug concentration during *in vitro* digestion executed with a liquid lipase.

4.1. Palatase® 20000 L as alternative to crude porcine pancreatin for real-time analytics in lipolysis experiments

Four liquid lipases have been studied in the present work, Palatase® 20000 L, Lipozyme® TL 100 L, Lipozyme® CALB L, and NovoCor® AD L. Palatase® 20000 L was found to be the most suitable alternative to porcine pancreatin based comparable digestion kinetics. For the medium-chain and long-chain formulations evaluated in this study, the extent of digestion was similar to porcine pancreatin (Figs. 1 and 3). The digestion rate profile of Palatase® 20000 L was approximately 30 % lower for the low lipid load and 10 % lower for the high lipid load medium-chain formulation, and approximately 55 % and 10 % lower for the long-chain formulation, respectively. This is reflecting a lower background digestion with Palatase® 20000 L relative to porcine pancreatin (Fig. 2 and supplementary material, Fig. S2). Importantly, in the context of UV probes, the media post-digestion with Palatase® 20000 L showed to be clearer and more transparent, which could make it compatible with UV detection (see supplementary material, Fig. S2). Further Palatase® 20000 L was found to be suitable for digestion across a range of LBFs and similar extent of digestion were observed for medium-chain and long-chain LBFs relative to porcine pancreatin. A comparison of fenofibrate drug concentration after one hour of digestion in the standard pH-stat set up between Palatase® 20000 L and porcine pancreatin demonstrated that the solubilisation capacity was similar in the digest media across three different formulations (Fig. 5).

Three immobilised lipases were investigated, Novozym® 435, Lipozyme® RM, and Sigma beads. Novozym® 435 has previously been used in lipolysis-permeation studies as an alternative to porcine pancreatin and has been reported to be compatible with Caco-2 cells, and UV detection (Alvebratt et al., 2020; Keemink et al., 2019; Keemink and Bergström, 2018). This study showed that digestion with Novozym® 435 was lower relative to porcine pancreatin, and that longer digestion times were needed to achieve the possible complete digestion in accordance with previous reports (Keemink et al., 2019; Keemink and Bergström, 2018; Phan et al., 2015). The digestion of the medium-chain LBF and long-chain LBF tested in this work with Novozym® 435 did not show a similar extent of digestion. The digestion experiments showed that Novozym® 435 had a substantially lower extent of digestion compared to porcine pancreatin (Figs. 1 and 3). One possible reason to explain the discrepancy may be that Novozym® 435 is a non-specific lipase whereas porcine pancreatin is an sn-1,3-specific lipase (May and Nesaretnam, 2014; Strem Chemicals, 2022a; Utama et al., 2019). Another explanation could be that the active site of the lipase had more difficulty reaching the lipids because it is adsorbed to a solid carrier (Murty et al., 2002).

4.2. Feasibility study in MicroDISS Profiler™

This study demonstrated the viability of using Palatase® 20000 L to simulate digestion and facilitate monitoring in-line real-time drug concentration using the MicroDISS Profiler™ upon one hour of dispersion and three hours of digestion. The benefit of this method includes in-line determination of drug concentration profile as a function of time, both during dispersion and digestion phase, in contrast to the classical pH-stat setup which involves extensive off-line analysis to determine drug concentrations. The obtained results showed similar results as obtained in the classical lipolysis setup where the concentration of the model compound, fenofibrate, decreased during digestion caused by digestion-induced decrease in solubility capacity (Fig. 5). While the main application of this method is seen in the context of high throughput screening and not as a direct alternative to the classical pH-stat method, the application presented herein demonstrates that a direct estimate of the

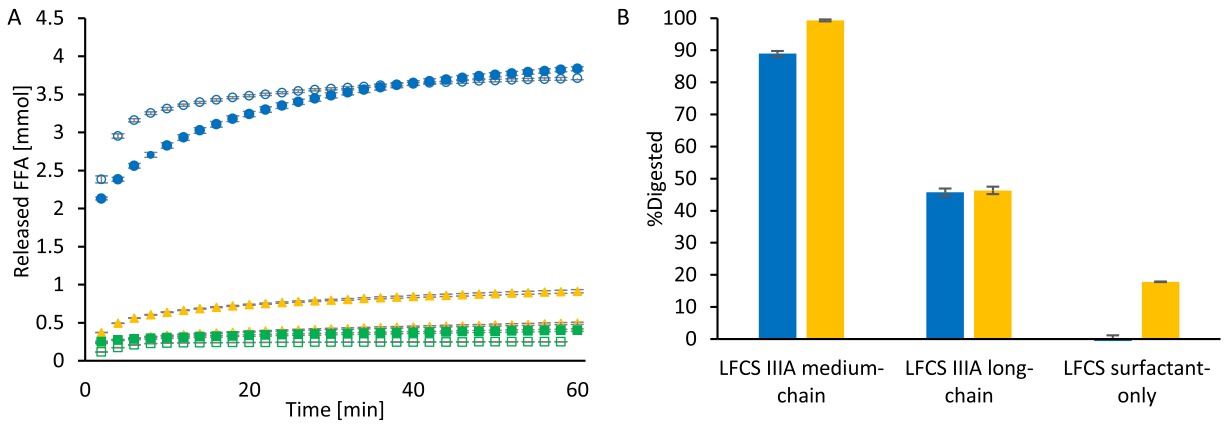

Fig. 4. Digestion results from the pH-stat experiment with the LFCS IIIA medium-chain (32.5 % Captex 30, 32.5 % Capmul MCM, and 35 % Cremophor EL), the LFCS IIIA long-chain (32.5 % Corn oil, 32.5 % Maisine CC, and 35 % Cremophor EL), and the LFCS surfactant-only (50 % Cremophor EL and 50 % Transcutol HP). A) shows the release of FFA upon digestion of LFCS IIIA medium-chain digested with porcine pancreatin (●), LFCS IIIA medium-chain digested with Palatase® 20000 L (○), LFCS IIIA long-chain digested with porcine pancreatin (▲), LFCS IIIA long-chain digested with Palatase® 20000 L (△), LFCS surfactant-only digested with porcine pancreatin (■), and LFCS surfactant-only digested with Palatase® 20000 L (□). Every 24th data point is shown in the graph (data point every 2 min) to improve graph legibility. B) shows the extent of digestion of the LFCS IIIA medium-chain, LFCS IIIA long-chain, and LFCS surfactant-only with porcine pancreatin (blue) and Palatase® 20000 L (yellow). All data shown is $n = 3 \pm$ SD.

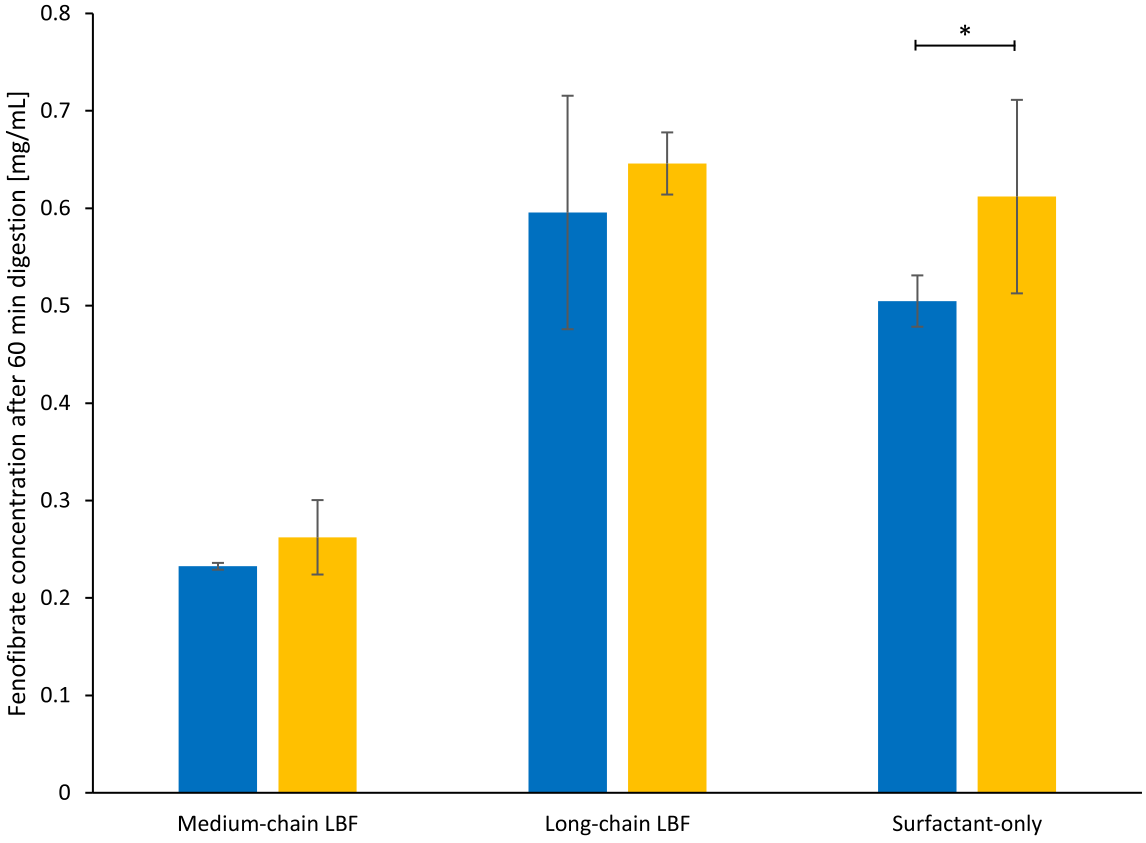

Fig. 5. Concentration of fenofibrate in the aqueous phase after one hour of digestion in the pH-stat setup of the medium-chain LBF (40 % Miglyol 812, 40 % Tween 85, and 20 % Cremophor RH40), the long-chain LBF (40 % Olive oil, 40 % Tween 85, and 20 % Cremophor RH40), and surfactant-only (67 % Tween 85 and 33 % Cremophor RH40). The formulations were loaded with 80 mg/g fenofibrate. All results shown are with calcium in the biorelevant media. The results are for digestion with porcine pancreatin (blue) and Palatase® 20000 L (orange). The data is $n = 3 \pm$ SD. The start (*) indicates significance ($p < 0.05$).

drug solubilisation capacity of lipid dispersion and digestion media can be obtained using in-line measurement. In contrast, in order to generate equivalent estimates of the solubilisation of lipid dispersion and/or digestion using the classical setup, this would entail a series of blank digestion experiments, sample extraction, addition of lipolysis inhibitors, addition of excess drug and followed by solubility studies over time to determine comparable solubilisation capacity. The benefit of the MicroDISS Profiler™ is readily apparent in terms of higher throughput and smaller sample size.

While the study demonstrated the feasibility of using purified liquid lipases there are limitations of the setup. This work has focused on mimicking intestinal digestion with porcine pancreatin, however, it is

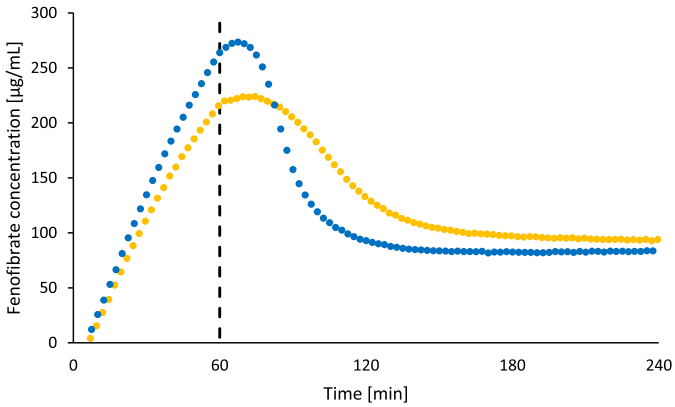

Fig. 6. Dispersion-digestion profile versus time profile conducted in the MicroDISS Profiler™. The dotted line represents the one-hour mark where lipase is added, and digestion is initiated. The formulations tested are medium-chain LBF (40 % Miglyol 812, 40 % Tween 85, and 20 % Cremophor RH40) (blue) and long-chain LBF (40 % Olive oil, 40 % Tween 85, and 20 % Cremophor RH40) (orange). Excess Fenofibrate was added to the 15 mL of FaSSIF containing 75 µL LBF at time point 0 min, and Palatase® 20000 L (125 PLU/mL) was added at time point 60 min. Every 5th data point is shown in the graph (data point every 2.5 min) to improve graph legibility. The results shown are $n = 3$.

also important to consider that *in vivo* the digestion is initiated in the stomach with the gastric lipase, and the pancreatic carboxyl ester hydrolase and pancreatin lipase-related protein-2 also contributes to digestion in the intestine (Carrière, 2016). Further studies may also be required to clarify the limitations of the setup. Firstly, a downside to this setup was the pH drop observed during/after digestion caused by the release of FFAs where the buffer capacity could not withstand the influence of the released FFAs. This change in pH could possibly influence the solubilisation capacity, especially for drug molecules with ionisation effects within this pH region. Secondly, the digestion of the surfactant-only formulations tested showed a significantly higher extent of digestion with Palatase® 20000 L when compared to porcine pancreatin (Fig. 4B and supplementary material Fig. S3). The reason for the higher digestibility of surfactants with this specific lipase was unclear and requires further investigation.

5. Conclusions

In summary, this study showed that the sn-1,3-specific liquid lipase, Palatase® 20000 L was found to be a suitable alternative to porcine pancreatin in terms of rate and extent of digestion of LBFs. Similar drug concentrations were observed using either Palatase® 20000 L or porcine pancreatin using the equivalent experimental set ups demonstrating the suitability of Palatase® 20000 L to simulate digestion during *in vitro* characterisation of drug loaded LBFs. The MicroDISS Profiler™ was demonstrated to be very useful for in-line UV detection of the fenofibrate concentration during Palatase® 20000 L induced digestion of LBFs. This adapted MicroDISS lipolysis model detected a reduction in solubilisation capacity caused by digestion, similar to what is observed in the classical pH-stat setup. However, the in-line analysis of the drug concentration made it possible to follow the dynamic change in concentration in real-time. This method offers opportunities to readily determine the real-time dynamic drug concentration during dispersion and digestion of LBFs in a small-scale setup, avoiding artifacts and the laboratory-intensive sample preparation that is required in the classical *in vitro* lipolysis setup.

CRediT authorship contribution statement

Lotte Ejskjær: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Patrick J. O'Dwyer:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Project administration, Supervision. **Callum D. Ryan:** Validation, Investigation. **René Holm:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Martin Kuentz:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Karl J. Box:** Methodology, Validation, Resources, Writing – review & editing. **Brendan T. Griffin:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Project administration, Supervision.

Declaration of Competing Interest

KJB is an employee of Pion Inc. The other authors disclosed no conflicts of interest related to this article.

Data availability

Data will be made available on request.

Acknowledgments

LE, PJOD, RH, MK, KJB, and BTG are part of the InPharma Industrial Training Network, which was funded by the European Union's Horizon 2020 Marie Sklodowska-Curie research and innovation program under grant agreement No. 955756.

The authors would like to thank Avril McCord, UCC, for training and support with the pH-stat experiments.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106681.

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