Research Article

Toward the Establishment of Standardized *In Vitro* Tests for Lipid-Based Formulations, Part 6: Effects of Varying Pancreatin and Calcium Levels

Philip Sassene,¹ Karen Kleberg,¹ Hywel D. Williams,² Jean-Claude Bakala-N’Goma,³ Frédéric Carrière,³ Marilyn Calderone,⁴ Vincent Jannin,⁵ Annabel Igonin,⁶ Anette Partheil,⁷ Delphine Marchaud,⁵ Eduardo Jule,⁸ Jan Vertommen,⁶ Mario Maio,⁷ Ross Blundell,⁴ Hassan Benameur,⁶ Christopher J. H. Porter,² Colin W. Pouton,⁹ and Anette Müllertz^{1,10}

Received 4 June 2014; accepted 9 September 2014; published online 2 October 2014

Abstract. The impact of pancreatin and calcium addition on a wide array of lipid-based formulations (LBFs) during *in vitro* lipolysis, with regard to digestion rates and distribution of the model drug danazol, was investigated. Pancreatin primarily affected the extent of digestion, leaving drug distribution somewhat unaffected. Calcium only affected the extent of digestion slightly but had a major influence on drug distribution, with more drug precipitating at higher calcium levels. This is likely to be caused by a combination of removal of lipolysis products from solution by the formation of calcium soaps and calcium precipitating with bile acids, events known to reduce the solubilizing capacity of LBFs dispersed in biorelevant media. Further, during the digestion of hydrophilic LBFs, like IIIA-LC, the un-ionized–ionized ratio of free fatty acids (FFA) remained unchanged at physiological calcium levels. This makes the titration curves at pH 6.5 representable for digestion. However, caution should be taken when interpreting lipolysis curves of lipophilic LBFs, like I-LC, at pH 6.5, at physiological levels of calcium (1.4 mM); un-ionized–ionized ratio of FFA might change during digestion, rendering the lipolysis curve at pH 6.5 non-representable for the total digestion. The ratio of un-ionized–ionized FFAs can be maintained during digestion by applying non-physiological levels of calcium, resulting in a modified drug distribution with increased drug precipitation. However, as the main objective of the *in vitro* digestion model is to evaluate drug distribution, which is believed to have an impact on bioavailability *in vivo*, a physiological level (1.4 mM) of calcium is preferred.

KEY WORDS: danazol; digestion; dispersion; drug precipitation; *in vitro* digestion; LFCS Consortium; lipid formulation.

Electronic supplementary material The online version of this article (doi:10.1208/s12248-014-9672-x) contains supplementary material, which is available to authorized users.

¹ Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100, Copenhagen, Denmark.

² Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria 3052, Australia.

³ UMR7282 Enzymologie Interfaciale et de Physiologie de la Lipolyse, Aix Marseille Université, CNRS, 13402, Marseille cedex 20, France.

⁴ Sanofi-Aventis R&D, 371 rue Pr. J. Blayac, 34184, Montpellier, France.

⁵ Gattefossé SAS, 36 chemin de Genas, 69804, Saint-Priest, France.

⁶ Capsugel R&D, Strasbourg, France.

⁷ Merck KGaA, Darmstadt, Germany.

⁸ Capsugel Product Development Center, Boston, Massachusetts, USA.

⁹ Pharmaceutical Biology, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria 3052, Australia.

¹⁰ To whom correspondence should be addressed. (e-mail: anette.mullertz@sund.ku.dk)

INTRODUCTION

The development of lipid-based formulations (LBF) for poorly soluble drugs is a recognized strategy to improve drug solubilization and absorption in the gastro-intestinal (GI) tract (1–3). Upon release into the gastrointestinal milieu, the LBF will mix first with gastric juice containing gastric lipase and subsequently with pancreatic juice and bile. Endogenous surfactants secreted in bile allow the emulsification of dietary lipids with a large surface area available for enzymatic hydrolysis by lipases from pancreatic juice. These surfactants can also interact with LBFs and products resulting from LBF lipolysis. Mixed micelles can thus be formed from the lipolysis products and exo- and/or endogenous surfactants. The drug initially present in LBF can partition between the colloidal phases/structures dispersed in solution until absorption through the intestinal epithelia (4). Studying the gastrointestinal lipolysis of LBFs and the various effectors involved in the enzymatic lipolysis process is therefore essential for understanding the fate of LBF in the GI tract and assessing their performance in drug solubilization/dispersion.

The most important digestive enzymes in the GI tract are secreted by the exocrine pancreas and released in the duodenum. Pancreatic lipase is thus present at levels of 150–600 units/ml in fasted human duodenal aspirates (5,6) and at much higher levels (1,700–7,000 U/ml) under fed conditions (7). Variations in lipase levels are mostly caused by inter-subject differences, by varying means of lipase quantification (USP vs. tributyrin units (TBU)), and by the fact that enzyme secretion and stability drastically differ between fasting and fed conditions. It is thus easier to assess digestive enzyme levels under fed conditions than under fasting conditions because the meal triggers enzyme secretions and protects them from proteolytic inactivation, by pepsin, which is primarily active at acidic conditions (8,9). Several lipase activity assays are used for determining lipolytic activity, varying with regard to type of substrate, pH value, choice of surfactants, and/or level of surfactants (10–12). All factors which are known to impact the rate of digestion hence make a direct comparison of the obtained values very difficult. Armand *et al.* determined a lipolytic level of pancreatic lipase in basal duodenal fluid of around 600 units/ml, using an assay which is very similar to the lipase assay of the United States Pharmacopeia's (USP) monograph for pancreatic extracts (12).

The most commonly used source of lipase for *in vitro* intestinal lipolysis models is pancreatin, a porcine pancreas extract (PPE) containing the main pancreatic digestive enzymes. This is due not only to limited commercial availability of human pancreatic lipase (HPL) and co-lipase but also to the fact that this mixture of enzymes contains additional lipolytic enzymes and thus better mimics the lipolytic potential of complete human pancreatic juice. Porcine pancreatic lipase (PPL) has furthermore been shown to have comparable digestive properties to that of HPL (13).

Human duodenal fluid contains pancreatic lipase and its co-lipase as the main lipases and other lipolytic enzymes such as carboxylester hydrolase, pancreatic lipase-related protein 2, and phospholipase A₂, most of these enzymes being also present in PPE along with proteases, amylases, and impurities consisting of inorganic and organic material (14–16).

Calcium, which is part of the inorganic material in PPE, has an impact on pancreatic lipase activity, at least *in vitro* (17). Calcium forms insoluble soaps with the liberated free fatty acids (FFA) and thus allows the removal of FFAs, which will hamper lipolysis, from the emulsion surface (18).

In the fasted state, the concentration of calcium in human duodenal fluids has been reported to be 0.5–3 mM (19,20). The rate of triacylglycerol lipolysis is highly dependent on the concentration of calcium up to at least 10 mM (17). It has also been shown that during *in vitro* lipolysis, the calcium addition scheme is a tool to control the lipolysis process in that continuous addition gives rise to a linear time-dependent hydrolysis (6). Thus, the complexity and diversity of the gastrointestinal fluids, including variable levels of pancreatic lipase and calcium, influencing lipolysis do, however, provide an obstacle to the standardized evaluation of LBFs.

To address this, the Lipid Formulation Classification System (LFCS) Consortium was formed in 2009 with the objective to develop and characterize standardized *in vitro*

methods for assessment of LBFs used for oral drug delivery (21).

The initial work of the consortium included tests and validation of an *in vitro* digestion test, ensuring reproducibility and comparable results between two participating laboratories in Monash and Copenhagen (21). The purpose of this article is to further characterize the LFCS digestion model in order to better understand the impact of the key parameters, pancreatin and calcium levels, on the digestion of eight different LBFs and the behavior of the incorporated drug. The LBFs used in this study are the same as those described and used in the previous LFCS publications (21–23).

MATERIALS AND METHODS

Materials

Danazol USP was purchased from Unikem (Denmark). Sodium taurodeoxycholate >95% (NaTDC), 4-bromobenzylboronic acid (4-BBBA), corn oil, Tween 85, and porcine pancreatin (8 × USP specifications activity) were all obtained from Sigma-Aldrich (Saint-Louis, USA). Phosphatidyl choline (Lipoid S-PC, approximately 99% pure, from soy) was bought from Lipoid (Ludwigshafen, Germany). Captex 300 and Capmul MCM were obtained from Abitec Corporation (Columbus, OH, USA). Maisine 35–1 and Transcutol were kindly donated by Gattefossé (St. Priest, France). Cremophor EL was obtained from BASF Corporation (Ludwigshafen, Germany). Purified water (18.2 MΩ cm) was obtained from an Ultra Clear UV water purification system from SG Wasseraufbereitung und Regenerierstation (Barsbüttel, Germany). All other chemicals and solvents used in this work were of analytical purity or high performance liquid chromatography (HPLC) grade.

Preparation of Lipid-Based Formulations

The LFCS LBFs were composed according to Table 3 (supplementary material) and previous reports (21–23).

The components of the LBF were weighed into glass vials and vortex-mixed to ensure homogeneity. In each formulation, danazol was loaded at 80% of its saturation solubility in each LBF, as previously reported, thoroughly mixed, and incubated at 37°C until all danazol was dissolved in the lipid base (21). All formulations were freshly prepared and used within 48 h from preparation.

Pancreatin Preparation

One gram of pancreatin was suspended in 5 ml of digestion buffer (without bile salt and phospholipid) for the reference conditions and approximately 17 µL of 5 M NaOH to obtain a pH of 6.5. After centrifugation for 7 min at 4,500 rpm (4°C) in a Megafuge 16R from Hereaus (Langensfeld, Germany), the lipase containing supernatant was used to digest the formulations. The activity in the digestion vessel upon addition of 4 ml pancreatin preparation was 600 USPU/ml (corresponding to 1,000 TBU/ml) in the reference protocol, although the amount of pancreatin added was varied during the lipase activity experiments in order to obtain different enzyme activities in the vessel.

LFCS Consortium *In Vitro* Digestion Test Conditions of LBFs

The reference conditions for the *in vitro* digestion of the formulations were similar to those established in the first LFCS paper (21). A digestion buffer consisting of 2 mM tris maleate, 150 mM NaCl, 1.4 mM Ca^{2+} , 3 mM NaTDC, and 0.75 mM phosphatidyl choline in purified water was used (pH=6.5). All the data obtained at reference conditions have been reproduced from the first LFCS paper (21).

One gram LBF was weighed and introduced into a thermostat-jacketed glass reaction vessel (Metrohm® AG, Herisau, Switzerland), which was placed in the experimental setup consisting of a pH-stat apparatus (Metrohm® AG, Herisau, Switzerland), comprising a Titrando 802 propeller stirrer/804 Ti Stand combination, a glass pH electrode (iUnitrode), and two 800 Dosino dosing units coupled to 10-ml autoburettes (Metrohm® AG, Herisau, Switzerland). The apparatus was connected to PCs and operated using Tiamo 2.0 and 1.3 software.

Digestion buffer (36 ml) was added, and stirring was initiated; the formulation was allowed to disperse and emulsify for 10 min before digestion was commenced by addition of 4 ml pancreatin preparation. During 30-min digestion, pH in the reaction vessel was kept constant at 6.5 by automatic addition of either 0.6 M (MC-formulations) or 0.2 M NaOH (type IV and LC formulations). After 30-min digestion, pH was rapidly elevated with 1 M NaOH to pH 9 in order to fully ionize the FFAs produced during the digestion of the LBF (10,11,21–24). The amount of NaOH added during the 30-min digestion and the amount needed to elevate pH to 9 were subtracted the corresponding values obtained during a blank lipolysis (no formulation) and used to calculate the extent of lipolysis in terms of FFAs formed. This was done to account for titration of impurities originating from the crude pancreatic extract and FFAs liberated from phosphatidyl choline. Titration to pH 9 was, however, not conducted during the dynamic addition of calcium, and the total extent of digestion can therefore not be accounted for in our current setup during these experiments.

Evaluation of Varying Pancreatin Levels

The reference conditions were applied with the following exception—pancreatin levels in the vessel were varied as follows: 150, 300, 600 (reference), and 900 USPU/ml.

Determination of Calcium Content in Pancreatin

A total of 200 mg of crude pancreatin was dissolved overnight in 67% nitric acid. This solution was diluted ten times in purified water, and the calcium concentration was determined by atomic absorption spectroscopy using a Perkin Elmer Atomic Absorption Spectrophotometer (model 2380; Waltham, MA, USA).

Evaluation of Varying Calcium Levels and Addition Schemes

Two different calcium addition schemes were applied: (1) The initial calcium level in the assay buffer was set at 0, 1.4, 5, or 10 mM, and lipolysis was carried out according to the

reference protocol. (2) No calcium was added to the digestion medium, but calcium was continuously added throughout the digestion period to obtain the following final concentrations: 0, 1.4, 5, and 10 mM.

Determination of Fatty Acid Ionization During the Course of Digestion

In vitro lipolysis tests of I-LC and IIIA-LC (Supplementary material, Table 3) were conducted at two different calcium concentrations: at 1.4 mM according to the LFCS Consortium *in vitro* digestion test conditions for digestion of LBFs and at 10 mM calcium. These two formulations were chosen in order to test whether the emulsification properties, as well as the amount of surfactant present in the formulation, affected the ratio of FFA ionization during the course of digestion. The digestions were inhibited at 1, 5, 10, and 20 min by addition of 200 μl of 1.0 M 4-BBBA to the reaction vessel, and “back-titrations” to pH 9 were conducted. A corresponding blank lipolysis (no formulation) was conducted for each time point.

Separation of Digestion Phases

At the end of digestion ($t=30$ min), a 4-ml sample was withdrawn and ultra-centrifuged at 100,000 rpm ($5.4 \times 10^5 g$ at r_{max}) for 30 min at 37°C in a Beckmann-Coulter Optima™ MAX Ultracentrifuge with a TLA-110 rotor (Beckmann-Coulter, Fullerton, CA, USA) in order to separate the digestion phases. In cases where a residual oil phase was formed, this was quantitatively removed using a hydraulic pump equipped with a silicone tube and a syringe. The oil phase was dissolved in methanol and diluted in mobile phase before HPLC analysis.

The aqueous phase was collected in the same manner and diluted in mobile phase. The pellet was dissolved in methanol and subsequently diluted in mobile phase prior to quantification by HPLC.

HPLC Detection of Danazol

All samples were analyzed by means of a Hitachi System from Merck with the following devices: D-7000 interface, L-7200 autosampler, L-7100 pump, L-7350 LaChrom column oven, and L-7400 LaChrom UV-detector (285 nm). The column was a C18, 150×4.6 mm, 5 μm , Phenomenex (Torrance, CA, USA), and equipped with a C18, 3.0×4 mm, Phenomenex guard column.

The mobile phase consisted of acetonitrile and water in a 60/40 v/v ratio which was pumped through the column at 1.25 ml/min flow rate.

RESULTS

In Vitro Lipolysis: Effect of Varying Pancreatin Levels

The liberation of FFAs at pH 6.5 during digestion of LBFs containing LC-fatty acids (FA) and LBF type IV at different levels of pancreatin is shown in Fig. 1.

For type I-LC, a fast initial lipolysis rate was observed at all enzyme levels; within the first 30 s, approximately 50% of

Fig. 1. Titrated FFA at pH 6.5 during 30-min digestion of LBF type I-LC*, II-LC, IIIA-LC, and IV at enzyme levels of 150, 300, 600, and 900 USPU/ml (filled square, filled diamond, filled triangle, and open circle, respectively) over time (min) ($n=2-3$). Only FFAs liberated from LBFs are depicted as contribution from background blank lipolysis (no LBF) has been subtracted

total hydrolysis had taken place. Afterwards, the lipolysis rate leveled off, and only a small fraction of the total hydrolysis, detected at pH 6.5, occurred in the final 20 min. The extent of lipolysis at 30 min increased by 33% from 150 to 300 USPU/ml; further addition of pancreatin led to no significant changes in the extent of lipolysis. The increase only accounted for ionized liberated FFAs, titrated at pH 6.5 (21,22). FFAs are known to have a higher apparent pK_a in micellar solutions than a typical carboxylic acid in water (e.g., acetic acid, pK_a 4.75) and are therefore not fully deprotonated at pH 6.5 (21,25–27). Thus, in order to quantify all liberated FFAs, it was necessary to perform a back-titration to pH 9 at the end of the lipolysis assay, as explained in previous publications, to also detect the FFAs that are un-ionized at pH 6.5 (10,11,21–24). An increase of 47% was achieved by elevating lipase activity from 150 to 900 USPU/ml, when all liberated FFAs are accounted for by a titration to pH 9 (Supplementary material, Table 4).

Even though a trend towards increased total digestion (FFAs titrated at 6.5 and 9) was observed at higher enzyme activity levels, only 40% of the FAs available for hydrolysis in the LBF were liberated during 30-min digestion at 900-USP U/ml activity level (Table I). Thus, complete digestion was not obtained during these test conditions of I-LC.

Digestion of II-LC had the most notable effect of increasing pancreatin from 150 to 300 USPU/ml, and the remaining increases of pancreatin levels only had a minor

impact on FFAs titrated at pH 6.5. During the digestion of IIIA-LC, an increase of 36% in FFAs detected at pH 6.5 was observed from 150 to 300 USPU/ml, and the remaining curves clustered together.

The amount of FFAs titrated at pH 6.5 did increase by 120 and 28% for II-LC and IIIA-LC, respectively, when pancreatin was increased from 150 to 900 USP U/ml, and the total extent of lipolysis increased by 79 and 28% II-LC and IIIA-LC, respectively (Supplementary material, Table 4).

The digestion of type IV was different from the other formulations. No fast initial lipolysis was observed, and the extent of lipolysis was very limited (approximately 0.028 mmol FFA was titrated at standard conditions). No triacylglyceride (TAG) is present in this formulation, and the only digestible excipient is Cremophor EL, which during digestion only released 0.36 mmol per gram of surfactant (28). Thus, the presence of additional pancreatin had negligible effect on lipolysis rate especially at activity levels 150 to 600 USPU/ml. An increase in extent of lipolysis was, however, observed from 600 to 900 USPU/ml.

By elevating pancreatin activity from 150 to 900 USPU/ml, the total extent of digestion on average for LC-LBFs increased by 51%, whereas the amount of FFAs detected at pH 6.5 increased by 55% on average (Supplementary material, Table 4). The ratio of un-ionized-ionized FFA, which was calculated by dividing the amount of FFAs titrated at pH 6.5 with FFAs quantified at pH 9, is therefore expected

to remain somewhat unchanged during digestion at the different pancreatin levels, which also can be observed in Table I.

Figure 2 shows the course of liberated FFAs from lipolysis of four LBFs containing MC-FA by varying the lipase activity levels at pH 6.5. The lipolysis curves at all activity levels and MC-LBFs showed a fast initial lipolysis, in agreement with lipolysis of LBFs containing LC-FAs. Approximately 70–85% of lipolysis occurred within the initial 10 min at standard conditions for the tested MC-LBFs. An increase in extent of digestion was observed with increasing pancreatin levels. However, for I-MC, IIIA-MC, and IIIB-MC formulations, the most noticeable increase was observed between activity levels 150 and 600 USPU/ml. Thus, no notable effect was obtained by elevating the lipase from 600 to 900 USPU/ml. Formulation II-MC was, however, affected by the varying activity levels, and it was possible to distinguish between the extents of digestion, detected at pH 6.5, for all four activity levels. For formulation I-MC and IIIA-MC, no increase of liberated FFAs was observed by elevating the activity level from 150 to 300 USP units/ml. However, an enhancement by 24 and 29%, respectively, was observed by elevating the level from 300 to 600 USPU/ml. This indicated that a somewhat threshold activity level is present between the two activity levels. All four MC formulations are completely digested at 600 USPU/ml lipase level, and the addition of further lipase would therefore have negligible effect on the extent of digestion (Table I). Hence, the lipolysis curves at activity levels 600 and 900 USPU/ml at pH 6.5 are expected to cluster together for all MC-containing formulations if the ratio of FFA ionization remains unchanged throughout the different activity levels. From Table I, it can be seen that the un-ionized-ionized FFA ratio seems to be more or less unaffected by the various lipase concentrations for all eight formulations (e.g., II-MC with a ratio of 0.71, 0.72, 0.65, and 0.74, respectively, for 150, 300, 600, and 900 USPU/ml), and no relationship between activity level and FFA ionization can be established. Thus, the assumption of a constant ratio of un-ionized-ionized FFAs throughout the lipolysis appears to be reasonable when the level of pancreatin was varied. The average total extent of digestion of MC-LBFs, when elevating pancreatin activity from 150 to 900 USPU/ml increased by 59%, whereas the

amount of FFAs detected at pH 6.5 increased by 41% on average (Supplementary material, Table 4).

Determination of the Calcium Content in Pancreatin

The calcium content in pancreatin was determined in order to assure that calcium in the medium predominantly originated from CaCl_2 added. This was necessary as the employed pancreatin is a crude extract obtained from the entire porcine pancreas that can contain calcium. The content of calcium in crude pancreatin dissolved in HNO_3 was determined to be $628 \pm 43 \mu\text{g/g}$. However, when pancreatin was added to the lipolysis vessel, the poorly water-soluble components had been removed by centrifugation. Thus, the appropriate concentration to determine in order to account for pancreatin's calcium contribution is the content in the supernatant. The calcium concentration in the supernatant of the pancreatin preparation was determined to be $28.8 \pm 2.2 \mu\text{g/ml}$, causing the total concentration in the lipolysis vessel to rise $0.07 \pm 0.005 \text{ mM}$. Thus, the concentration of calcium is low in the pancreatin solution compared to the calcium levels tested in this paper and does not therefore influence the experiments significantly.

In Vitro Lipolysis: Effect of Varying Calcium Levels and Addition Schemes

Initial Addition of Calcium's Effect on Digestion of LC-FA-Based LBFs

The impact of different calcium levels present at time zero on the digestion rate of LC-LBFs and LBF type IV, at pH 6.5 and 600 USPU/ml of pancreatin, is depicted in Fig. 3a. A fast and substantial initial lipolysis was observed for I-LC, II-LC, and IIIA-LC: within the first 10 min, 89, 74, and 74% of the total amount of liberated FFAs, at pH 6.5, respectively, at standard conditions (1.4 mM Calcium). However, as calcium concentration increased, so did the extent of titrated FFAs at pH 6.5 through the concentrations tested. A linear relationship can be established between calcium concentration and titrated FFAs at pH 6.5 for the three LC-FA-based

Table I. Influence of Pancreatin at 150, 300, 600, and 900 USPU/ml on the Extent of Digestion and Its Influence on the Un-ionized–Ionized FFA Ratios. The Un-ionized–Ionized FFA Ratio Is Calculated by Dividing FFAs Detected at pH 9 with the Sum of FFAs at pH 6.5 and pH 9. The Extent of Digestion Is Based on Calculations from Williams *et al.* for the Total Amount of FFAs Available for Digestion in the LBFs (21)

LBF type	Titrated FFA ionized/total FFA released (mmol)				Un-ionized–ionized FFA ratio				Extent of digestion (%)			
	150 ^a	300 ^b	600 ^c	900 ^a	150 ^b	300 ^d	600 ^d	900 ^c	150 ^c	300 ^d	600 ^c	900 ^d
I-LC	0.06/0.60	0.08/0.73	0.09/0.54	0.07/0.88	9.00	8.13	5.00	11.57	27	33	25	40
II-LC	0.05/0.61	0.10/1.09	0.12/1.00	0.11/1.09	11.20	9.90	7.33	8.91	31–43	55–76	50–70	55–76
IIIA-LC	0.25/1.49	0.34/1.85	0.36/1.32	0.32/1.91	4.96	4.44	2.67	4.97	73–104	91–129	65–92	94–134
I-MC	1.89/2.90	1.96/3.62	2.30/3.44	2.46/5.50	0.53	0.85	0.50	1.24	74	93	88	141
II-MC	1.44/2.46	1.63/2.80	1.82/3.00	2.01/3.49	0.71	0.72	0.65	0.74	79–97	90–110	96–118	112–137
IIIA-MC	1.31/2.33	1.46/2.60	1.88/2.67	1.84/3.92	0.78	0.78	0.42	1.13	78–92	88–102	90–105	132–154
IIIB-MC	0.59/1.01	0.73/1.08	0.88/1.28	0.90/1.38	0.71	0.48	0.45	0.53	64–105	69–113	82–133	88–144
IV	0.03/0.19	0.03/0.16	0.03/0.10	0.05/0.60	5.33	4.33	2.33	11.00	31	26	16	98

Maximum relative standard deviation (RSD) for the parameter (type IV excluded due to low extent of digestion resulting in high RSDs)

^a RSD <40

^b RSD <30

^c RSD <20

^d RSD <10

Fig. 2. The course of FFAs titrated, at pH 6.5, during 30-min digestion of formulation type I-MC, II-MC, IIIA-MC, and IIIB-MC at enzyme levels of 150, 300, 600, and 900 USPU/ml (filled square, filled diamond, filled triangle, and open circle, respectively) depicted against time (min) ($n=2-3$). Only FFAs liberated from LBFs are depicted as contribution from background blank lipolysis (no LBF) has been subtracted

Fig. 3. **a** The course of FFAs titrated, at pH 6.5, during 30-min digestion of formulation type I-LC, II-LC, IIIA-LC, and IV at initial addition of calcium; levels of 0, 1.4, 5, and 10 mM (filled square, filled diamond, filled triangle, and open circle, respectively) depicted against time (min) ($n=2-3$). **b** The course of FFAs titrated, at pH 6.5, during 30-min digestion of formulation type I-LC, II-LC, IIIA-LC, and IV at dynamic addition of calcium; levels of 0, 1.4, 5, and 10 mM (filled square, filled diamond, filled triangle, and open circle, respectively) depicted against time (min) ($n=2-3$). Only FFAs liberated from LBFs are depicted as contribution from background blank lipolysis (no LBF) has been subtracted

formulations with correlation factors of 0.999, 0.999, and 0.996, respectively. The linear correlation is however only based on four data points and is therefore not irrefutable but suggests that FFAs titrated at pH 6.5 increase with increasing calcium levels. Thus, by additional elevation of calcium concentration, further digestion is likely to occur as only 42 and 49–69% of the FFAs available for liberation have been titrated at a 10-mM calcium level for I-LC and II-LC (Table II). Type IIIA-LC had, on the other hand, been completely digested, and further addition of calcium would not benefit the extent of digestion. The digestion of formulation type IV was, however, promoted by the increasing calcium levels, but no linear relationship could be established.

It is furthermore worth noticing that the un-ionized–ionized FFA ratios in Table II decreased with increasing calcium concentrations. This can also be observed in Table 5 (Supplementary material) where the amount of ionized FFAs detected at pH 6.5 from LC-LBFs on average increased by 346%, when elevating calcium levels from 0 mM to 10 mM, but the total release of liberated FFAs (ionized+un-ionized) only appeared to increase >19%.

Dynamic Addition of Calcium's Effect on Digestion of LC-FA-Based LBFs

The impact of continuous calcium addition on the rate of digestion of LC-LBFs and LBF type IV is depicted in Fig. 3b. A fast initial lipolysis did occur at reference conditions (1.4 mM calcium) for formulation I-LC, II-LC, and IIIA-LC, and 66, 55, and 55% of the hydrolysis detected at pH 6.5 occurred within the first 10 min.

Even though a fast initial lipolysis did occur, the pace of digestion within the initial 10 min was reduced compared to when calcium was added at time point zero for all three LBFs. An increase in calcium addition rate led to more titrated FFAs at pH 6.5, and a linear relationship can be established between calcium concentration and FFA titrated with correlation factors 0.992, 0.995, and 0.996 for I-LC, II-LC, and IIIA-LC, respectively.

Formulation type IV did again vary from the lipid-based formulations as no fast initial lipolysis did occur, and only a very limited amount of FFAs was liberated during the digestion. The digestion was however promoted by the increasing calcium levels, but no linear relationship could be established here either.

Initial Addition of Calcium's Effect on Digestion of MC-FA-Based LBFs

The impact of calcium by the initial addition scheme and its impact on digestion rate of LBFs containing MC-FFAs, at pH 6.5, are depicted in Fig. 4a. LBFs I-MC, II-MC, IIIA-MC, and IIIB-MC followed the same trend as described earlier: a fast initial lipolysis rate, followed by a subsequent slower digestion pace for all the tested calcium levels. Within the first 10 min of digestion of I-MC, II-MC, IIIA-MC, and IIIB-MC at standard conditions (1.4 mM calcium), 71.3, 81.4, 80.2, and 77.7%, respectively, of the total hydrolysis detected at pH 6.5 had taken place. The amount of titrated FFAs at pH 6.5 was not greatly affected by increasing calcium levels from 0 to 5 mM for I-MC, II-MC, and IIIA-MC, and at 30 min, increases of 10.3, 7.5, and 10.6% of titrated FFAs at pH 6.5 were obtained, respectively. However, by elevating the calcium level from 5 to 10 mM, increases of 14.5, 19.8, and 19.1% were evident at 30 min, suggesting that there might be a threshold concentration between 5 and 10 mM, above which the degree of lipolysis is increased. This was also confirmed by the total extent of digestion (including titration to pH 9) (Table II), where increases of 12.8, 8.7, and 15.6% were obtained when elevating calcium levels from 5 to 10 mM for I-MC, II-MC, and IIIA-MC, respectively, and only an increase of 16.8, 3.1, and 9.0% when calcium level was elevated from 0 to 5 mM, respectively, for the three LBFs. Formulation IIIB-MC did not seem to be affected by a threshold calcium concentration; a more pronounced increase of digestion was actually observed when elevating the calcium level from 0 to 5 mM (18.1% increase) than from 5 to 10 mM (11.0% increase).

Table II. Calcium Influence at 0, 1.4, 5, and 10 mM on the Extent of Digestion and Its Influence on Un-ionized–Ionized FA Ratios. The Un-ionized–Ionized FFA Ratio Is Calculated by Dividing FFAs Detected at pH 9 with the Sum of FFAs at pH 6.5 and pH 9. The Extent of Digestion Is Based on Calculations from Williams *et al.* for the Total Amount of FFAs Available for Digestion in the LBFs (21)

LBF type	Titrated FFA ionized/total FFA released (mmol)				Un-ionized–ionized FFA ratio				Extent of digestion (%)			
	0 ^a	1.4 ^c	5 ^b	10 ^b	0 ^a	1.4 ^d	5 ^c	10 ^b	0 ^d	1.4 ^c	5 ^c	10 ^d
I-LC	0.05/0.79	0.09/0.54	0.19/0.53	0.35/0.93	14.84	5.00	1.74	1.63	35	25	24	42
II-LC	0.08/1.00	0.12/1.00	0.21/1.01	0.33/0.98	11.50	7.33	3.81	1.97	50–70	50–70	51–71	49–69
IIIA-LC	0.31/1.44	0.36/1.32	0.53/2.41	0.70/1.86	3.65	2.67	3.55	1.66	71–101	65–92	118–169	91–130
I-MC	2.25/3.15	2.30/3.44	2.48/3.68	2.84/4.15	0.40	0.50	0.48	0.46	81	88	94	106
II-MC	1.71/2.91	1.82/3.00	1.84/2.86	2.20/3.11	0.70	0.65	0.55	0.41	94–115	96–118	92–113	100–122
IIIA-MC	1.69/2.45	1.88/2.67	1.87/2.75	2.23/3.18	0.45	0.42	0.47	0.43	83–96	90–105	93–108	107–125
IIIB-MC	0.79/1.32	0.88/1.28	0.93/1.32	1.04/1.38	0.67	0.45	0.42	0.33	84–138	82–133	84–138	88–144
IV	0.01/0.24	0.03/0.10	0.05/0.37	0.06/0.53	23.00	2.33	6.40	7.83	39	16	61	87

Maximum relative standard deviation (RSD) for the parameter (type IV excluded due to low extent of digestion resulting in high RSDs)

^a RSD <50

^b RSD <30

^c RSD <20

^d RSD <10

Fig. 4. **a** The course of FFAs titrated, at pH 6.5, during 30-min digestion of formulation type I-MC, II-MC, IIIA-MC and IIIB-MC at initial addition of calcium levels of 0, 1.4, 5, and 10 mM (filled square, filled diamond, filled triangle, and open circle, respectively) depicted against time (min) ($n=2-3$). **b** The course of FFAs titrated, at pH 6.5, during 30-min digestion of formulation type I-MC, II-MC, IIIA-MC, and IIIB-MC at dynamic addition of calcium levels of 0, 1.4, 5, and 10 mM (filled square, filled diamond, filled triangle, and open circle, respectively) depicted against time (min) ($n=2-3$). Only FFAs liberated from LBFs are depicted as contribution from background blank lipolysis (no LBF) has been subtracted

The total extent of digestion (FFA detected both at pH 6.5 and 9) of MC-LBFs on average increased by >31% when elevating calcium levels from 0 to 10 mM, whereas the amount of FFAs detected at pH 6.5 increased by 30% (Supplementary material, Table 5).

Dynamic Addition of Calcium's Effect on Digestion of MC-FA-Based LBFs

The impact of the dynamic calcium addition scheme on rate of digestion of LBFs containing MC-FAs is depicted in Fig. 4b. As for the initial calcium addition, a fast initial lipolysis was seen, and within the first 10 min, 74.1, 80.8, 80.4, and 78.6% of the total hydrolysis occurred for LBF I-MC, II-MC, IIIA-MC, and IIIB-MC, respectively, at reference calcium level (1.4 mM calcium). However, the rate and extent of lipolysis of I-MC, II-MC, and IIIB-MC did not seem to be greatly affected by the increasing levels of calcium added by the dynamic addition scheme as the digestion curves for each formulation clustered together regardless of the calcium addition rate. For LBF type IIIA-MC, changing the calcium concentration from 0 to 5 mM did not appear to affect the rate or extent of lipolysis; however, when increasing calcium level from 5 to 10 mM, the extent of digestion was enhanced by 18.5%. Thus, the digestion of MC formulations, except IIIA-MC, did not appear to be promoted by increasing calcium levels added by the dynamic calcium scheme.

In Vitro Lipolysis: Change of Fatty Acid Ionization During the Course of Digestion

Figure 5 shows the un-ionized-ionization FFA ratio during *in vitro* digestion of I-LC and IIIA-LC at two calcium

levels: 1.4 and 10 mM. The ratio increased for formulation I-LC at 1.4 mM Ca^{2+} over time; initially (1 min of digestion), the ratio was 0.83; corresponding to 55% of the total amount of liberated FFAs are titrated at pH 6.5. During the course of digestion, the ratio increased and was 3.83 after 20 min, which corresponds to a detection of 21% of the total liberated FFA at pH 6.5. Hence the “back titration” at pH 9 accounted for a more substantial amount of the liberated FFAs as digestion progressed.

The ratio was, however, influenced by the calcium concentration. The FFA ionization ratio during the digestion of I-LC at 10 mM Ca^{2+} remained somewhat unchanged from 0.61 after 1 min to 0.76 after 20 min, which corresponds to a decline in titrated FFAs at pH 6.5 from 62 to 57%. It is furthermore worth noticing that the ionization ratio is below 1 throughout the tested time points, indicating that the majority of liberated FFAs are titrated at pH 6.5.

The ionization ratio for IIIA-LC decreased slightly from 2.54 to 1.91 during the course of digestion, corresponding to an increase in detected FFA from 28 to 34% at pH 6.5. The same trend was observed when calcium level was elevated to 10 mM; the ratio drops from 1.31 to 0.71, equal to an increase of FFA detected at pH 6.5 from 43 to 58%.

Both formulations did in general have higher un-ionized-ionized ratios at 1.4 mM than 10 mM, suggesting that calcium had a major influence on the un-ionized-ionized FFA equilibrium.

In Vitro Lipolysis: Distribution of Danazol During Digestion

The impact of varying pancreatin and calcium levels and addition schemes on danazol distribution after 30 min digestion of I-LC, II-LC, IIIA-LC, and IV can be seen in Fig. 6a–c.

Fig. 5. FFA ionization during the course of lipolysis of ■—I-LC at 1.4 mM Ca^{2+} , ▨—I-LC at 10 mM Ca^{2+} , ■—IIIA-LC at 1.4 mM Ca^{2+} , and ▨—IIIA-LC at 10 mM Ca^{2+} . The dashed line indicates the un-ionized-ionized ratio 1. When the ratio is above 1, the majority of FFAs are detected by back titration to pH 9 and continuous titration at 6.5 is the dominant way of detection below 1

Pancreatin's Effect on Drug Distribution for LC-FA-Based LBFs (Fig. 6a)

For I-LC, the amount of danazol in the oil phase was decreasing, with increasing pancreatin levels. The decrease in danazol present in the oil phase corresponded to the increase in the pellet phase, and the danazol concentration in the aqueous phase remained somewhat unchanged. The digestion of LBFs II-LC, IIIA-LC, and IV, with increasing levels of pancreatin, did not affect danazol distribution, and it remains similar throughout the tested conditions.

Initial Addition of Calcium's Effect on Drug Distribution (Fig. 6b)

The danazol distribution for I-LC and IV at different calcium levels seemed to be more or less unchanged. For II-LC and IIIA-LC, a decline in danazol situated in the oil phase was observed with increasing calcium levels. This decline resulted in a higher amount of danazol in both aqueous and pellet phase.

Dynamic Addition of Calcium's Effect on Drug Distribution for LC-FA-Based LBFs (Fig. 6c)

LBF type I-LC, II-LC, and IV were unaffected by modifications in calcium concentrations.

For IIIA-LC, danazol content in the oil phase was decreasing with higher calcium concentrations, and the amount in the pellet had increased from 0 mM calcium to 10 mM. The concentration of danazol in the aqueous phase did furthermore increase for IIIA-LC.

For the MC-LBFs, the impact of varying pancreatin (Fig. 7a) and calcium levels and addition schemes (Fig. 7b, c) on danazol distribution after 30-min digestion can be seen in Fig. 7a–c.

Pancreatin's Effect on Drug Distribution for MC-FA-Based LBFs (Fig. 7a)

For I-MC, the amount of danazol in the oil phase increased from 0 to 600 USPU/ml and decreased from 600 to 900 USPU/ml. The increase and subsequent decrease in danazol distributed to the oil phase corresponded to the

Fig. 6. Drug distribution during digestion of LC-LBFs and IV under varying **a** pancreatin levels, **b** fixed calcium addition levels, and **c** dynamic addition levels. ■—oil phase, ■—aqueous phase, ■—pellet phase. The four formulations seem to be unaffected by the different pancreatin or calcium levels. Formulation IIIA-LC does, however, seem to be slightly affected by the varying calcium levels in **b** and **c**

Fig. 7. Drug distribution during digestion of MC-LBFs under **a** varying pancreatin levels, **b** varying fixed calcium addition levels and **c**; varying dynamic addition levels. ■—oil phase, ■—aqueous phase, □—pellet phase. The four formulations seemed to be unaffected by the different pancreatin levels. Formulations I-MC, II-MC, and IIIA-MC does however seem to be slightly affected by the varying calcium levels in **b** and **c**. More precipitation is experienced with increasing calcium levels

decrease from 0 to 600 USPU/ml and increases from 600 to 900 USPU/ml in the amount of danazol situated in the aqueous phase. The changes are however small. The pellet remained unaffected by the varying pancreatin levels. II-MC and IIIB-MC were somewhat unaffected by the variation in levels of pancreatin. The danazol content in the aqueous phase, during digestion of IIIA-MC, increased slightly from 0 to 600 USPU/ml and then dropped from 600 to 900 USPU/ml. The pellet phase decreased from 0 to 600 USPU/ml and increases from 600 to 900 USPU/ml. The digestion phases are interconnected. Thus, when danazol content in the aqueous phase decreased, content in the pellet phase increased and *vice versa*.

Initial Addition of Calcium's Effect on Drug Distribution for MC-FA-Based LBFs (Fig. 7b)

During the digestion of I-MC, increasing levels of calcium resulted in a decline in the amount of danazol located in the oil phase. This decline was reflected in an increase of danazol precipitating to the pellet, while the aqueous phase was unaffected. However, during the digestion of II-MC and IIIA-MC, the amount of danazol in the aqueous phase was decreasing with higher calcium levels. The variation in calcium levels did not affect the danazol distribution during digestion of IIIB-MC.

Dynamic Addition of Calcium's Effect on Drug Distribution for MC-FA-Based LBFs (Fig. 7c)

The danazol situated in the oil phase during digestion of I-MC was unaffected by varying calcium levels. The

concentration in the aqueous phase was declining with higher calcium concentrations, resulting in more danazol precipitating to the pellet. The same trend was also observed during digestion of II-MC and IIIA-MC. During digestion of IIIB-MC, the danazol distribution was unaffected by the varying levels of calcium.

It was generally observed that when the oil phase was physically disappearing, which happened at elevated pancreatin or calcium levels, the amount of danazol distributed to the oil phase decreased. Since the solubility in the oil phase most likely did not change dramatically throughout the different test conditions, whereas the oil phase itself was diminished during the 30-min digestion, the reduced amount of danazol is probably due to the disappearance of the oil phase.

DISCUSSION

In Vitro Lipolysis: Effect of Varying Pancreatin and Calcium Levels on Digestion

As evident from the data described above, varying pancreatin and calcium levels appear to affect the rate and extent of lipolysis. The manner in which they do this is, however, different. Elevation of pancreatin levels from 150 to 900 USPU/ml seems to influence the extent of total digestion for both LC- and MC-LBFs, with increases of 51 and 59% on average, respectively, whereas an elevation in calcium from 0 to 10 mM only results in increases of >19 and >31%, respectively. This is, however contrary to what previously has been reported by Zangenberg *et al.*, where calcium was the most efficient promoter of *in vitro* digestion (6,29). If the

detection of FFAs at pH 6.5 is looked at isolated, the increases in digestions are 55 and 41% for LC-LBFs and MC-LBFs, respectively, when elevating the level of pancreatin from 150 to 900 USPU/ml, which is similar to the increase in total digestion (51 and 59%). However, when the level of calcium is raised from 0 to 10 mM, increases in detected FFAs at pH 6.5 of 346 and 30% were observed for LC-LBFs and MC-LBFs, respectively, which do not correlate well with the increases in total extent of digestion (>19 and >31%). Thus, calcium, contrary to pancreatin, does seem to greatly affect the amount of FFA detected at pH 6.5, but only slightly on the total extent of lipolysis. Zangenberg *et al.* is using a LC-LBF consisting of soybean oil, Tween 80, and Span 80 for the digestion experiments and only quantifies the liberated FFAs by titration at pH 6.5 (6). Hence, the discrepancy between this recent finding and that of Zangenberg *et al.* can probably be explained by the lack of an elevation to pH 9 at the end of the experiment in the model of Zangenberg *et al.* (21).

A possible explanation for the calcium-promoted detection of FFAs at pH 6.5, but only to a small degree in terms of the total extent of digestion, is that calcium affects the equilibrium between un-ionized and ionized FFAs. This is schematically displayed in Fig. 8. Calcium pushes the equilibrium between unionized and ionized FFAs towards ionized FFAs according to Le Chatelier's principle by removing ionized FFAs by formation of insoluble calcium soaps (6). Hence, the more calcium that is present, the more that the equilibrium is affected.

Calcium is furthermore known to precipitate with bile acids, impurities from crude pancreatic extract, as well as liberated FFAs (30,31). Hence, at reference calcium levels (1.4 mM), the level of calcium could be expected to drop significantly during lipolysis, resulting in a change of the unionized-ionized FFA ratio during the course of digestion (where unionized FFAs are detected by titration to pH 9 and ionized FFAs quantified by continuous titration at pH 6.5;

thus, the amount of ionized FFAs is actually a combination of FFAs deprotonated in solution and FFAs precipitated as soaps.). This is evident for I-LC at 1.4 mM (Fig. 5). In this case, one should be very careful when interpreting the lipolysis curve at pH 6.5 as it is not representative for the course of digestion. After 1 min, 77% of the FFAs detected at pH 6.5 has been titrated, but only 16% of the total amount of liberated FFAs has been hydrolyzed. However, when the level of calcium is increased to 10 mM, the ratio of un-ionized-ionized FFAs remains somewhat unchanged during the course of digestion of I-LC (Fig. 5). Hence, the digestion curve at pH 6.5 is representative of the digestion occurring throughout the *in vitro* lipolysis experiment.

The micro-milieu in which the FFA is situated is known to influence the apparent pK_a value (22,32,33). Thus, another explanation for the changing un-ionized-ionized ratio could be the shift in colloidal structures during the course of digestion, from initial emulsion droplets to the formation of unilamellar and multilamellar vesicles and finally predominantly mixed micelles (34). This explanation does not, however, seem to be plausible in our studies as the un-ionized-ionized ratio seems to be somewhat unchanged at different pancreatin levels even though more digestion is occurring.

Calcium precipitating with bile acids could also be expected to affect the apparent pK_a as bile concentration affects the pK_a of FFAs (22). Again, this seems only to have a minor contribution in our studies as the un-ionized-ionized ratio is unchanged during digestion at 10 mM Ca^{2+} , which, since it is the highest tested concentration, is expected to be the level where most bile acids would precipitate.

For LBF IIIA-LC, the pattern of the ratio appears to be slightly different at both 1.4 and 10 mM as it drops during the course of digestion. The drop is, however, small (2.54 to 1.91 at 1.4 mM Ca^{2+} and 1.31 to 0.71 at 10 mM Ca^{2+}), and the curves at pH 6.5 are more or less representative of the

Fig. 8. Step 1: lipase digests TAG and FFAs are liberated. Step 2: FFAs are solubilized in aqueous phase until saturation solubility is reached. Afterwards, FFAs will form their own liquid phase. Step 3: equilibrium between ionized and unionized FFAs exists and only the ionized FFAs are being detected by titration at pH 6.5. Step 4: FFAs are getting removed from the oil/water interface by calcium and the equilibrium in step 3 is pushed towards ionized FFAs. Step 5: FFAs_(aq) and FFAs_(l) are being accounted for by an elevation of pH to 9

digestion occurring (data not shown). The decrease in the ratio during the course of digestion can probably be linked to the formation of lipolysis products (diacylglycerides (DAGs) and monoacylglycerides (MAGs)), which together with surfactant Cremophor EL help solubilize FFAs in the aqueous phase. The more hydrophilic nature of IIIA-LC compared to I-LC explains why the un-ionized-ionized ratio pattern is different.

It is furthermore worth noticing that the un-ionized-ionized ratio is only slightly affected for MC-LBFs. This is probably due to a combination of several things: (1) Calcium soaps of MC-FAs are more water soluble, and the equilibrium at step 4 in Fig. 8 is pushed less towards soap formation (35,36); (2) The amount of FFAs released during digestion of MC-LBFs is higher than the amount released from LC-LBFs. Thus, calcium will have a relatively less effect on the equilibrium as calcium will be depleted at all the calcium concentrations tested, e.g., at 10-mM calcium level, which is the highest level tested; the maximal theoretical amount of formable calcium soaps is 0.8 mmol based on a digestion medium volume of 40 ml and a stoichiometric relationship of 2:1 of FFAs to Ca^{2+} .

***In Vitro* Lipolysis: Effect of Varying Pancreatin and Calcium Levels on Drug Distribution**

The level of pancreatin was shown to have a major impact on the extent of lipolysis. Thus, it could be expected that varying levels of pancreatin might affect drug distribution during digestion of a LBF. This, however, did not seem to be the case for the eight LBFs tested in this work. The danazol distribution in the different digestion phases is only slightly affected by the increasing level of digestion. One explanation for this behavior might be the lack of absorption in the model. *In vivo*, liberated FFAs, MAGs, and drug would be absorbed from the lumen and hence be removed from solution. The solubilizing capacity of the dispersed LBF might therefore be reduced as less colloidal structures would be formed. This is, however, not the case in this model as lipolytic products are kept in the medium and are able to be incorporated into various colloidal structures and in turn maintain the solubilizing capacity of the formulation. Hence, changing levels of pancreatin is not very useful if it is desired to stress the solubilizing capacity of a LBF in the model, even though more digestion is occurring. Therefore, a pancreatin level resembling *in vivo* activity under fasting conditions is preferable (e.g., 600 USPS/ml), as a standard condition, as higher activities do not help to differentiate between LBFs.

In contrast, the level of calcium does have an effect on drug distribution during digestion of IIIA-LC, I-MC, II-MC, and IIIA-MC LBFs and to some extent also I-LC and II-LC LBFs. IIIB-MC and IV LBFs are not affected as the majority of drug is precipitated before digestion is even initiated. This is due to the more hydrophilic nature of the formulations, resulting in a decrease in solubilizing capacity of the LBF during dispersion by partitioning of the hydrophilic components of the LBFs into the aqueous phase (37). A general observation for the remaining six LBFs is that more drug is precipitating with increasing levels of calcium, which has been observed in previous publications as well (29). Calcium is known to precipitate as calcium soaps with FFAs, thereby

removing some of the lipolysis product from the solution. Thus, as digestion is progressing and FFAs are liberated and precipitated as calcium soaps, the solubilizing capacity for the LBFs is decreasing. It is furthermore worth keeping in mind that no absorption is present in this *in vitro* model, and all liberated FFAs will therefore not be removed from the system, at physiological levels of calcium, as it might be depleted. This can result in an overprediction of solubilization capacity in the aqueous phase, as mentioned earlier. Thus, by introducing non-physiological calcium levels, this problem might be addressed. It might be beneficial to increase calcium levels if a stress test of a LBF is desired. During standard testing of LBFs, a physiological level of calcium (e.g., 1.4 mM in the fasted state) is however preferred as calcium also precipitates bile acids, which might result in an overestimation of precipitating drug (22).

CONCLUSIONS

It can be concluded that both calcium and pancreatin affect the extent of LBF lipolysis. Pancreatin is the most potent promoter of the two, which appears contrary to what previous studies, using other formulations and a different model setup, have found, *i.e.*, calcium was the most important factor for lipolysis (6). However, pancreatin contains the enzymes responsible for lipolysis, and pancreatic lipase is not a calcium-dependent enzyme (38). Calcium mainly plays an indirect effect by interacting with the reaction products (FFAs) and thus shifts the lipolysis reaction equilibrium *in vitro*. Calcium affects the ratio between un-ionized and ionized FFAs; elevated calcium levels lead to lower ratios, and therefore a higher amount of ionized FFAs can be titrated directly at pH 6.5, whereas the overall release of FFA only shows a small increase in total digestion. The discrepancy between previous studies and the current study might thus be attributed to the means of FFA detection as lipolysis was only estimated from the titration of ionized FFAs at pH 6.5 in previous studies and was thus largely underestimated (6).

Interpretation of lipolysis curves of lipophilic LBFs as I-LC at pH 6.5 with standard levels of calcium (1.4 mM) should be conducted with caution as the un-ionized-ionized ratio might change during the course of digestion. Increasing calcium levels does, however, result in increased precipitation of drug by removal of FFAs, but possibly also bile acids. Thus, an overestimation of precipitating drug might occur at non-physiological levels of calcium. Hence, for standard *in vitro* testing of LBFs, physiological levels of calcium are desirable as drug distribution is believed to be predictive of *in vivo* bioavailability. It is not sufficient to assume that only solubilized drug will be available for absorption, when applying *in vitro* lipolysis models, as recent studies have shown that precipitated drug depending on its solid state might be available for absorption (39–41). More studies are, however, needed in order to fully elucidate how drug distribution data from *in vitro* lipolysis are interpreted as recent studies have found discrepancies between drug distribution data and bioavailability (42,43). For the more hydrophilic LBFs, as IIIA-LC, the un-ionized-ionized ratio does only change slightly, both at physiological (1.4 mM) and non-physiological (10 mM) calcium levels. Thus, the digestion curve at pH 6.5 is representative for the total lipolysis

occurring. All in all, this work has provided a better understanding of the impact of different parameters on the rate and extent of *in vitro* lipolysis. These are important tools when optimizing *in vitro* lipolysis in order to achieve a correlation with *in vivo* performance.

ACKNOWLEDGMENTS

This study results from a joint collaboration between members of the LFCS Consortium, which received funding from Capsugel, Gattefosse, Sanofi, Merck Serono, NiCox, Roche, Bristol-Myers Squibb, and Actelion.

REFERENCES

- Araya H, Nagao S, Tomita M, Hayashi M. The novel formulation design of self-emulsifying drug delivery systems (SEDDS) type O/W microemulsion I: enhancing effects on oral bioavailability of poorly water soluble compounds in rats and beagle dogs. *Drug Metab Pharmacokinet.* 2005;20(4):244–56.
- Araya H, Tomita M, Hayashi M. The novel formulation design of self-emulsifying drug delivery systems (SEDDS) type O/W microemulsion II: stable gastrointestinal absorption of a poorly water soluble new compound, ER-1258 in bile-fistula rats. *Drug Metab Pharmacokinet.* 2005;20(4):257–67.
- Kang BK, Lee JS, Chon SK, *et al.* Development of self-microemulsifying drug delivery systems (SMEDDS) for oral bioavailability enhancement of simvastatin in beagle dogs. *Int J Pharm.* 2004;274(1–2):65–73.
- Larsen AT, Ohlsson AG, Polentarutti B, *et al.* Oral bioavailability of cinnarizine in dogs: relation to SNEDDS droplet size, drug solubility and *in vitro* precipitation. *Eur J Pharm Sci.* 2013;48(1–2):339–50.
- Di Maio S, Carrier RL. Gastrointestinal contents in fasted state and post-lipid ingestion: *in vivo* measurements and *in vitro* models for studying oral drug delivery. *J Control Release.* 2011;151(2):110–22.
- Zangenberg NH, Mullertz A, Kristensen HG, Hovgaard L. A dynamic *in vitro* lipolysis model. I. Controlling the rate of lipolysis by continuous addition of calcium. *Eur J Pharm Sci.* 2001;14(2):115–22.
- Carriere F, Grandval P, Renou C, *et al.* Quantitative study of digestive enzyme secretion and gastrointestinal lipolysis in chronic pancreatitis. *Clin Gastroenterol Hepatol.* 2005;3(1):28–38.
- Ville E, Carriere F, Renou C, Laugier R. Physiological study of pH stability and sensitivity to pepsin of human gastric lipase. *Digestion.* 2002;65(2):73–81.
- Johnston N, Dettmar PW, Bishwokarma B, *et al.* Activity/stability of human pepsin: implications for reflux attributed laryngeal disease. *Laryngoscope.* 2007;117(6):1036–9.
- Gargouri Y, Pieroni G, Riviere C, *et al.* Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology.* 1986;91(4):919–25.
- Carriere F, Barrowman JA, Verger R, Laugier R. Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology.* 1993;105(3):876–88.
- Armand M, Borel P, Pasquier B, *et al.* Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am J Physiol.* 1996;271(1 Pt 1):G172–83.
- Capolino P, Guérin C, Paume J, *et al.* *In vitro* gastrointestinal lipolysis: replacement of human digestive lipases by a combination of rabbit gastric and porcine pancreatic extracts. *Food Dig.* 2011;2:43–51.
- Fernandez S, Jannin V, Rodier JD, *et al.* Comparative study on digestive lipase activities on the self emulsifying excipient Labrasol, medium chain glycerides and PEG esters. *Biochim Biophys Acta.* 2007;1771(5):633–40.
- Armand M. Lipases and lipolysis in the human digestive tract: where do we stand? *Curr Opin Clin Nutr Metab Care.* 2007;10(2):156–64.
- Xiao X, Ross LE, Sevilla WA, *et al.* Porcine pancreatic lipase related protein 2 has high triglyceride lipase activity in the absence of colipase. *Biochim Biophys Acta.* 2013;1831(9):1435–41.
- Alvarez FJ, Stella VJ. The role of calcium ions and bile salts on the pancreatic lipase-catalyzed hydrolysis of triglyceride emulsions stabilized with lecithin. *Pharm Res.* 1989;6(6):449–57.
- Patton JS, Carey MC. Watching fat digestion. *Science.* 1979;204(4389):145–8.
- Lindahl A, Ungell AL, Knutson L, Lennernas H. Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharm Res.* 1997;14(4):497–502.
- Hofmann AF, Mysels KJ. Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca²⁺ ions. *J Lipid Res.* 1992;33(5):617–26.
- Williams HD, Sassene P, Kleberg K, *et al.* Toward the establishment of standardized *in vitro* tests for lipid-based formulations, part 1: method parameterization and comparison of *in vitro* digestion profiles across a range of representative formulations. *J Pharm Sci.* 2012;101(9):3360–80.
- Williams HD, Anby MU, Sassene P, *et al.* Toward the establishment of standardized *in vitro* tests for lipid-based formulations. 2. The effect of bile salt concentration and drug loading on the performance of type I, II, IIIA, IIIB, and IV formulations during *in vitro* digestion. *Mol Pharm.* 2012;9(11):3286–300.
- Williams HD, Sassene P, Kleberg K, *et al.* Toward the establishment of standardized *in vitro* tests for lipid-based formulations, part 3: understanding supersaturation versus precipitation potential during the *in vitro* digestion of type I, II, IIIA, IIIB and IV lipid-based formulations. *Pharm Res.* 2013;30:3059–76.
- Carriere F, Moreau H, Raphael V, *et al.* Purification and biochemical characterization of dog gastric lipase. *Eur J Biochem.* 1991;202(1):75–83.
- Fernandez S, Rodier JD, Ritter N, *et al.* Lipolysis of the semi-solid self-emulsifying excipient Gelucire 44/14 by digestive lipases. *Biochim Biophys Acta.* 2008;1781(8):367–75.
- Kanicky JR, Shah DO. Effect of degree, type, and position of unsaturation on the pKa of long-chain fatty acids. *J Colloid Interface Sci.* 2002;256(1):201–7.
- Cistola DP, Hamilton JA, Jackson D, Small DM. Ionization and phase behavior of fatty acids in water: application of the Gibbs phase rule. *Biochemistry.* 1988;27(6):1881–8.
- Cuine JF, McEvoy CL, Charman WN, *et al.* Evaluation of the impact of surfactant digestion on the bioavailability of danazol after oral administration of lipidic self-emulsifying formulations to dogs. *J Pharm Sci.* 2008;97(2):995–1012.
- Devraj R, Williams HD, Warren DB, *et al.* *In vitro* digestion testing of lipid-based delivery systems: calcium ions combine with fatty acids liberated from triglyceride rich lipid solutions to form soaps and reduce the solubilization capacity of colloidal digestion products. *Int J Pharm.* 2013;441(1–2):323–33.
- Zangenberg NH, Mullertz A, Kristensen HG, Hovgaard L. A dynamic *in vitro* lipolysis model. II: evaluation of the model. *Eur J Pharm Sci.* 2001;14(3):237–44.
- Bodansky O. Are the phosphatases of bone, kidney, intestine, and serum identical? The use of bile acids in their differentiation. *J Biol Chem.* 1937;118(2):341–62.
- Salentinig S, Sagalowicz L, Glatter O. Self-assembled structures and pK(a) value of oleic acid in systems of biological relevance. *Langmuir.* 2010;26(14):11670–9.
- Salentinig S, Sagalowicz L, Leser ME, *et al.* Transitions in the internal structure of lipid droplets during fat digestion. *Soft Matter.* 2011;7(2):650–61.
- Fatouros DG, Bergenstahl B, Mullertz A. Morphological observations on a lipid-based drug delivery system during *in vitro* digestion. *Eur J Pharm Sci.* 2007;31(2):85–94.
- Jenkins TC, Palmquist DL. Effect of added fat and calcium on *in vitro* formation of insoluble fatty-acid soaps and cell-wall digestibility. *J Anim Sci.* 1982;55(4):957–63.
- Marounek M, Skrivanova E, Rada V. Susceptibility of *Escherichia coli* to C2–C18 fatty acids. *Folia Microbiol (Praha).* 2003;48(6):731–5.

37. Sassene PJ, Knopp MM, Hesselkilde JZ, *et al.* Precipitation of a poorly soluble model drug during in vitro lipolysis: characterization and dissolution of the precipitate. *J Pharm Sci.* 2010;99(12):4982–91.
38. Winkler FK, D'Arcy A, Hunziker W. Structure of human pancreatic lipase. *Nature.* 1990;343(6260):771–4.
39. Thomas N, Holm R, Mullertz A, Rades T. In vitro and in vivo performance of novel supersaturated self-nanoemulsifying drug delivery systems (super-SNEDDS). *J Control Release.* 2012;160(1):25–32.
40. Thomas N, Holm R, Garmer M, *et al.* Supersaturated self-nanoemulsifying drug delivery systems (Super-SNEDDS) enhance the bioavailability of the poorly water-soluble drug simvastatin in dogs. *Aaps J.* 2013;15(1):219–27.
41. Stillhart C, Durr D, Kuentz M. Toward an improved understanding of the precipitation behavior of weakly basic drugs from oral lipid-based formulations. *J Pharm Sci.* 2014;103(4):1194–203.
42. Thomas N, Richter K, Pedersen TB, *et al.* In vitro lipolysis data does not adequately predict the in vivo performance of lipid-based drug delivery systems containing fenofibrate. *Aaps J.* 2014;16(3):539–49.
43. Griffin BT, Kuentz M, Vertzoni M, *et al.* Comparison of in vitro tests at various levels of complexity for the prediction of in vivo performance of lipid-based formulations: case studies with fenofibrate. *Eur J Pharm Biopharm.* 2014;86(3):427–37.