

Toward the Establishment of Standardized *In Vitro* Tests for Lipid-Based Formulations. 5. Lipolysis of Representative Formulations by Gastric Lipase

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ABSTRACT

Purpose Lipid-based formulations (LBF) are substrates for digestive lipases and digestion can significantly alter their properties and potential to support drug absorption. LBFs have been widely examined for their behaviour in the presence of pancreatic enzymes. Here, the impact of gastric lipase on the digestion of representative formulations from the Lipid Formulation Classification System has been investigated.

Methods The pHstat technique was used to measure the lipolysis by recombinant dog gastric lipase (rDGL) of eight LBFs containing either medium (MC) or long (LC) chain triglycerides and a range of surfactants, at various pH values [1.5 to 7] representative of gastric and small intestine contents under both fasting and fed conditions.

Results All LBFs were hydrolyzed by rDGL. The highest specific activities were measured at pH 4 with the type II and IIIA MC formulations that contained Tween®85 or Cremophor EL respectively. The maximum activity on LC formulations was

recorded at pH 5 for the type IIIA-LC formulation. Direct measurement of LBF lipolysis using the pHstat, however, was limited by poor LC fatty acid ionization at low pH.

Conclusions Since gastric lipase initiates lipid digestion in the stomach, remains active in the intestine and acts on all representative LBFs, its implementation in future standardized *in vitro* assays may be beneficial. At this stage, however, routine use remains technically challenging.

KEY WORDS digestion · drug delivery · lipid formulation · lipolysis · stomach

INTRODUCTION

Modern drug discovery has led to an increasing number of poorly water-soluble drug candidates, with current estimates

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suggesting that up to 75% of drugs in development have low aqueous solubility (i.e. BCS Class II and IV) (1,2). These molecules often suffer from low oral absorption, and despite their pharmacological activity, commonly fail to proceed to advanced stages of research and clinical development (3). A great challenge facing the pharmaceutical industry is therefore to develop formulations that support the bioavailability of poorly water soluble drugs following oral administration (4). One popular approach to improve oral bioavailability is the utilization of lipid-based formulations (LBF) (5,6). LBFs are mixtures of components including non-polar and polar oils, surfactants and cosolvents. Depending on their composition, LBF may form oil-in-water emulsions, microemulsions or nanoemulsions upon mild agitation in aqueous media such as gastrointestinal (GI) fluids (7). LBFs increase drug absorption and oral bioavailability via several physicochemical and biological mechanisms, including avoidance of traditional drug dissolution processes, increased drug solubility in the intestine (6,8,9), increased intestinal permeability (10–12), decreased pre-systemic metabolism in the intestine (13) or enhancement of lymphatic transport (14).

Whilst LBFs offer great potential for enhancing drug absorption and oral bioavailability, their use has been limited by the lack of standardized *in vitro* tests for LBFs that are predictive of *in vivo* performance, and the fact that relatively few *in vivo* studies in humans have been reported in the literature. Hence, there is a need for standardized *in vitro* methods that support the rational selection of LBF composition for a given drug candidate. Standard dissolution/dispersion methods are not easily adapted for testing LBFs, since many of the excipients present in LBFs are potential substrates for digestive lipolytic enzymes, and a dynamic variation in LBF composition is likely to occur in the GI tract (15). Combining dissolution/dispersion studies with *in vitro* digestion is therefore required to better simulate the fate of LBFs in the GI tract and the effects of LBF digestion on drug dispersion, solubilisation and absorption. In that context, a consortium of academic laboratories and pharmaceutical industry representatives, the Lipid Formulation Classification System (LFCs) Consortium, has been established to develop common methods for the *in vitro* assessment of LBFs (see <http://www.lfcsconsortium.org/>). A standardized method using pancreatic enzymes (pancreatin) and the pHstat technique has been developed as a static *in vitro* model for testing LBF lipolysis (16) and the *in vitro* performance of type I, II, IIIA, IIIB, and IV LBFs using danazol, fenofibrate and tolfenamic acid as model poorly water-soluble drugs has been reported previously (17,18). So far, however, this method has only included a single digestion step mimicking the fasted upper small intestine condition in terms of pH (6.5), enzyme (pancreatic lipase from pancreatin) and bile salt concentration.

In most *in vitro* digestion models, it is generally assumed that pancreatic lipase is the main enzyme involved in the GI

lipolysis of LBFs and therefore, that the gastric step of lipolysis by gastric lipase can be neglected. Another and more practical reason is that gastric lipase is not commercially available and this limits its use to only a few laboratories. The role of gastric lipase and intragastric lipolysis in the overall process of dietary fat digestion is however well documented. In the fed state, gastric lipase has a significant contribution to the digestion of long-chain dietary triacylglycerols (TAG), both by acting inside the stomach and also in the upper small intestine where it has been shown to be stable and active (19,20). Gastric lipase can release up to 10 to 25% of dietary TAG acyl chains in the stomach depending on the type of meal. More recently, it has also been demonstrated that gastric lipase is able to hydrolyse both the acylglycerol and PEG ester fractions of lipid excipients such as Labrasol® (21) and Gelucire® 44/14 (22). Gastric lipase could therefore play an important role in the digestion of LBFs and this is supported by recent reports of *in vitro* studies of Labrasol® and Gelucire® 44/14 digestion using a two-step digestion model and lipolysis product analysis (23,24).

The aim of this study was therefore to evaluate the digestion of eight representative LBFs (the intestinal digestion properties of which have been well reported recently (16,18)) by gastric lipase. For this purpose, *in vitro* experiments were carried using similar conditions to those already used by the LFCs Consortium (16,18), except that pancreatin was replaced by recombinant dog gastric lipase (rDGL) and pH values covering both fasted and fed conditions, as well as intragastric and duodenal conditions, were tested. Both direct titration and end-point titration to pH 9 were investigated as means of assessing gastric lipase specific activity on LBFs as well as the degree of fatty acid ionization at various pHs. The latter data allowed better definition of the pH range in which the pHstat technique can be used for the direct and continuous assessment of LBF digestion, and also helped to identify potential limitations of the technique.

MATERIALS AND METHODS

Chemicals

Tris-maleate, 2-(N-morpholino)ethanesulfonic acid (MES), sodium taurodeoxycholate (NaTDC, 97% TLC) and calcium chloride hydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) were purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France). Sodium chloride (NaCl) was purchased from VWR international (Fontenay-sous-Bois, France). Phosphatidylcholine (Lipoid E PC S, approximately 99.2% pure, from egg yolk) was obtained from Lipoid GmbH (Ludwigshafen, Germany). One molar sodium hydroxide (Titrisol Merck, Darmstadt, Germany) stock solution was diluted with water to obtain 0.1 N NaOH titration solution. The lipid formulations

were made using various triglycerides (corn oil, Captex 300EP/NF, Maisine 35–1, Capmul MCM EP), surfactants (Polysorbate 85 (Tween®85), Cremophor EL) and co-solvents (Transcutol HP). Details of all lipidic excipients used during *in vitro* digestion testing in the framework of LFCs Consortium can be found in (16,18).

Enzymes

Recombinant dog gastric lipase (rDGL) produced in transgenic maize was a generous gift from Meristem Therapeutics (Clermont-Ferrand, France) and was used as a model gastric lipase. This enzyme has similar properties to human gastric lipase (HGL) and has been used in previous studies on the *in vitro* digestion of lipid formulations (21,22,24). The batch used here (rDGLm 19) was a lyophilized powder which contains pure lipase (>98% proteins) mixed with lactose and salts (50% *w/w*). A stock solution of rDGL at 1 mg rDGL/mL was prepared by dissolving 20 mg rDGLm 19 powder in 20 mL of 10 mM MES, 150 mM NaCl, pH 6.0 buffer. Aliquots of 500 μ L were kept frozen at -20°C prior to use. The stability of rDGL thawed aliquots was confirmed by measuring the activity on tributyrin according to dog gastric lipase standard assay conditions (25).

Pancreatin (P7545, 8 \times USP specifications activity) was purchased from Sigma-Aldrich-Fluka Chimie (St-Quentin-Fallavier, France).

Preparation of Lipid-Based Formulations

Table 1 shows the composition of 8 LBFs investigated in this study. These formulations were chosen to cover the four classes defined in the Lipid Formulation Classification System (26). They were prepared using either long-chain lipids (LC; corn oil triglyceride in combination (1:1 *w/w*) with mixed glycerides of predominantly linoleic acid (Maisine™ 35–1))

or medium-chain lipids (MC; tricaprates/tricaprylate triglycerides (Captex 300 EP/NF) in combination (1:1 *w/w*) with mixed glycerides of capric/caprylic acid (Capmul MCM EP) as the oil phase, mixed with polysorbate 85 (Tween® 85) or polyethoxylated castor oil (Cremophor® EL) as surfactants, and di-ethylene glycol monoethyl ether (Transcutol® HP) as a co-solvent (16,18). These formulations show wide differences in emulsification properties, digestion properties and drug solubilization capacity. The oil to surfactant ratio was kept constant among these formulations in order to allow effective inter-formulation comparison. For example, Type II and IIIA formulations differed only by the choice of surfactant and not the surfactant concentration. Excipient ratios in LC and MC formulations were also held constant.

In Vitro Lipolysis of Lipid Formulations by Gastric Lipase

The experiments were performed with a pHstat device (Methrom® Titrino 718 apparatus) equipped with a 20–90 mL Thermostat 20 EA 876–20 titration vessel (Methrom® ref 61418220), a pH electrode (Methrom® ref 6.0234.100, 3 M KCl) and a propeller (25 mm in diameter) set at 450 rpm (“speed 2.5”) for mechanical stirring. Fatty acid titration was performed with 0.1 N NaOH. The pH stat vessel, thermostated at 37°C , was filled with 40 mL of the digestion buffer (2 mM Tris-maleate, 1.4 mM CaCl_2 , 150 mM NaCl, 3 mM NaTDC, 0.75 mM phosphatidylcholine) and 1 g LBF added per assay. Stirring was initiated for 10 min before enzyme addition and the initial pH was adjusted to pH 1.5, 2.0, 3.0, 4.0, 5.0, 5.5, 6.0, 6.5 or 7.0. The enzyme (10 μ L of rDGL stock solution; 10 μ g) was then added and direct titration of free fatty acids (FFA) was performed for 5 min at the selected pH value which was set as the pH end-point for the pHStat device. The pH-endpoint was then shifted to pH 9.0 for back-titration in order to ensure full ionization and titration of FFA, which was performed for 5 additional minutes (Total duration

Table 1 Compositions of Representative LBFs and Maximum Specific Activities of Gastric Lipase on These LBFs

LBF type	Composition (% <i>w/w</i>)	Maximum gastric lipase specific activity (U/mg)	Optimum pH
I-LC	Corn oil: mixed glycerides of predominantly linoleic acid (50/50)	137 \pm 3	5.0
II-LC	Corn oil: mixed glycerides of predominantly linoleic acid: Tween 85 (32.5/32.5/35)	104 \pm 3	4.0
IIIA-LC	Corn oil: mixed glycerides of predominantly linoleic acid: polyethoxylated castor oil (32.5/32.5/35)	165 \pm 4	5.0
I-MC	Tricaprate/tricaprylate triglycerides: mixed glycerides of capric/caprylic acid (50/50)	761 \pm 16	5.5
II-MC	Tricaprate/tricaprylate triglycerides: mixed glycerides of capric/caprylic acid : Tween 85 (32.5/32.5/35)	1035 \pm 65	4.0
IIIA-MC	Tricaprate/tricaprylate triglycerides: mixed glycerides of capric/caprylic acid : polyethoxylated castor oil (32.5/32.5/35)	1268 \pm 62	4.0
IIIB-MC	Mixed glycerides of capric/caprylic acid : polyethoxylated castor oil: di-ethylene glycol monoethyl ether (25/50/25)	407 \pm 28	4.0
IV	Polyethoxylated castor oil: di-ethylene glycol monoethyl ether (50/50)	28 \pm 2	5.0

A more detailed description of glyceride mixtures can be found in reference 16

LBF lipid-based formulation, LC long chain triglycerides, MC medium chain triglycerides

of the assay=10 min). A blank assay without any enzyme was performed for each initial pH in order to determine the amounts of NaOH required to increase the pH from the initial value to pH 9.0 in the absence of FFAs released by the lipase. These amounts of NaOH were subtracted from the total amounts of NaOH delivered in presence of the lipase. In this way, it was possible to determine the amounts of NaOH required to titrate FFAs released by the lipase and therefore, lipase activity. The total volume of NaOH delivered during the assays ranged from 500 μL to a maximum of 3,000 μL (i.e., 7.5% of the initial reaction volume (40 mL)) depending on the initial pH and enzyme activity. However, the maximum volume of NaOH added during the 5-min lipolysis period did not exceed 200 μL (i.e., 0.5% of the initial reaction volume), and most of the NaOH volume was delivered during the back titration at pH 9. This was achieved in less than 30 s for all assays. Since the lipase is totally inactive at pH 9 the main changes in volume did not occur during the critical period for enzyme activity. As such the dilution of the reaction mixture by NaOH is unlikely to have had an effect on enzyme activity and lipolysis rate. Adding lipase inhibitors to block lipolysis upon pH shifting is not necessary since this latter process is much faster and effective for inactivating gastric lipase than the complete enzyme inhibition process by specific inhibitors (27). Experiments were performed in triplicate at each pH value. Activities are expressed here as international units: 1 U=1 μmole FFA released per minute. Specific activities were expressed as U per mg of pure enzyme.

In Vitro Lipolysis of Lipid Formulations by Pancreatin

Additional lipolysis experiments at pH values ranging from 6.5 to 9 were performed using pancreatin (porcine pancreatic extracts) instead of gastric lipase. All other experimental conditions were as described in the “*In vitro* lipolysis of lipid formulations by gastric lipase” section.

Statistical Analysis

The specific activities of rDGL on various LBFs were compared using Student's *t* test.

RESULTS

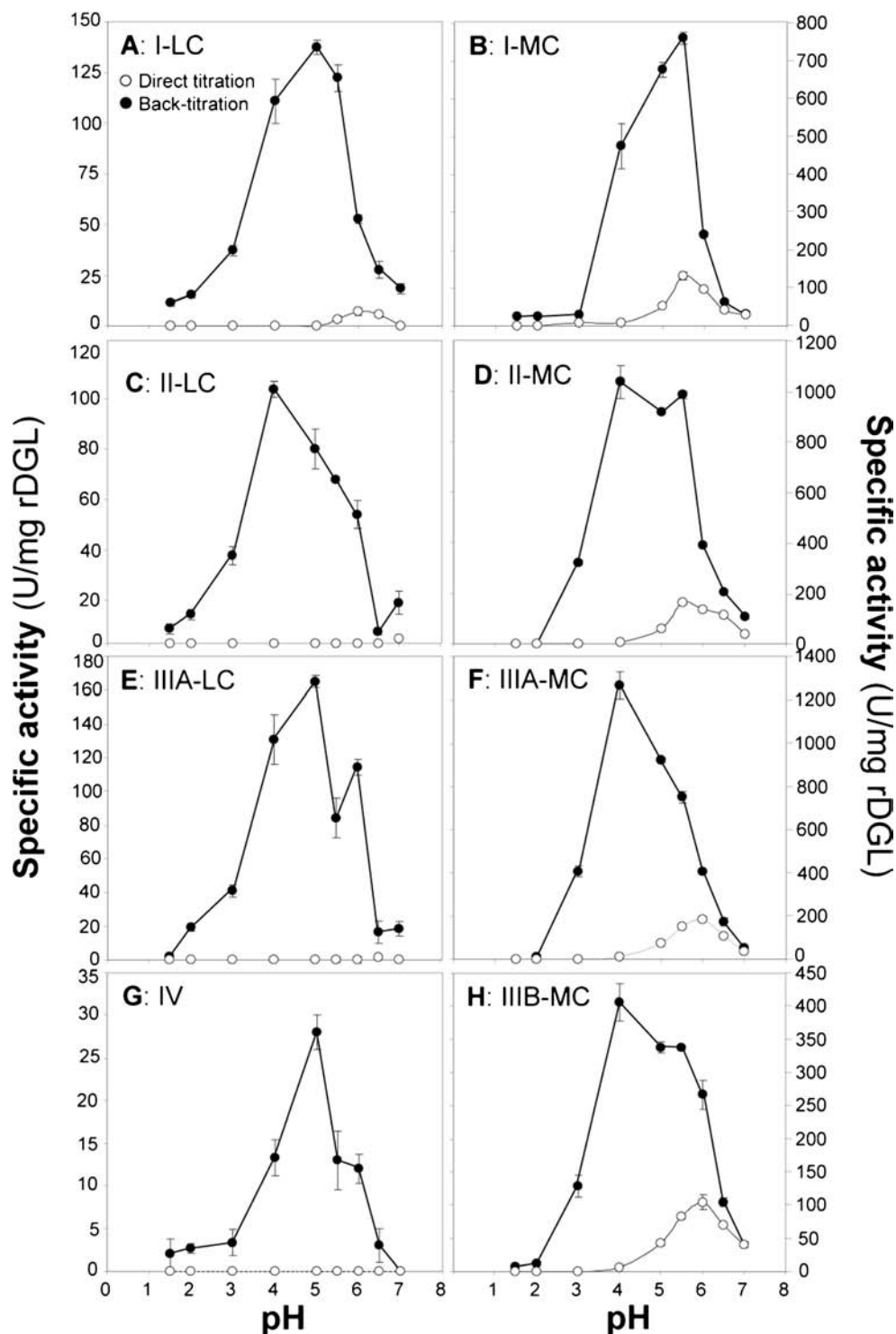
The lipolysis of LBFs by gastric lipase was evaluated *in vitro* using the standard conditions established by the LFCS Consortium for testing LBF lipolysis by pancreatin (16,18). This allowed comparison of gastric and pancreatic lipases under similar assay conditions. Nonetheless, various pH conditions were also screened, instead of using a single pH and lipolysis experiments were performed at pH values ranging from 1.5 to

7.0 to cover gastric and small intestine pH values, as well as fasted and fed conditions. The LFCS digestion buffer contains bile salts (3 mM NaTDC) and phospholipids (0.75 mM) in order to mimic intestinal conditions. The choice was made to keep these parameters for testing gastric lipase since gastric lipase acts both in the stomach and the small intestine (19,28) and its activity is not impaired by bile salts (20) and phospholipids (29). Using the pHstat device, it was possible to undertake direct titration and back-titration of the fatty acids released during lipolysis and thus to determine rDGL specific activities based on both total fatty acid titration and fatty acid ionization levels at the various pH values tested.

rDGL was active on all LBFs tested (Fig. 1), including LC and MC formulations, with optimum activities recorded at acidic pH values. The maximum apparent specific activities of rDGL were recorded within the 4 to 5.5 pH range for all formulations (Table 1). The highest specific activities of rDGL were measured with the Type II MC formulations ($1,035.0 \pm 65.0$ U/mg) and Type IIIa MC formulations ($1,267.7 \pm 62.5$ U/mg) at pH 4, while the lowest activity was recorded with the Type IV formulation, a formulation that only contains polyethoxylated castor oil as potential lipase substrate (Cremophor® EL). The maximum activity of rDGL on LC formulations reached 165 ± 4 U/mg with the Type IIIa LBF and was 7.7-fold lower than the maximum activity on MC LBFs. For all LBF, rDGL had no significant activity at pH values ≤ 2 and ≥ 7 . The presence of surfactant (Tween® 85 or Cremophor® EL) in the LBF composition resulted in a higher activity of rDGL on MC LBFs and a shift of the optimum pH value from 5.5 (Fig. 1b) to 4 (Fig. 1d and f). rDGL activity on Type II and Type IIIa MC LBFs at pH 4 were thus increased 2.2-fold ($P < 0.001$) and 2.7-fold ($P < 0.001$) when compared with the activity on Type I MC formulation. For the LC LBFs, only Tween® 85 shifted the optimum pH from 5 (Fig. 1a) to 4 (Fig. 1c). Unlike the MC formulations, rDGL activity on Type II and Type IIIa LC LBF at pH 4 was not significantly different ($P > 0.05$) to that on Type I LC formulations.

Figure 1 also shows the comparison between direct and back-titration of the fatty acids released by rDGL at various pHs. As expected, the apparent activity of rDGL obtained by direct titration was much lower than the activity measured by back-titration. Indeed titration could only be detected using the direct titration method for the MC formulations (Fig. 1, panels b, d, f, h). The apparent pH optima were shifted towards the 5.5 to 6 pH range for the MC formulations. In contrast, for the LC formulations and the Type IV formulation, no or very low direct titration was observed (Fig. 1, panels a, c, e, g), probably due to low FA ionization at low pH values and low activity at higher pH values. It was thus impossible to estimate the levels of FFA ionization under these conditions. Additional experiments with pancreatin, that contains lipolytic enzymes that are active at higher pH values,

Fig. 1 Variation with pH in rDGL specific activity (U/mg of enzyme) on lipid-based formulations. **(a)** Type I-LC; **(b)** Type I-MC; **(c)** Type II-LC; **(d)** Type II-MC; **(e)** Type IIIA-LC; **(f)** Type IIIA-MC; **(g)** Type IV; **(h)** Type IIIB-MC. The *open circles* indicate the direct titration of ionized FFAs. The *dark circles* indicate the back-titration at pH 9 of total FFAs. Data are means \pm SD ($n = 3$). Formulation compositions can be found in Table 1. Assays were performed using 1 g LBF in 40 ml digestion medium containing 3 mM NaTDC, 0.75 mM phosphatidylcholine, 1.4 mM CaCl_2 and 150 mM NaCl. Abbreviations: rDGL recombinant dog gastric lipase, LC long chain, MC medium chain, FFA free fatty acids, LBF lipid-based formulation, NaTDC sodium taurodeoxycholate.



were performed with the same LBFs in order to compare direct and back-titrated data for FFA released at pH values above 6.5 and to establish FFA ionization levels at pH values ranging from 6.5 to 9. Results obtained with rDGL and pancreatin were combined and used to plot the variations in FFA ionization levels as a function of pH (Fig. 2). It is apparent that the FFAs released from the MC formulations and the LC

formulations form two distinct groups, as might be expected based on their differing FFA chain lengths and ionization properties. It was possible to estimate a mean apparent pKa of 6.25 ± 0.15 [6.1–6.4] for the FA of MC LBFs and of 7.75 ± 0.15 [7.6–7.9] for LC LBFs, respectively.

Correction factors to convert direct titration data into total titration of FFAs were obtained from the plots of FFA

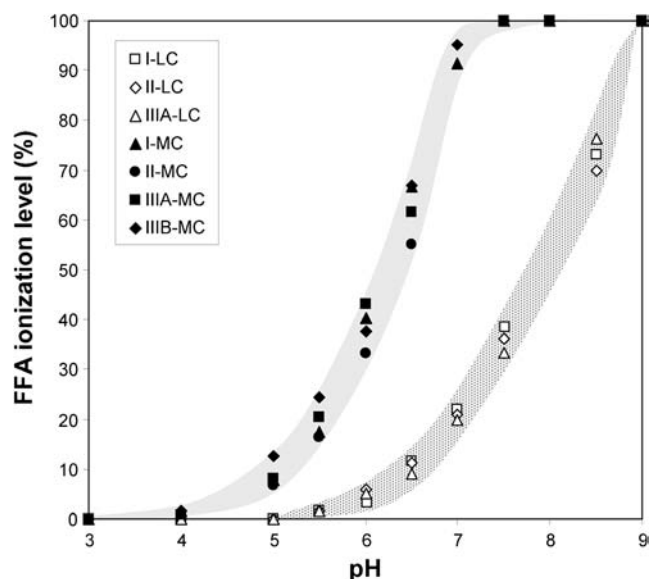


Fig. 2 Variations with pH in the ionization level of fatty acids released from LBFs. Values were deduced from direct and back-titration of FFAs released upon lipolysis of LBFs by rDGL (pH 1.5 to 6.5) and pancreatin (pH 6.5 to 9). Formulation compositions can be found in Table 1. Assays were performed using 1 g LBF in 40 ml digestion medium containing 3 mM NaTDC, 0.75 mM phosphatidylcholine, 1.4 mM CaCl₂ and 150 mM NaCl. Abbreviations: LBF lipid-based formulation, FFA free fatty acids, rDGL recombinant dog gastric lipase, NaTDC sodium taurodeoxycholate.

ionization *versus* pH. These conversion factors can be subsequently utilised to allow continuous monitoring of LBF lipolysis using the pHstat technique as shown previously (16). However, it is evident that some direct titration must be detectable to allow correction of the data and thereby to estimate total FFA release as a function of time. Since the optimum activity of gastric lipase on LC formulations is in the acidic pH range (where ionisation is low), and poor enzyme activity is apparent at pH 6.5 and above where ionisation is reasonable (Fig. 1), it appears that the direct and continuous assessment of LC LBFs lipolysis by gastric lipase cannot be performed at the pH values at which the enzyme mainly shows its activity (Fig. 1). Under these circumstances back titration must be employed to provide an indication of digestion.

DISCUSSION

In previous studies of the lipolysis of lipid-based formulations (LBFs), it has been shown that gastric lipase may exhibit high lipolytic activity against Labrasol® and Gelucire® 44/14 (21,22), two lipidic excipients containing lipase substrates (i.e. acylglycerols and PEG esters) with various acyl chain lengths. The activity of gastric lipase on these excipients was found to be optimum at acidic pH conditions (pH 5), as previously observed with dietary triglycerides (20,25,30). It is therefore likely that, *in vivo*, the digestion of these LBFs can commence in

the stomach under the action of gastric lipase, at least under gastric pH conditions corresponding to the fed state i.e. ranging from 3 to 6 and (24). These studies were extended here to a larger selection of LBFs spanning the four formulation classes as outlined by the Lipid Formulation Classification System (26). Recombinant dog gastric lipase (rDGL) was used as a model gastric lipase because it is more readily available than human gastric lipase (HGL) but retains many similar characteristics. The two enzymes have similar lipolytic activities on triglycerides with various acyl chain lengths (25,31), they share 85% amino acid identity (32) and their 3D structures obtained by X-ray crystallography are superimposable (33,34). As in previous studies of the LFCS consortium, the assays were performed using 1 g LBF in 40 ml reaction volume for direct comparison with dog pre-clinical studies and to allow comparison across the literature. Such an amount might be considered as too high for mimicking conditions in humans. Nevertheless, the LBF dose in a capsule for human use is typically 0.5 g–1 g and it is not uncommon for more than one capsule to be needed. A 1-g amount is therefore found within the normal dose range for a human. Moreover, the fluid volume commonly used for mimicking human conditions (250 mL) is overestimated compared to the usual gastric content volume under fasting conditions (19,20).

The eight representative LBFs selected by the LFCS Consortium were hydrolyzed by rDGL *in vitro*, regardless of the composition and acyl chain length of the excipients, at pH values above 2 and below 7, with optimum activities in the 4.0 to 5.5 pH range (Fig. 1 and Table 1). These results suggest that gastric lipase could also act on these LBFs *in vivo*, particularly in the stomach under fed conditions. Since the FFA released by gastric lipase can trigger the action of pancreatic lipase on triglyceride emulsions *in vitro* (29), it may be useful to include a gastric step in *in vitro* models of lipid digestion. Moreover, it has been shown that gastric lipase is still active in the proximal small intestine during test meals in humans (19) and dogs (35). Therefore, gastric lipase could be used in combination with pancreatic enzymes to better reproduce *in vitro* the duodenal conditions that are present during lipid digestion (15). To this point, however, the work of the LFCS consortium has focused on an *in vitro* digestion method corresponding to fasting conditions in the small intestine. Under these conditions it is likely that intragastric lipolysis will be minimal since lipolysis by gastric lipase is negligible at pH <2 and under fasting conditions the pH in the stomach is expected to be low.

The specific activities of rDGL were higher with MC than with LC formulations. It is possible that the difference in digestibility between MC and LC formulations may be explained by a difference in the dispersion properties of the formulations rather than by an acyl chain length preference of the enzyme. Indeed, rDGL usually shows higher activity on LC triglycerides (Intralipid®) than on short (tributyrin) and medium (trioctanoin) triglycerides (25). Thus, MC lipids in

combination with the surfactant employed are often better dispersed than LC and this could lead to higher levels of available lipid surface area for lipase binding and activity. The mean particle size of the dispersed type IIIA-MC and IIIB-MC LBFs were found to be 29.1 ± 0.6 and 21.4 ± 0.5 nm, respectively, which is consistent with good self-emulsification properties and the formation of ultrafine dispersions (16). In contrast, the Type I-LC and Type II-LC LBFs slowly dispersed to form a coarse emulsion (16). The emulsification properties of the type I-MC (opaque emulsion) and type I-LC (coarse emulsion) LBFs could not be characterized quantitatively by particle size analysis (16), but the Type I-MC LBF appeared to be a better substrate for gastric lipase than Type I-LC LBF (Table 1), consistent with slightly better dispersion properties. The addition of surfactant to form type II and IIIA LC LBFs enhanced emulsification properties significantly. Type IIIA-LC thus gave a near translucent dispersion of 61.8 ± 0.4 nm particle size (16), which was a slightly preferred substrate for gastric lipase among LC LBFs (Fig. 1 and Table 1).

Another possible explanation for the higher activity of rDGL on MC than on LC formulations could reside in the properties of the lipolysis products. Those produced from MC are more soluble in water and can readily enter the aqueous phase, while LC products usually remain at the oil-water interface and in doing so can inhibit access of lipase to the interface. When MC lipid formulations with or without surfactants were compared, differences in digestibility were apparent. Thus the rate and extent of lipolysis by rDGL was higher for the MC lipid formulations with surfactants (Fig. 1d and f) than for those without surfactants (Fig. 1b). This suggests that surfactants positively impact on both the dispersion properties of the MC lipid formulation and favour the solubilisation of MC digestion products, in turn promoting lipolysis.

Initiation of a gastric lipolysis step, prior to incubation with pancreatic enzymes may therefore be justified to most faithfully reproduce *in vitro* the combined actions of gastric and pancreatic lipases *in vivo*. A two-step static digestion model including a gastric phase and a duodenal phase has been reported previously for testing the *in vitro* lipolysis of two lipid excipients, Labrasol® and Gelucire® 44/14 (23,24), meals (36–38), food emulsions (39) and CITREM, a food emulsifier (40). The time-course of liberation of lipolysis products including FFAs were however measured after lipid extraction and analysis at various incubation times.

A limitation of the pHstat technique in a two-step static digestion model is the fact that long chain FFA may not be significantly ionized at the low pH value of the gastric step to allow direct titration. To assess this issue, FFA ionization levels were determined at various pH values for each LBF and mean apparent pKa values were estimated for MC (pKa=6.25) and LC (pKa=7.75) fatty acids. A shift of 1.5 pH unit in apparent

pKa was thus observed between MC and LC fatty acids. The mean apparent pKa value obtained with LC is in the same range as that reported for free oleic acid present in an aqueous emulsion of olive oil (41,42). Previous studies have shown that the apparent pKa value of fatty acids is dependent not only on the alkyl chain length but also on the colloidal environment (43,44). Bile salts, gum Arabic, calcium ions, phospholipids, the quantity of lipids dispersed in the water phase and the presence of differing colloidal structures (membranes, micelles, oil-in-water emulsions, vesicles) therefore have significant effects on apparent pKa (41–44). This dictates that it is important to determine apparent pKa values and FA ionization levels under the conditions employed in individual tests since changes in pKa are likely to influence direct titration data obtained under, for example, different bile salt concentrations (41,42). With this caveat it is important to realise that the results reported here have been obtained in the presence of 3 mM NaTDC, 0.75 mM phosphatidylcholine, 1.4 mM CaCl₂ and 150 mM NaCl and that some differences may be apparent at the lower bile salt levels expected in the stomach. However, it is also apparent that quantifying lipolysis using the direct titration pH stat technique is restricted by both the pH-dependent activity profile of lipases and incomplete fatty acid ionization, particularly with LC lipids. It is therefore not always possible to use direct and continuous titration of FFAs for monitoring the lipolysis of LBFs under *in vitro* conditions that reflect the physiological conditions in the GI tract (especially under acidic conditions). An end-point value of lipolysis at any pH can be easily obtained by back-titration, but the time-course assessment of lipolysis at low pH still requires additional assays such as lipid extraction and analysis for quantifying FFA.

Bearing in mind these complexities, the LFCS Consortium has, to this point, employed a standardized *in vitro* assay that includes a single incubation step with pancreatic enzymes at pH 6.5 (16,18). This one-step assay is simple, mimics the conditions during digestion in the upper small intestine, and allows the direct and continuous measurement of both MC and LC LBFs lipolysis using the pHstat technique. Under these conditions (pH 6.5) the FFA released from MC LBFs are ionized to a large extent at (55 to 67% ionization of medium chain fatty acids) and even long chain FFA released from LC LBFs are to some extent ionized (10 to 20% at pH 6.5) and can therefore be directly measured and quantified using a correction factor obtained during back titration. The standard LFCS assay conditions have also been deliberately developed to use commercially available equipment and pancreatic digestive enzymes. In contrast, recombinant gastric lipase is only available in a few laboratories limiting broader application. In conclusion, the use of gastric lipase for *in vitro* assay of LBF digestion is today restricted by both its production as a commercial product and the technical limitation of the pHstat technique for a direct measurement of LC

triglyceride lipolysis at low pH. Since gastric lipase is at the start of lipid digestion in the GI tract and has been shown to act on all representative LBFs tested here, the implementation in future iterations of the LFCS standardized *in vitro* assay may be envisaged but remains a challenging outcome.

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