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RESEARCH PAPER

In Vitro Digestion of the Self-Emulsifying Lipid Excipient Labrasol® by Gastrointestinal Lipases and Influence of its Colloidal Structure on Lipolysis Rate

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ABSTRACT

Purpose Labrasol® is a self-emulsifying excipient used to improve the oral bioavailability of poorly water-soluble drugs. It is a mixture of acylglycerols and PEG esters, these compounds being substrates for digestive lipases. The characterization of Labrasol® gastrointestinal lipolysis is essential for understanding its mode of action.

Methods Labrasol® lipolysis was investigated using either individual enzymes (gastric lipase, pancreatic lipase-related protein 2, pancreatic carboxyl ester hydrolase) or a combination of enzymes under *in vitro* conditions mimicking first the gastric phase of lipolysis and second the duodenal one. Specific methods for quantifying lipolysis products were established in order to determine which compounds in Labrasol® were preferentially hydrolyzed.

Results Gastric lipase showed a preference for di- and triacylglycerols and the main acylglycerols remaining after gastric lipolysis were monoacylglycerols. PEG-8 diesters were also hydrolyzed to a large extent by gastric lipase. Most of the compounds initially present in Labrasol® were found to be totally hydrolyzed after the duodenal phase of lipolysis. The rate of Labrasol® hydrolysis by individual lipases was found to vary significantly with the dilution of the excipient in water and the resulting colloidal structures (translucent dispersion; opaque emulsion; transparent microemulsion), each lipase displaying a distinct pattern depending on the particle size.

Conclusions The lipases with distinct substrate specificities used in this study were found to be sensitive probes of phase transitions occurring upon Labrasol® dilution. In addition to their use for developing *in vitro* digestion models, these enzymes are interesting tools for the characterization of self-emulsifying lipid-based formulations.

KEY WORDS carboxyl ester hydrolase · enzyme · gastric lipase · gastrointestinal lipolysis · macrogolglycerides · oral drug delivery · pancreatic lipase-related protein 2 · particle size

ABBREVIATIONS

BSA	Bovine serum albumin
CEH	Carboxylester hydrolase
CMC	Critical micellar concentration
DAG	Diacylglycerol
FFA	Free fatty acid
HPJ	Human pancreatic juice
HPL	Human pancreatic lipase
MAG	Monoacylglycerol
NaTDC	Sodium taurodeoxycholate
PCS	Photon correlation spectroscopy
PPE	Porcine pancreatic extracts
PPL	porcine pancreatic lipase
PSD	Particle size distribution
rDGL	Recombinant dog gastric lipase
rHPLRP2	Recombinant human pancreatic lipase-related protein 2
SEDDS	Self Emulsifying Drug Delivery Systems
SMEDDS	Self MicroEmulsifying Drug Delivery Systems
SNEDDS	Self NanoEmulsifying Drug Delivery Systems
TAG	Triacylglycerol

INTRODUCTION

Oral administration is the most convenient way for drug uptake. However, most of new drugs developed by the phar-

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maceutical industry are poorly soluble in gastrointestinal fluids which impairs their oral bioavailability (1). Innovative lipid-based formulations such as SEDDS (Self Emulsifying Drug Delivery Systems), SMEDDS (Self MicroEmulsifying Drug Delivery Systems) and recently, SNEDDS (Self NanoEmulsifying Drug Delivery Systems) are developed to improve the oral bioavailability of poorly-water soluble drugs (2–8). SEDDS, SMEDDS, and SNEDDS are isotropic mixtures of oil, surfactant, co-surfactant, and drug that form oil-in-water emulsions, microemulsions and nanoemulsions, respectively, under gentle stirring (2,9,10).

The oral bioavailability of the drug is improved by either increasing the drug dissolution rate or by presenting and maintaining the drug in solution in the gastrointestinal tract. The interaction of lipid-based formulations and their digestion products with endogenous biliary amphiphilic molecules and dietary lipids in the gastrointestinal tract could explain the enhanced bioavailability of some poorly water-soluble drugs (11). Since lipid-based formulations contain various esters (acylglycerols, PEG esters…) that are substrates for gastrointestinal lipolytic enzymes (12–14), their lipolysis products may have a major influence on the fate of the drug in the gut (15). Based on these hypothesis, new *in vitro* dissolution assays including lipolytic enzymes are currently in development for a better prediction of drug solubility and bioavailability (16–18).

A new interest for lipid digestion has raised in this context and is now extended to the substrates found in lipid-based formulations. Digestive lipases are soluble enzymes which are able to hydrolyze ester bonds of insoluble substrates such as long chain triacylglycerols (TAG) but some of these enzymes are also able to hydrolyze ester bonds of various soluble or partially soluble substrates (14). Acylglycerols are known to be the natural substrates of digestive lipases (Fig. 1a), gastric and classical pancreatic lipases showing a substrate preference for TAG and diacylglycerols (DAG) while pancreatic lipase-related protein 2 (PLRP2) and pancreatic carboxyl ester hydrolase (CEH) have a substrate preference for monoacylglycerols (MAG) and more generally substrates forming micellar structures in water (14). It was shown however that gastric lipase, PLRP2 and CEH can hydrolyze at high rates the synthetic PEG mono- and diesters (Fig. 1b) present in lipid-based formulations (12,13).

In recent years, the importance of gastrointestinal lipolysis on the bioavailability of poorly water-soluble drugs has been investigated. In most of the lipolysis and/or solubility studies related to the drug absorption, the gastric digestion step has not been taken into consideration. Porcine pancreatic extracts (PPE; pancreatin) has often been used to mimic human pancreatic juice (HPJ) and it is generally assumed that the lipolytic activity is due to the action of the classical pancreatic lipase and its cofactor, colipase (19–24). Although classical pancreatic lipase is the main lipase involved in the lipolysis of dietary TAG in humans, the digestion of dietary lipids actually starts in the stomach with the action of gastric lipase (25). Intragastric lipolysis (10% to 25%) is quantitatively lower than intestinal lipolysis but the free fatty acids (FFA) produced in the stomach are qualitatively important for triggering bile and pancreatic juice secretions, as well as for the activity of pancreatic lipase (26). In addition to human pancreatic lipase (HPL) (27), the exocrine pancreas produces other lipolytic enzymes that play significant roles in lipid digestion such as human pancreatic lipase-related protein 2 (HPLRP2), carboxyl ester hydrolase (CEH or bile salt stimulated lipase or cholesterol esterase) and phospholipase A2 (14). Some of these human enzymes like PLRP2 have not been identified in PPE so far and these digestive enzyme preparations from porcine origin might not be always equivalent to HPJ. Recent studies on the lipolysis of Labrasol® and Gelucire® 44/14 have well illustrated the necessity to take into account the various lipolytic enzymes found in the GI tract. These lipid excipients were not hydrolyzed by HPL whereas they were hydrolyzed by recombinant dog gastric lipase (rDGL), recombinant HPLRP2 (rHPLRP2) and bovine CEH (12,13). Labrasol® and Gelucire® 44/14 were also used for investigating the gastrointestinal lipolysis of lipid-based formulations of cinnarizine and piroxicam under conditions mimicking first the gastric phase of digestion in presence of gastric lipase, and second the duodenal phase of digestion after adding PPE previously mixed with bile salts (15). This is the first time to our knowledge that this two-step model of *in vitro* digestion developed with test meals (28–31) was used for testing lipid-based drug delivery systems.

As previously mentioned, digestive lipases have different substrate specificities and studies with Labrasol® and Gelucire® 44/14 allowed to identify which compounds in these excipients were substrates for each lipase (12,13). Moreover, it was thus shown that most compounds found in these excipients were hydrolyzed by lipases. Labrasol® is a macrogolglyceride which is able to form microemulsions in gastrointestinal fluids. It is a mixture of PEG-8 caprylocaproyl macrogolglycerides, which is obtained by performing polyglycolysis of medium chain TAG with PEG-8 (molecular weight = 400). Labrasol® is composed of C8-C10 mono-, di-, and triacylglycerols, C8-C10 mono- and diesters of PEG-8, and free PEG-8. The main fatty acids present are caprylic and capric acids. Many studies have shown that Labrasol® significantly increases the oral bioavailability of various drugs such as insulin (32), gentamicin and glycyrrhizin (33), vancomycin (34), low molecular weight heparin (35), ezetimibe (36), fizetin (37), flurbiprofen (38), dexibuprofen (39), olmesartan medoxomil (40), curcumin (41), ganciclovir (42), anti-cancer agent SR13668 (43), etodolac (44), buparvaquone (45), coenzyme Q10 (46), or nimodipine (47).

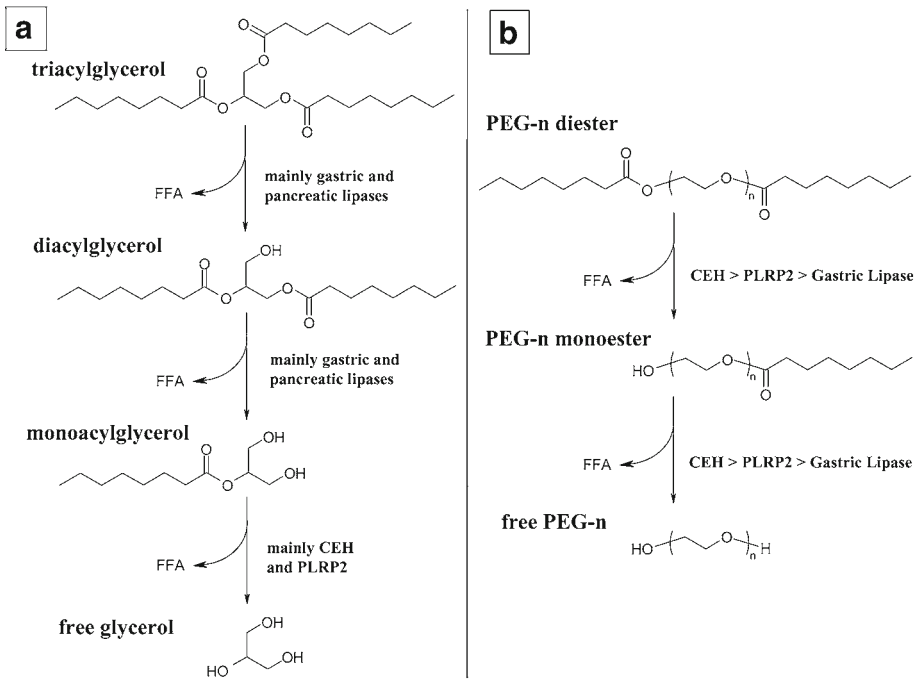

Fig. 1 Schematic representation of Labrasol® constituent hydrolysis by digestive lipases. (**a**) hydrolysis of tricapryloylglycerol. (**b**) hydrolysis of PEG-n dicaprylate. n is the number of monomeric units of PEG, PEG-8 being the main form of PEG in Labrasol®.

The main gastrointestinal lipases acting on Labrasol® (gastric lipase, PLRP2, CEH) have been previously identified (12). The aim of this novel study was to go further in the characterization of their substrate specificity by measuring changes occurring in Labrasol® composition during lipolysis. These investigations were performed with both individual lipases and lipase mixtures in a two-step *in vitro* digestion model mimicking the gastric and duodenal phases. In this later case, HPJ and PPE were compared to support the use of PPE for replacing HPJ. The influence of Labrasol® particle size and colloidal structure on lipolyis by individual lipases was also investigated.

MATERIALS AND METHODS

Chemicals

Sodium taurodeoxycholate (NaTDC, 97% TLC), bovine serum albumin (BSA), and calcium chloride dihydrate ($CaCl_2$, $2H_2O$; minimum 99%), were purchased from Sigma-Aldrich-Fluka Chimie (Saint-Quentin-Fallavier, France). Tris-(hydroxymethyl)-aminomethane (Tris) and sodium chloride (NaCl) were purchased from Euromedex (Mundolsheim, France) and from VWR International (Fontenay-sous-Bois, France), respectively. Organic solvents were purchased from Carlo Erba-SDS (Val de Reuil, France) and were of HPLC grade. Labrasol® (PEG-8 caprylocaproyl macrogolglycerides, batch 34756, Gattefossé SAS, Saint-Priest, France) is composed of mono-, di- and triacylglycerols (10%), PEG-8 mono- and diesters and free PEG-8 (90%). The main fatty acids are caprylic (57%) and capric (43%) acids. A stock solution of 1 M NaOH (Tritrisol, Merck, Darmstadt, Germany) was diluted with water to obtain 0.1 M NaOH titration solution.

Enzymes

Lyophilised HPJ and recombinant dog gastric lipase (rDGL) were generous gifts from Dr. A. De Caro (EIPL, Marseille) and Meristem Therapeutics (Clermont-Ferrand, France), respectively. Lipid-free PPE and bovine carboxyl ester hydrolase (CEH) were purchased from ID bio SAS (Limoges, France) and Sigma-Aldrich-Fluka (Saint-Quentin-Fallavier, France) respectively. Recombinant human PLRP2 (rHPLRP2) was produced in the yeast *Pichia pastoris* and purified from culture media as described in (48). Porcine colipase was partly purified from lipid-free pancreatic powder using the procedure described in (12). Protein concentrations were determined using Bradford's procedure (49) with Bio-Rad dye reagent and BSA as the standard protein.

Lipolysis of Labrasol® by Individual Lipases

From previous experiments (12), it emerged that all lipases acting on Labrasol® showed a significant activity at pH 6.0, the mean pH value recorded in the small intestine during meal digestion (25). This pH value was therefore chosen for performing continuous and direct assays of lipase activity on Labrasol® using the pHstat technique. The release of FFAs upon Labrasol® hydrolysis was measured with a 718 STAT Titrino pH-stat apparatus (Metrohm, Switzerland). A

dispersion of Labrasol® in the assay solution (NaCl 150 mM; NaTDC 4 mM; $CaCl_2$ 1.4 mM; Tris–HCl 1 mM) was mechanically stirred in a temperature-controlled reaction vessel at 37°C. After addition of the lipase sample (rHPLRP2, CEH or rDGL), the pH was kept constant at pH 6.0 using an automated burette to titrate FFAs with a 0.1 M NaOH solution. A 2-fold molar excess of colipase was added when the lipase activity of rHPLRP2 was measured. Since the ionisation rate of the FFA released from Labrasol® is 38% at pH 6.0, a correction factor of 2.63 had to be used for estimating the effective amounts of FFAs released with time upon Labrasol® hydrolysis (12). Lipase activities were expressed in either U per mg of enzyme (1 U = 1 μmole of FFA released per min) or percentage of the maximum activity measured with each lipase. Labrasol® hydrolysis levels were deduced from the effective amounts of FFAs and the known amounts of esterified fatty acids in Labrasol®.

A first series of experiments was performed using 1.0 g of Labrasol® in a 30 mL of the assay solution for the subsequent analysis of Labrasol® lipolysis products. Samples of 1,000 μL were taken at various times ranging from 0 to 15 min and immediately frozen using liquid N_2 so as to stop the hydrolysis reaction. The collected samples were stored at −20°C until lyophilisation. For each lipase, the experiments were performed twice using two different lipase concentrations.

A second series of experiments was performed using various amounts of Labrasol® (0.5 to 70 g/L) in 15 mL of the assay solution for determining lipase activity as a function of colloidal structure and particle size in Labrasol® aqueous dispersions (see the following section on particle size analysis). For each Labrasol® concentration, experiments were performed in triplicates.

In Vitro Simulation of Gastrointestinal Lipolysis of Labrasol®

Experimental conditions were adapted from the *in vivo* data recorded at 50% gastric emptying of test meals both in the stomach (pH 5.5) and in the duodenum (pH 6.25), and enzymatic solutions were prepared according to *in vivo* secretions of lipases during a meal (28). The following simulation was adapted from our previous publication (15) with a lower amount of lipid excipient (300 mg instead of 1 500 mg in a total assay volume of 30 mL; 10 g/L) corresponding to the usual lipid formulations of poorly water-soluble drugs with this self-emulsifying excipient.

The gastric enzyme solution was prepared using rDGL. Lyophilized rDGL powder was dissolved in the assay solution (150 mM NaCl, 4 mM NaTDC, 1.4 mM $CaCl_2$, 1 mM Tris–HCl) so as to obtain a solution at 100 μg/mL. Concerning pancreatic enzyme solutions, they were prepared using either HPJ or PPE. The solution of HPJ was prepared so as to obtain a concentration of 614 μg HPL/mL. To determine the amount of PPE necessary to reach the same amounts of pancreatic lipase contained in HPJ, we assessed first the lipolytic activity of HPJ and PPE using tributyrin as substrate and used a specific activity of 8,000 U/mg for determining the mass concentration of active pancreatic lipase in both HPJ and PPE (50). Both gastric and pancreatic enzymatic solutions were stored at -20°C until their use.

Experiments were performed during 90 min to simulate gastrointestinal digestion of lipids occurring in the upper part of the GI tract (stomach and duodenum). An emulsion of 300 mg of Labrasol® in 30 mL of the assay solution (NaCl 150 mM; NaTDC 4 mM; $CaCl_2$ 1.4 mM; Tris–HCl 1 mM) was mechanically stirred in a temperature-controlled reaction vessel at 37°C. At $t = 0$ min, 6.0 mL of gastric enzyme solution freshly prepared were added to the reaction vessel to obtain a final concentration of 17 μg/mL of rDGL and the pH was kept constant at 5.5 for 30 min (gastric lipolysis phase), via an automated titration of FFAs with 0.1 M NaOH using a pH-stat device. At $t = 30$ min, 22.0 mL of pancreatic enzyme solution freshly prepared with either PPE or HPJ, were added to the mixture and the pH was shifted to 6.25 and kept constant for 60 min. After adding the pancreatic enzyme solution to the reaction vessel, the final pancreatic lipase concentration was 250 μg/mL and the dilution of the gastric phase was 1.7-fold. At $t = 0$, 15, 29, 35, 40, 45, 60, and 90 min, 1,000-μL samples were taken and immediately frozen using liquid N_2 so as to stop the lipolysis. The samples collected were stored at -20°C until lyophilization. In order to check that there was no spontaneous hydrolysis of the excipients, similar experiments were performed without adding enzyme solutions but using similar dilutions.

The analytical method developed by Gattefossé to assay its pure excipients was adapted to the samples resulting from the lipolysis of Labrasol® (15). Frozen lipolysis samples were freeze-dried for 1 day (Heto Drywinner FD 3). Aliquots containing the freeze-dried samples were mixed with 3 times with 1 mL of chloroform (Carlo Erba RS for HPLC) and the organic phase was filtered on 0.45 μm glass filters. After evaporation of the solvent, samples were dissolved in 0.5 mL of chloroform and solid phase separation (Chromabond SiOH, Macherey-Nagel, Germany) was performed in order to collect separately the fraction of polar compounds (free PEG, mono and diesters of PEG) and the fraction of apolar compounds (TAG, DAG and MAG). Free PEG and PEG diesters were analyzed by HPLC-ELS (HPLC LaChrom, Merck Hitachi; detector ELSD PL-ELS 1000, Polymer Laboratories, United-Kingdom). Acylglycerols were assayed by GPC-FID method (6890 Agilent Technologies, France). The quantification of PEG monoesters was not possible using this method, explaining why they are not represented on Figs. 2 and 3.

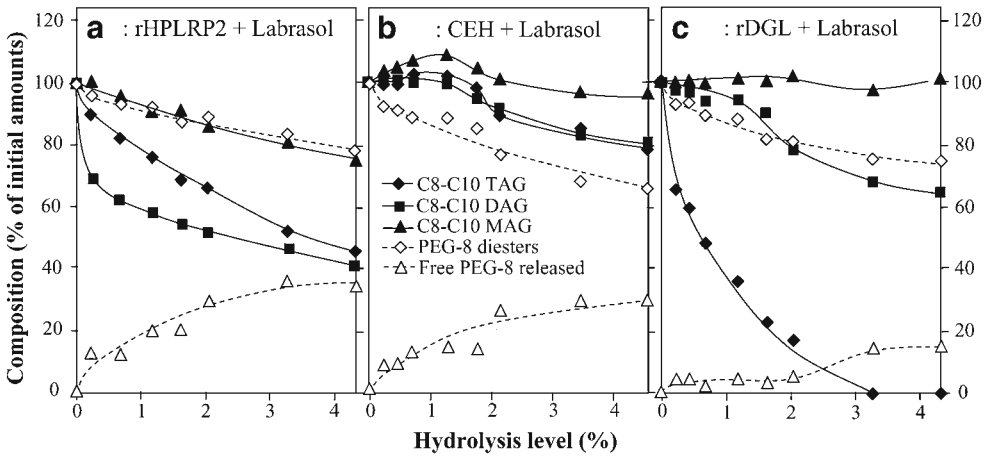

Fig. 2 Initial variations in the composition of Labrasol® upon hydrolysis by individual lipases. (**a**) rHPLRP2, (**b**) CEH and (**c**) rDGL. The hydrolysis level was defined as the ratio between the micromoles of FFAs released and the total micromoles of fatty acids present in the excipient. The variations in lipolysis products are expressed in percent of TAG, DAG, MAG and PEG diesters initially present in the excipient. For free PEG, only the amounts generated upon hydrolysis are shown and expressed in percent of the amounts of PEG initially present in the excipient.

Particle Size Analysis of Aqueous Dispersions of Labrasol®

Labrasol® dispersions were prepared using increasing amounts of the self-emulsifying excipient in the assay solution (150 mM NaCl, 4 mM NaTDC, 1.4 mM CaCl2 1 mM Tris–HCl) at pH 6.0 or in water. The masses of Labrasol® introduced in 1 L of assay solution were 0.5, 1, 2, 3, 4, 5, 10, 20, 25, 30, 31, 32, 40, 50 and 70 g. Dissolution bath USP type II paddle apparatus were used to keep the dispersions at 37°C and stirred them at 100 rpm for 30 min.

The droplet size distribution of the dispersions obtained after 30 min was determined by photon correlation spectroscopy (PCS) using a PSS Nicomp 380 ZLS (PSS Nicomp, Santa Barbara, USA). This equipment enables measuring particle sizes between 1 and 5,000 nm, as well as fluctuation with time of the particle size distribution (PSD). This is particularly useful when unstable phases are formed and the droplet size steadily increases with time. PCS analyzes the fluctuations in light scattering due to Brownian motion of particles. Light scattering was monitored at 37°C, at a scattering angle of 90° and λ =632.8 nm. PSD was expressed in Nicomp intensity weighted distribution after 2 runs of 10 min in order to compare PSD obtained with the same method and after the same time.

RESULTS

Identification of Substrate Preference in the Course of Labrasol® Lipolysis by Individual Lipases

These experiments were performed using a large excess of Labrasol® (30 g/L) so as to identify the families of compounds

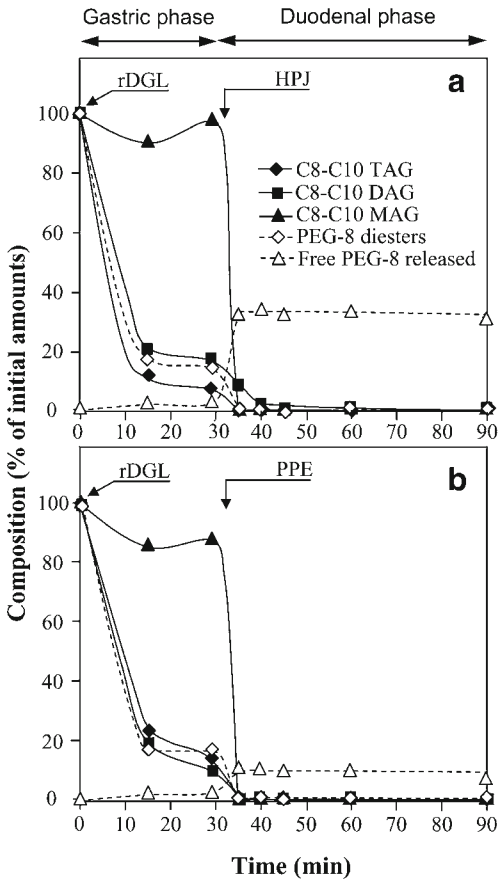

Fig. 3 Variation with time in the composition of Labrasol® in a two-step digestion model including gastric and duodenal phases of lipolysis. (**a**) Combined action of rDGL and HPJ; (**b**) combined action of rDGL and PPE. Arrows indicate the successive additions of rDGL (t = 0 min) and either HPJ or PPE (t = 30 min). The variations in lipolysis products are expressed in percent of TAG, DAG, MAG and PEG diesters initially present in the excipient. For free PEG, only the amounts generated upon hydrolysis are shown and expressed in percent of the amounts of PEG initially present in the excipient.

(TAG, DAG, MAG and PEG diesters) that were preferentially hydrolyzed by each lipase. Kinetics of Labrasol® hydrolysis by individual lipases (1–10 μg added to the reaction vessel) were found to be linear under these conditions where the overall hydrolysis level did not exceed 5%. The hydrolysis level was deduced from the continuous titration of FFA released upon hydrolysis and the known amounts of fatty acids initially esterified in Labrasol® compounds. Variations in these compounds were expressed in percent of their initial amounts, except for free PEG-8, for which only the amounts generated during the reaction were expressed as a percent of total PEG. The variations in PEG monoesters were not directly accessible using the methods applied in this study but the measurement of free PEG-8 released during the reaction was used as an indirect estimation of PEG monoester hydrolysis (Fig. 1b). These variations in Labrasol® compounds were plotted as a function of the hydrolysis level (Fig. 2) rather than time because this representation is not dependent on the reaction rate and amount of enzyme used in the reaction. It is commonly used for comparing the substrate specificity of various lipases acting on a complex substrate with several ester bonds (51).

As shown in Fig. 2, the digestive lipases acting on Labrasol® exhibited different apparent substrate specificities. With rHPLRP2, DAG levels decreased faster than those of TAG and the decrease in MAG levels was even slower. Since DAG and MAG can be produced in the course of the reaction by the hydrolysis of TAG and DAG, respectively, it is not obvious to estimate the relative rates of hydrolysis for each compound without using mathematical model (51) and this estimation was not performed in the present study. The decrease in PEG-8 diesters levels (−22% at 4.3% of hydrolysis of Labrasol®; Fig. 2a) was similar to that observed with MAG. The release of free PEG-8 reached +34% at 4.3% of Labrasol® hydrolysis and one can deduce that PEG-8 monoesters were a better substrate for rHPLRP2 than PEG-8 diesters.

With bovine CEH, PEG-8 esters of Labrasol® were better hydrolyzed than C8-C10 acylglycerols (Fig. 2b). PEG-8 diesters showed a significant decrease (−35% at 4.7% of Labrasol® hydrolysis) whereas free PEG-8 released due to the lipolysis of PEG-8 monoesters increased (+29% at 4.7% of Labrasol® hydrolysis). The C8-C10 DAG and TAG presented a decrease (−20% at 4.7% of Labrasol® hydrolysis) significantly higher than that of C8-C10 MAG (−3% at 4.7% of Labrasol® hydrolysis) which showed a transient increase between 1 and 2% hydrolysis (Fig. 2b).

rDGL showed a clear preference for C8-C10 TAG of Labrasol® which were totally hydrolyzed at 3.1% of hydrolysis (Fig. 2c). A slower decrease in DAG levels was observed and the levels of C8-C10 MAG remained stable (101% remaining at 4.1% of hydrolysis of Labrasol®), suggesting that MAG were hydrolyzed at the same rate they were produced by rDGL (Fig. 2c). A continuous decrease of PEG-8 diesters (−25% at 4.1% hydrolysis of Labrasol®) was observed, whereas free PEG-8 levels slightly increased, suggesting that PEG-8 diesters were a better substrate for rDGL than PEG-8 monoesters.

In Vitro Simulation of Gastrointestinal Lipolysis of Labrasol®

In order to predict the fate of Labrasol® in the GI tract, the lipid excipient was first incubated with gastric lipase (rDGL) for 30 min at pH 5.5 before pancreatic enzymes (HPJ or PPE) were added to simulate duodenal lipolysis at pH 6.25 (Fig. 3). Experimental conditions such as enzymes concentrations and pH values reproduced the physiological conditions encountered in the human GI tract at 50% meal gastric emptying (28). The initial Labrasol® concentration (10 g/L) was deduced from the amounts of this lipid excipient usually added to lipid formulations of poorly water-soluble drugs. Under these conditions, Labrasol® forms an unstable emulsion with a particle size distribution in the 1,000-nm range (see zone 2 in Fig. 4a).

C8-C10 TAG and DAG were rapidly and almost totally hydrolyzed during the gastric lipolysis step (−86% and −89% on average at $t=29$ min; Fig. 3a and b). C8-C10 MAG remained at high levels (90-100%) after a slight decrease during the 0 to 15-min period of time, indicating that the rate of MAG production from DAG hydrolysis was similar to the rate of MAG hydrolysis by rDGL during the gastric lipolysis step (Fig. 3a and b). Only 16% of the PEG-8 diesters initially present at $t=0$ min remained at the end of the gastric lipolysis step ($t=29$ min). The increase in free PEG-8 levels was weak, indicating that PEG-8 monoesters were not significantly hydrolyzed.

PEG-8 monoesters were however hydrolyzed during the following step of duodenal lipolysis as indicated by a large release of free PEG-8. Different results were obtained however using HPJ (Fig. 3a) and PPE (Fig. 3b). PEG-8 monoesters were found to be better hydrolyzed by HPJ (+31% of free PEG-8 released at $t=90$ min; Fig. 3a) than PPE (+7% of free PEG-8 released at $t=90$ min; Fig. 3b). The C8-C10 TAG, MAG, and PEG-8 diesters remaining after the gastric step were totally hydrolyzed by pancreatic enzymes in less than 5 min after the addition of either HPJ or PPE. C8-C10 DAG were totally hydrolyzed in less than 15 min after addition of HPJ (Fig. 3a) and in less than 10 min after addition of PPE (Fig. 3b). Overall, the only significant difference observed between experiments performed with HPJ and PPE was the less efficient hydrolysis of PEG-8 monoesters by PPE.

Particle Size Analysis of Aqueous Dispersions of Labrasol®

The particle size distribution of Labrasol® dispersions in either lipolysis medium or water was measured by PCS for

various overall mass concentrations of Labrasol® (Fig. 4a). In both cases, three different zones were optically identified: a translucent dispersion (zone 1), a whitish opaque dispersion (zone 2) and a transparent solution (zone 3). Zone 1 is observed for Labrasol® concentrations below 2 g/L. The particle size is roughly constant in this zone with a mean particle size of 210±40 nm and 189±30 nm in lipolysis medium and water, respectively. Zone 2 is observed for concentrations of Labrasol® ranging from 2 to 31 g/L. The particle size distribution of these dispersions increases from 300 to 1,100 nm with the amounts of Labrasol®. This zone is composed of unstable emulsions as indicated by the particle size increase during measurement by PCS. Zone 3 is observed for concentrations of Labrasol® above 31 g/L. Particle size analysis showed that this phase is formed by a microemulsion which is stable up to 70 g/L of Labrasol®. The microemulsion particle sizes were 15±1 nm and 22±1 nm in lipolysis medium and water, respectively.

Lipase Activity on Various Labrasol® Aqueous Dispersions

The overall mass concentration of Labrasol® and consequently its particle size had a significant effect on the activity of rHPLRP2, bovine CEH, and rDGL (Fig. 4b). Under the same experimental conditions, rHPL did not show any activity on Labrasol® at any of the concentrations tested (data not shown). It is worth noticing that similar amounts of lipase (25 μg) were used in these assays for the sake of comparison. Maximum activities were found to be 48±2 U/mg for rDGL using 32 g/L Labrasol®, 116±6 U/mg for rHPLRP2 using 10 g/L Labrasol® and 226±12 U/mg for CEH using 10 g/L Labrasol®.

rHPLRP2 and CEH showed similar activity profiles as a function of Labrasol® concentration. Indeed, rHPLRP2 and CEH presented a higher relative activity in zone 1 compared to rDGL. rHPLRP2 and CEH activities rapidly increased in zone 2 to reach a maximum at 10 g/L of Labrasol® for both enzymes. In this zone 2, the particle size reached a plateau with values close to 1,000 nm when Labrasol® was dispersed in the lipolysis medium (Fig. 4a). A continuous decrease in lipase activity, slight for CEH and larger for rHPLRP2 was then observed until a drastic drop in activity occurred at the transition between zones 2 and 3, before returning to a slight decrease for both enzymes in zone 3 (31–70 g/L Labrasol®) where Labrasol® forms a fine microemulsion of 15±1 nm.

The activity of rDGL was also affected by the particle size distribution of Labrasol® aqueous dispersions but in a distinct manner compared to rHPLRP2 and CEH activities (Fig. 4b). rDGL activity increased continuously with Labrasol® concentration in zones 1 (translucent dispersion) and 2 (emulsion). It reached a maximum at 30 g/L of Labrasol® and remained stable up to 32 g/L, i.e. at the transition between zones 2 and 3. In zone 3 (microemulsion), rDGL presented a slight decrease in activity, like CEH and rHPL, but this activity still remained higher than 80% of the maximum activity at 70 g/L Labrasol®. In contrast, CEH and rHPLRP2 only displayed 54±3% and 24±2% of their respective maximum activities at the same Labrasol® concentration.

DISCUSSION

Lipase Activities on Labrasol® Compounds

We previously studied the enzyme activity of several digestive lipases on Labrasol® as well as on the various acylglycerols and PEG esters present in this excipient and taken individually (12). Lipase activities on the entire Labrasol® were only based on the titration of the FFA released upon lipolysis and the compounds hydrolyzed within this mixture of esters were not identified. Changes in the composition of Labrasol® during lipolysis were investigated here using the same digestive lipases, namely rHPLRP2, CEH and rDGL, to determine whether these lipases exhibit the same substrate preference for individual compounds of Labrasol® when these compounds are isolated or mixed to form Labrasol®. The results obtained with the entire Labrasol® indicate that C8-C10 DAG and PEG-8 monoesters are hydrolyzed by rHPLRP2 more rapidly than the other compounds (Fig. 2a), in agreement with the specific activities on individual compounds recorded with rHPLRP2 (13). Similarly, PEG-8 mono- and diesters are the best substrates for CEH in Labrasol (Fig. 2b), in agreement with the fact that bovine CEH shows the highest specific activities on PEG-8 mono- and dicaprylates compared to the other digestive lipases (13). Finally, among all three lipases tested in this study, rDGL displays a clear preference for the hydrolysis of C8-C10 TAG present in Labrasol® (Fig. 2c) which reflects its substrate specificity for TAG. rDGL was previously shown to be much more active on tricapryloylglycerol than rHPLRP2 and CEH, and poorly active on monocapryloylglycerol (12,13). The lipase substrate specificities characterized with individual compounds are therefore preserved when Labrasol® is used as a complex substrate. Overall, all the compounds contained in Labrasol® can be hydrolyzed by at least one digestive lipase and one can therefore imagine that Labrasol® can be totally hydrolyzed *in vivo* during the digestion process. Moreover, some of the products generated by rDGL, rHPLRP2 and CEH can be substrates for classical pancreatic lipase (HPL in humans) that is inactive on Labrasol® taken as initial substrate for this enzyme (12).

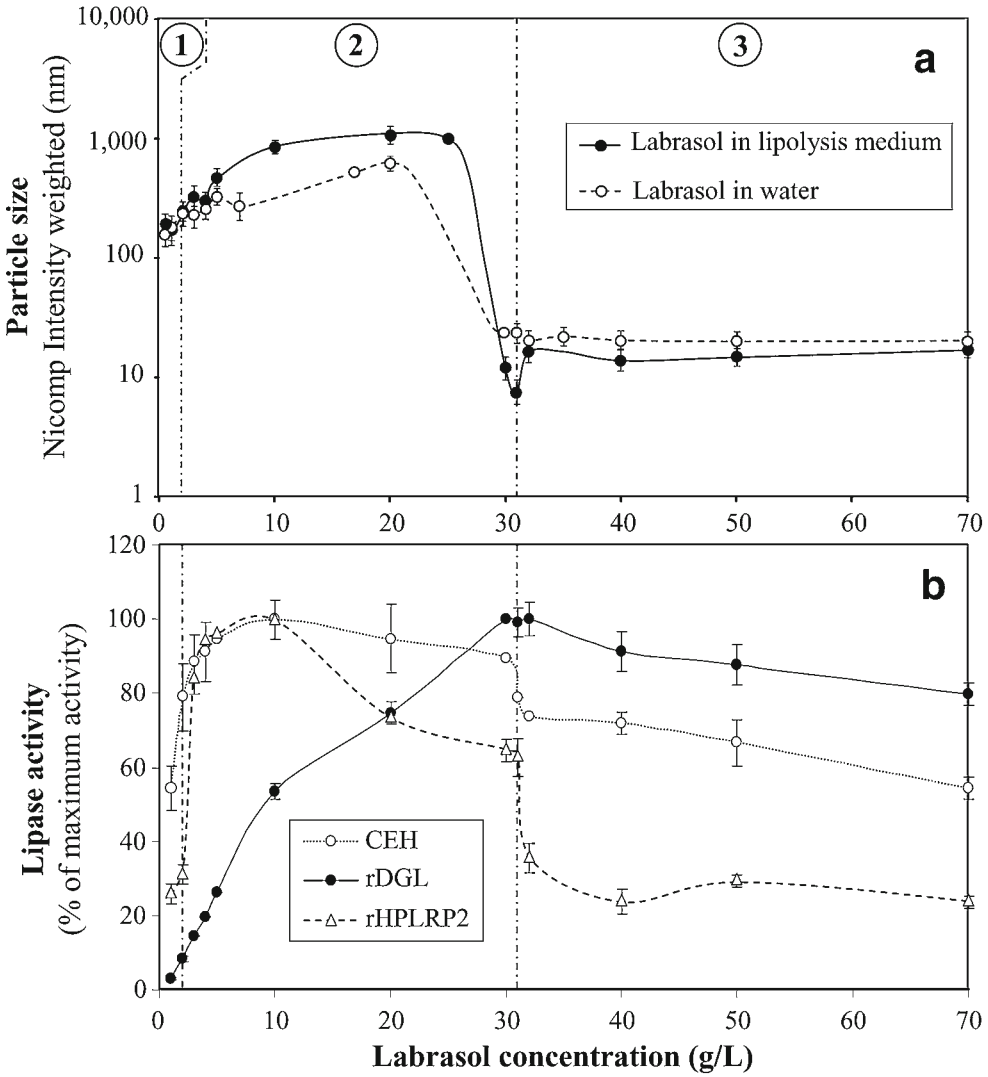

Fig. 4 Variations in particle size and lipase activity as a function of Labrasol® concentration in aqueous dispersions. (**a**) Evolution of the particle size distribution (nm). Zone 1: translucent dispersion; Zone 2: opaque emulsion; Zone 3: transparent microemulsion. Values are mean ± SD ($n=3$) and expressed in Nicomp Intensity weighted; (**b**) Variations in rDGL, rHPLRP2, bovine CEH activities with Labrasol® concentration. For each enzyme, the lipase activity is expressed as percent of the maximum activity. Values are mean ± SD ($n=3$).

In Vitro Simulation of Gastrointestinal Lipolysis of Labrasol®

A two-step *in vitro* digestion model reproducing intragastric and duodenal lipolysis was used for testing the extent of Labrasol® hydrolysis under conditions close to the physiological ones. Intragastric lipolysis was simulated with rDGL because dog gastric lipase is known to be a good model of human gastric lipase (52–55). Concerning duodenal lipolysis, natural mixtures of pancreatic enzymes, namely human pancreatic or porcine pancreatic extracts, were used instead of purified enzymes. This allowed having all lipolytic enzymes and cofactors (colipase for classical pancreatic lipase) delivered as a single reagent. Moreover, the equivalence of PPE and HPJ could be tested in separated experiments after the preliminary action of rDGL in both cases (Fig. 3a and b). It was confirmed that PPE could be used for replacing HPJ, with similar variations in Labrasol® composition upon lipolysis, with the exception of the release of free PEG-8 that appeared to be lower with PPE than with HPJ (Fig. 3a and b). This finding reflects a lower lipolysis of PEG-8 monoester by PPE enzymes and this is probably due to the absence of PLRP2 in PPE. So far, no porcine PLRP2 could be identified in either pancreatic extracts or juice (56).

C8-C10 TAG, DAG and PEG-8 diesters of Labrasol® were found to be largely hydrolyzed during the gastric step of lipolysis whereas C8-C10 MAG remained at a rather constant level (Fig. 3). MAG and PEG-8 monoesters were therefore the main hydrolyzable compounds of Labrasol® remaining at the end of the gastric lipolysis phase. These finding suggest that MAG and PEG monoesters play a major role in the dispersion/solubilisation of poorly water-soluble drugs for which the bioavailability is increased by Labrasol® *in vivo*. MAG and PEG-8 monoesters are more polar compounds than TAG, DAG and PEG-8 diesters. These surfactant molecules, initially present as such in Labrasol® or generated during lipolysis, allow the formation of colloidal dispersions. One can therefore assume that the

physicochemical organisation of the dispersion will be modified upon intragastric lipolysis of Labrasol®, as previously shown during the *in vitro* lipolysis of a SNEDDS by pancreatin (57,58). These results underline the importance of the gastric digestion step on the fate of lipid-based excipients in the gut. However, this has been taken into account in only one *in vitro* lipolysis study on lipid formulations of cinnarizin and piroxicam (15).

Concerning the duodenal step of lipolysis, it is worth noticing that C8-C10 TAG, DAG, MAG and PEG-8 diesters were found to be totally hydrolyzed 15 min after pancreatic enzymes were added (Fig. 3). This finding suggests that Labrasol® lipolysis products might be as efficient as the whole lipid-based excipient for drug solubilization. Alternatively, the remaining PEG-8 monoesters could play an important role in maintaining lipophilic drugs in solution. In this case, one would have to be cautious in using HPJ or PPE for the *in vitro* testing of drug solubility because a higher lipolysis of PEG-8 monoesters is achieved with HPJ.

Influence of Particle Size Distribution on the Hydrolysis Rate of Labrasol® Aqueous Dispersions by Individual Lipases

Labrasol® has the ability to form various phases in water or in lipolysis medium depending on its concentration. Two main changes occur at concentrations near the critical micellar concentration (CMC) of the surfactants found in Labrasol® (PEG-8 monocaprylate and glyceryl monocaprylate) and the characteristic concentration c_1 (31 g/L) where the amounts of surfactants become sufficient to finely disperse the oily components of Labrasol® and form a microemulsion phase. The binary phase diagrams of Labrasol® exhibit three distinct zones in terms of optic appearance and colloidal organization. Below 2 g/L of Labrasol®, the system organizes as a fine suspension of insoluble apolar components in water because all surfactants are below their CMC and thus soluble. The presence of bile salts in the lipolysis medium (4 mM NaTDC) does not affect this organization since particle sizes of about 200 nm are observed in both water and lipolysis medium (Fig. 4a). The CMC values of PEG-8 monocaprylate and glyceryl monocaprylate in water at 20°C were measured by tensiometry with a Wilhelmy plate and were found to be 5 mg/L and 8 mg/L, respectively (data not shown). The maximum activities of rHPLRP2 and CEH being reached in the 5 to 10 g/L range of Labrasol® concentrations (Fig. 4b), these data suggest that rHPLRP2 and CEH preferentially act on micellar forms of PEG-8 monoesters and C8-C10 MAG. This would be in perfect agreement with the substrate specificity of these enzymes and the fact they usually display their highest activities on substrates forming mixed micelles with bile salts such as phospholipids, galactolipids, MAG and PEG esters (48,59,60).

rDGL, an enzyme showing a preference for TAG and DAG, clearly displays a distinct activity profile compared to rHPLRP2 and CEH. Its maximum activity is reached at much higher Labrasol® concentrations (30 g/mL) whereas it is only about 50% at 5–10 g/L Labrasol® (Fig. 4b). TAG and DAG form oil-in-water emulsions, thus creating an oil–water interface where gastric lipase preferentially acts (61). At Labrasol® concentrations ranging from 2 to 31 g/L, particles with sizes ranging from 300 to 1,100 nm are formed and they coexist with some micelles of pure surfactants. These micelles are usually not detected by PCS because the scattering of emulsion droplets is too intense, but PCS was able to detect a micellar solution of 1.0 ± 0.1 nm (8.0% by intensity) coexisting with the emulsion at 10 g/L Labrasol® (data not shown). The emulsion particles are mainly formed by a TAG-DAG core, are stabilized by surfactants and are probably the preferred supramolecular substrate of rDGL in Labrasol® dispersions. rDGL activity increased with the amounts of substrate up to 31 g/L, the characteristic concentration c_1 where the amount of surfactants is sufficient to finely micro-emulsify all apolar components of Labrasol®. Above c_1, the increase in rDGL activity stopped when the phase transition occurred and the particle size was drastically reduced (15 nm *vs.* 1,100 nm). Since the activity of true triacylglycerol lipase depends more on the interfacial area offered by the substrate than on the substrate amounts (62), a significant increase in rDGL activity was expected at the phase transition but this was not the case. One possible explanation is that the specific surface provided by Labrasol® microemulsion at 31 g/L was already high enough for measuring the maximum activity of rDGL. It is also possible that the changes in interfacial quality occurring at the phase transition had an impact on rDGL turnover.

The transition from emulsion to microemulsion occurring at 31 g/L Labrasol® also induced a drastic drop in rHPLRP2 and CEH activities (Fig. 4b). Since these enzymes preferentially act on micellar substrate, it is not obvious to understand why a change in emulsion particle size has an effect on enzyme turnover. The formation of a microemulsion however provides a larger specific area where surfactant molecules can adsorb. The microemulsification process can thus displace the apparent CMC of Labrasol® surfactants (MAG and PEG-8 monoesters) towards higher concentrations and reduce the micellar substrate available for rHPLRP2 and CEH.

In conclusion, the lipases with distinct substrate specificities used in this study were found to be sensitive probes of phase transitions occurring upon Labrasol® dilution. In addition to their use for developing *in vitro* digestion models, these enzymes are interesting tools for the characterization of self-emulsifying lipid-based formulations.

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