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Research Paper

In Vitro Gastrointestinal Lipolysis of Four Formulations of Piroxicam and Cinnarizine with the Self Emulsifying Excipients Labrasol® and Gelucire® 44/14

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Purpose. Labrasol® and Gelucire® 44/14 are defined admixtures of acylglycerols and PEG esters which are substrates for digestive lipases.
Methods. We investigated their *in vitro* gastrointestinal lipolysis to understand which compounds are, after digestion, responsible for keeping poorly water-soluble drugs in solution. The precipitation of piroxicam and cinnarizine formulated in these excipients during the gastrointestinal lipolysis was also studied.
Results. Monoacylglycerols and PEG monoesters are the largest compounds present at the end of gastric phase whereas PEG-monoesters are the largest compounds after the duodenal phase. The precipitation of piroxicam is mainly due to the gastric lipolysis. In the control experiments performed without digestive lipases, cinnarizine formulated in Labrasol® was found to precipitate upon dilution of the gastric medium to form the solution mimicking the duodenal medium. In the presence of gastric lipase, Labrasol® was hydrolyzed and the precipitation of cinnarizine was not observed in this case. When the cinnarizine was formulated with Gelucire® 44/14 the precipitation was only due to the dilution of the gastric medium.
Conclusion. Our study highlights the importance of the gastrointestinal lipolysis and the associated phenomena such as the dilution of chyme by biliary and pancreatic secretions *in vivo*, on the solubilisation of poorly water-soluble drugs formulated with lipid-based excipients.

KEY WORDS: cinnarizine; gastrointestinal lipolysis; macrogolglycerides; oral drug delivery; piroxicam.

INTRODUCTION

The oral administration of drugs exhibiting poor solubility in gastrointestinal fluids is one of the main challenges of the pharmaceutical industry. In order to improve oral bioavailability of hydrophobic drugs, innovative formulations such as SEDDS (Self Emulsifying Drug Delivery Systems), SMEDDS (Self MicroEmulsifying Drug Delivery Systems), and SNEDDS (Self NanoEmulsifying Drug Delivery Systems) are developed (1–5).

SEDDS, SMEDDS, and SNEDDS are isotropic mixtures of oil, surfactant, co-surfactant, and drug that form, under stirring, oil-in-water emulsions, microemulsions, and nanoemulsions, respectively (6,7).

Among the different excipients available so far, Labrasol® and Gelucire® 44/14 are macrogolglycerides able to form microemulsions in contact with gastrointestinal fluids (8). Labrasol® and Gelucire® 44/14 are obtained by polyglycolysis of medium or medium and long chain triacylglycerols and PEG-8 or PEG-32, respectively. These excipients are a mixture of mono-, di-, and triacylglycerols and mono- and diesters of PEG, and free PEG. Although Labrasol® and Gelucire® 44/14 are already used in commercial formulations of fenofibrate, ibuprofen, and piroxicam and significantly increase the bioavailability of various poorly water-soluble drugs (9–19); the mechanisms underlining these effects still remain to be elucidated. This improvement is probably due to the administration of the drug in a pre-dissolved state which corresponds to a reduction of the energy associated with the solid-to-liquid phase transition process. Moreover, the interactions between the formulation and the endogenous lipids such as bile salts, phospholipids and dietary lipids will increase the solubilisation of the drug in the colloidal structures and consequently enhances the oral bioavailability of the poorly water-soluble drug (20,21). We have already shown that, *in vitro*, Labrasol® and Gelucire® 44/14 were hydrolyzed by several digestive lipases (22,23). These findings support that *in vivo*, these excipients are hydrolyzed by digestive lipases and their lipolytic products might play an important role in the transport of the drug from the formulation to the mixed micelles and/or the unstirred water layer next to the enterocytes.

Piroxicam (Fig. 1A) belongs to the oxicam group of non steroidal anti-inflammatory drugs. According to the Biopharmaceutics Classification System (BCS) (24), Piroxicam is a

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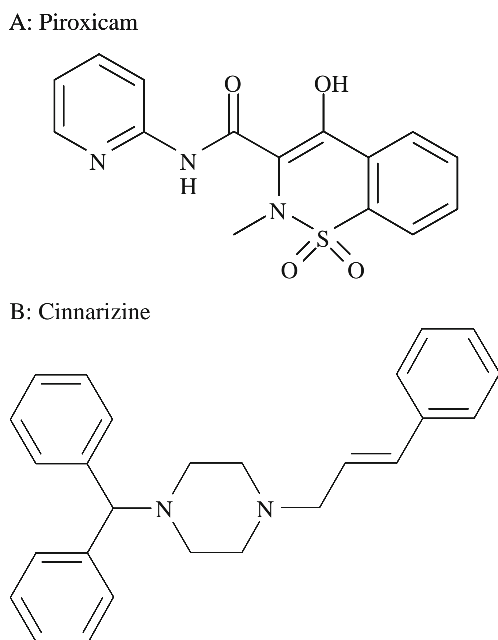

Fig. 1. Chemical structures of A: piroxicam and B: cinnarizine.

class 2 compound which corresponds to low solubility and high permeability. Karataş *et al.* (25) showed that Labrasol® and Gelucire® 44/14 improved 20- and 50-fold the *in vitro* apparent solubility of piroxicam in water, respectively. In addition, mixtures of Labrasol® and Gelucire® 44/14 increased the dissolution rate of piroxicam in all media tested: pH 1.2 Simulated Gastric Fluid, pH 4.5 buffer, pH 6.8 buffer, and water when compared to the dissolution in the absence of the excipients. Yüksel *et al.* (19) have already demonstrated that a combination of Labrasol® and Gelucire® 44/14 increased the bioavailability of piroxicam in healthy human volunteers. However, these studies did not consider the influence that the lipolysis of these formulations might have on the fate of piroxicam.

Cinnarizine (Fig. 1B) is a piperazine derivative with antihistaminic activity and mainly used for the control of vomiting due to motion sickness which belongs to the class 2 of BCS (24). Kossena *et al.* (26) studied the *in vitro* release of cinnarizine from a cubic phase of medium chain lipid in a simulated endogenous intestinal fluid composed of 4 mM bile salts, 1 mM lysophosphatidylcholine, and 0.25 mM cholesterol.

In humans, the digestion of dietary triacylglycerols starts in the stomach with the gastric lipase (27,28). The lipolysis products generated in the stomach are essential for the stimulation of biliary and pancreatic secretions, as well as for the subsequent action of pancreatic lipases (29,30). Thanks to the gastric emptying, the lipolysis continues in the duodenum where the chyme is mixed with pancreatic enzymes produced by the exocrine pancreas. Pancreatic juice contains the classic pancreatic lipase and its co-factor, colipase. In addition there are two human pancreatic lipase-related proteins (1 and 2) in pancreatic juice (31–33). Whereas human pancreatic lipase-related protein 1 does not show any lipolytic activity up to now, human pancreatic lipase-related protein 2 is able to hydrolyze various natural substrates such as triacylglycerols, phospholipids, galactolipids and vitamin esters (32,34). Carboxyl ester hydrolase, also known as bile salt-stimulated lipase, is also produced by the exocrine pancreas and presents broad substrate specificity (35).

We have already shown that acylglycerols and PEG esters contained in both Labrasol® and Gelucire® 44/14 were bad substrates of the classic human pancreatic lipase but good ones for the other digestive lipases (22,23). So as to mimic the gastrointestinal lipolysis, we developed an *in vitro* method that takes in consideration both the gastric and the duodenal steps of digestion, the first step being not taken into consideration in most of the studies on bioavailability and/or solubility of poorly water-soluble drugs (36,37). Using this model, we studied the changes in the composition of both Labrasol® and Gelucire® 44/14 during their *in vitro* lipolysis.

The aim of this study was to perform similar experiments in the presence of two poorly water-soluble drugs, piroxicam and cinnarizine, and to follow the concentration of piroxicam and cinnarizine dissolved in the aqueous phase during the lipolysis of the excipients composed of either Labrasol® or Gelucire® 44/14.The changes in the composition of the formulation were studied in parallel so as to determine which compounds in these excipients were important to maintain the poorly water-soluble drug in solution.

MATERIALS AND METHODS

Materials

Sodium taurodeoxycholate (NaTDC, 97% TLC), bovine serum albumin (BSA), tributyrylglycerol, 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. Piroxicam and cinnarizine were chosen as the poorly-water soluble model drugs and purchased from AMSA (batch 109107001, Milan, Italy) and Sigma-Aldrich (batch 127 K1457, Saint-Louis, USA), respectively.

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. Gelucire® 44/14 (PEG-32 lauroyl macrogolglycerides, batch 105656, Gattefossé SAS, Saint-Priest, France) is composed of C8-C18 mono-, di- and triacylglycerols (20%), PEG-32 mono- and diesters and free PEG-32 (80%). The main fatty acid present is lauric acid (which accounts for 45% on average of the total fatty acids). A stock solution of 1 M NaOH (Tritrisol, Merck, Darmstadt, Germany) was diluted with water to obtain 0.1 M NaOH titration solution. All organic solvents used (chloroform, acetonitrile) were HPLC grade and were purchased from local suppliers.

Recombinant dog gastric lipase (rDGL) was a generous gift from Meristem Therapeutics (Clermont-Ferrand, France). DGL provides a good model for mimicking the action of Human Gastric Lipase (HGL): both HGL and DGL show similar activities on triacylglycerols with various acyl chain lengths (30,38,39). In addition, DGL shows 85% amino acid identity with HGL (40,41), and the corresponding 3D

structures are superimposable (42,43). Moreover dogs are often used to predict the *in vivo* performance of a drug because of the similarities between dog and human gastrointestinal tracts. Lipid-free porcine pancreatic extracts (PPE) was purchased from ID bio SAS (Limoges, France).

Protein concentrations were determined using Bradford's procedure (44) with Bio-Rad dye reagent, with BSA as the standard protein.

Identification of the Changes in the Composition of Labrasol® and Gelucire® 44/14 During Their *In Vitro* Gastrointestinal Lipolysis

Experimental conditions were adapted from *in vivo* data recorded at 50% gastric emptying of test meals, both in the stomach and in the duodenum, and enzymatic solutions were prepared according to *in vivo* secretions of lipases during a meal (45).

The gastric enzymatic solution was prepared using rDGL. The lyophilised 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. Pancreatic enzymatic solution was prepared using PPE. The solution of PPE was prepared so as to obtain a solution at 614 μg/ml of porcine pancreatic lipase (PPL). 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. An emulsion of 1.5 g of either Labrasol® or Gelucire® 44/14 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 enzymatic 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 during 30 min (gastric step of lipolysis), 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 enzymatic solution freshly prepared with PPE, were added to the mixture and the pH was shifted to 6.25 and kept constant for 60 min. After adding the pancreatic enzymatic solutions to the reaction vessel, the final PPL concentration was 250 μg/ml and the gastric phase was diluted 1.7-fold. At t=0 min, 15 min, 29 min, 35 min, 40 min, 45 min, 60 min, 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 lyophilisation. In order to check that there was no spontaneous hydrolysis of the excipients, the same experiments were performed without adding enzymatic solutions, which were replaced by the same volume of the assay solution to respect dilutions.

The analytical method developed by Gattefossé to assay its pure excipients was adapted to lipolysis samples of Labrasol® and Gelucire® 44/14. Frozen lipolysis samples were freeze-dried for 1 day (Heto Drywinner FD 3). Aliquots containing the freeze-dried samples were rinsed with 3×1 ml of chloroform and filtered on 0.45 μm glass filters. After evaporation, 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 (mono-, di- and triacylglycerols). Free PEG and PEG diesters were analyzed by HPLC-ELS (HPLC LaChrom, Merck-Hitachi, USA; 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, thus their amount was calculated based on weight balance of all other species quantified in the sample (monoacylglycerols, diacylglycerols, triacylglycerols, PEG diesters, free PEG, glycerol and free fatty acids).

Solubility Measurements of Piroxicam and Cinnarizine in the Assay Solution

To determine the solubility of piroxicam and cinnarizine in the assay solution (NaCl 150 mM; NaTDC 4 mM; $CaCl_2$ 1.4 mM; Tris-HCl 1 mM), solubility measurements were performed by dispersing a large amount of either piroxicam or cinnarizine in the assay solution at pH 5.5 or at pH 6.25 at 37°C. After 24 h, 1 week or 2 weeks at 37°C under stirring, samples were taken and filtered on 0.45 μm polypropylene membrane. The solubility of both piroxicam and cinnarizine were determined by HPLC.

Piroxicam concentration in the aqueous phase was assayed by HPLC using a 150×4.6 mm Uptisphere Strategy 100 Å C18-2 (5 μm) analytical column (Interchim, Montluçon, France). The HPLC system consisted of an Alliance 2695D (Waters, Saint-Quentin en Yvelines, France) and an UV detector (2487, Waters, Saint-Quentin en Yvelines, France) operated at 40°C. The mobile phase, consisting of a 50 mM potassium dihydrogen phosphate buffer adjusted at pH 3.0 with phosphoric acid (50% v/v) and acetonitrile (50% v/v), was eluted at a flow rate of 1 ml/min. The injection volume was 20 μl, and the detection wavelength was set at 327 nm. Under these conditions, the retention time of piroxicam was 4.0 min. The assay was validated in terms of specificity, accuracy and precision using standard methodologies.

Cinnarizine concentration in the aqueous phase was assayed by HPLC using a 125×4 mm SuperSpher RP 18 (4 μm) analytical column (Agilent Technologies, Massy, France). The HPLC system consisted of Lachrom 1 (VWR, Fontenay-sous-Bois, France) and an UV detector (L-7400, VWR, Fontenay-sous-Bois, France) operated at 30°C. The mobile phase, consisting of a 20 mM ammonium dihydrogen phosphate buffer (40%, v/v) and acetonitrile (60%, v/v), was eluted at a flow rate of 1 ml/min. The injection volume was 10 μl, and the wavelength was set at 251 nm. Under these conditions, the retention time of cinnarizine was 12.1 min. The assay was validated in terms of specificity, accuracy and precision using standard methodologies.

Preparation of Different Formulations of Piroxicam and Cinnarizine

We prepared two formulations of either piroxicam or cinnarizine with each excipient (Labrasol® and Gelucire® 44/14). Either 20 mg of piroxicam or 25 mg of cinnarizine were weighed and mixed with 1.5 g of either Labrasol® or Gelucire® 44/14. Gelucire® 44/14 was previously melted for 1 min at 800 W using a microwave oven (Samsung) and was used as a liquid to prepare formulations. To facilitate the

solubilisation of both piroxicam and cinnarizine, the formulations were then placed in an ultrasonic bath at 40 kHz and 45°C for 90 min and used immediately thereafter.

In Vitro Gastrointestinal Lipolysis of Formulations of Either Piroxicam or Cinnarizine

Experiments were performed as described in "Identification of the Changes in the Composition of Labrasol® and Gelucire® 44/14 During Their *In Vitro* Gastrointestinal Lipolysis". At $t=$ 0 min, 15 min, 29 min, 35 min, 40 min, 45 min, 60 min, and 90 min, 1,000-µl samples were taken and filtered on 0.45 µm polypropylene membrane. The filtered samples were immediately diluted 25-fold in acetonitrile (50-fold for the sample taken at $t=0$ min) in the case of piroxicam and 2-fold in the case of cinnarizine to stop the lipolysis. The dilution of cinnarizine samples was less important according to the concentration range of the validated analytical method but was sufficient to stop the lipolysis. Concerning samples taken during the duodenal lipolysis ($t=35$ min, 40 min, 45 min, 60 min, and 90 min), the dilution in acetonitrile induced the precipitation of proteins. Consequently, these samples were filtered again on 0.45 µm polypropylene membrane. In order to check the piroxicam and cinnarizine precipitation in absence of lipolysis, the same experiments were performed without adding enzymatic solutions which were replaced by the same volume of assay solution to respect the dilution. The same experiments were also performed using inactive enzymatic solutions so as to check if the precipitation of poorly water-soluble drugs is due to the presence of proteins. Enzymatic solutions were heated 2×30 s at 800 W using a microwave oven (Samsung). The absence of lipolytic activity was checked using the pH-stat technique and an emulsion of 0.5 mL tributyrylglycerol in 29.5 mL of 150 mM NaCl, 4 mM NaTDC, 1.4 mM $CaCl_2$, 1 mM Tris-HCl at pH 6.0 as previously described in (22,23).

Piroxicam and cinnarizine concentrations in the aqueous phase were assayed by HPLC as described in "Solubility Measurements of Piroxicam and Cinnarizine in the Assay Solution".

RESULTS

Identification of the Changes in the Composition of Labrasol® and Gelucire® 44/14 During Their *In Vitro* Gastrointestinal Lipolysis

We studied the changes in the composition of both Labrasol® and Gelucire® 44/14 during their gastrointestinal lipolysis without any poorly water-soluble drugs.

The experiments performed here, were based on *in vivo* data recorded during test meals. The gastric lipolysis step was simulated using rDGL, for 30 min at pH 5.5, the average pH value found in the stomach at 50% of gastric emptying. A solution of PPE was then added to the reaction mixture in order to perform the duodenal step of lipolysis. The pH value was increased to 6.5, the mean pH value found in the duodenum during digestion of a meal. Adding PPE solution resulted in a 1.7-fold dilution of the reaction mixture as compared to the gastric step of lipolysis (45). This is important to mention since dilution can also affect drug solubility.

Fig. 2 presents the variations in the composition of Labrasol® and Gelucire® 44/14 during their *in vitro* gastrointestinal lipolysis. For each family of compounds, the results are expressed in percent of the initial composition and as a function of time. The variation of PEG monoesters composition was not directly accessible but the variation of free PEG released allowed us to deduce the hydrolysis of PEG monoesters. Indeed, the hydrolysis of one molecule of PEG monoester leads to the release of one molecule of free PEG and one molecule of free fatty acid. Since free PEG was already present in the formulation, the results shown here are

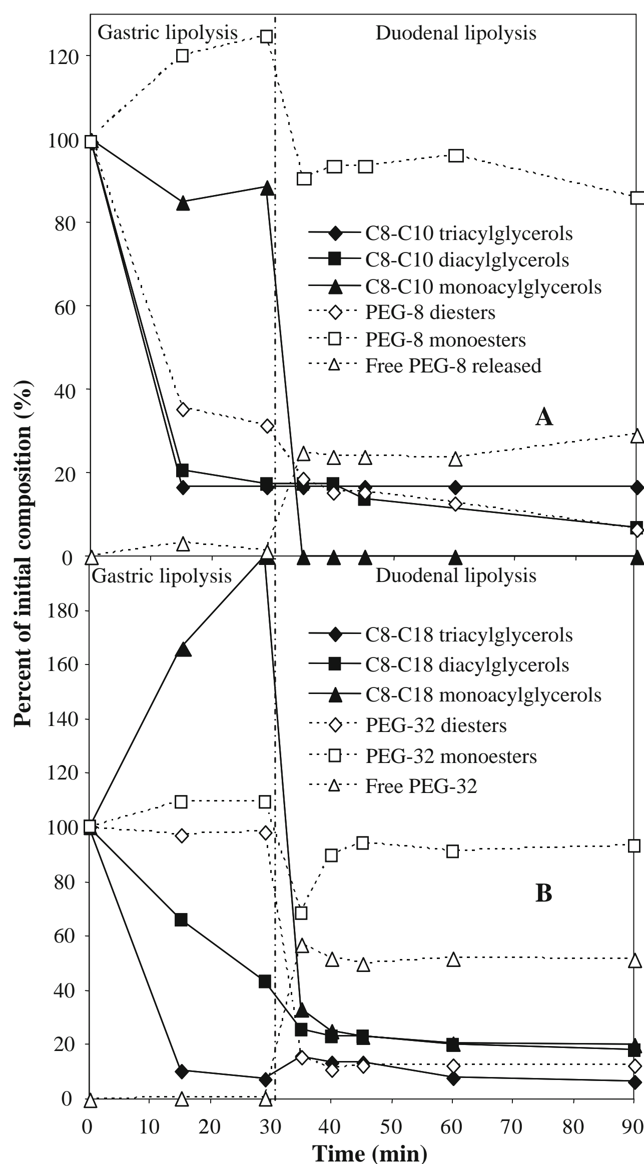

Fig. 2. Variation in the concentration of Labrasol® and Gelucire® 44/14 lipolysis products by rDGL and pancreatic enzymes as a function of time. The vertical line at 30 min of elapsed time marks the end of the gastric phase and the beginning of the pancreatic phase. These variations are expressed in percent of mono-, di-, and triacylglycerols, PEG mono- and diesters initially present in the excipient. For free PEG, only the amounts generated upon the lipolysis are shown and expressed in percent of the amounts of PEG present in the excipient. A: Labrasol® lipolysis by rDGL and PPE. B: Gelucire® 44/14 lipolysis by rDGL and PPE.

the difference in free PEG *versus* the initial amounts of free PEG (%).

During the gastric lipolysis step of Labrasol® (Fig. 2A), C8-C10 di- and triacylglycerols were rapidly hydrolyzed to reach 17% of their initial composition at the end of the gastric phase (t= 29 min), whereas C8-C10 monoacylglycerols were slightly hydrolyzed (−13% at t=29 min). PEG-8 diesters were also well hydrolyzed (−68% at t=29 min), whereas PEG-8 monoesters were not significantly hydrolyzed since only 1% of additional free PEG-8 was released at the end of gastric lipolysis.

The addition of pancreatic enzymes led to major changes. Indeed, C8-C10 monoacylglycerols were rapidly and totally hydrolyzed within 5 min after the addition of pancreatic enzymes. Contrary to C8-C10 monoacylglycerols, the residual C8-C10 diacylglycerols were slightly hydrolyzed (−10%) and residual C8-C10 triacylglycerols were not hydrolyzed during the duodenal step of lipolysis. The percentage of residual PEG-8 diesters decreased to reach 7% of their initial amounts at the end of the duodenal lipolysis step. Free PEG-8 increased during the duodenal phase (+29% at t=90 min), thus showing that PEG-8 monoesters generated from PEG-8 diesters or initially present in the excipient were hydrolyzed.

During the gastric lipolysis step of Gelucire® 44/14, results were quite different from those found with Labrasol® (Fig. 2B). Indeed, during the gastric phase, C8-C18 triacylglycerols were rapidly hydrolyzed (−92% at t=29 min) whereas C8-C18 diacylglycerols decreased linearly to reach 43% of their initial amounts at the end of gastric phase. An accumulation of C8-C18 monoacylglycerols was observed simultaneously which were not hydrolyzed (200% at t=29 min). PEG-32 mono- and diesters were not hydrolyzed during the gastric phase.

Five minutes after adding pancreatic lipases, C8-C18 monoacylglycerols and PEG-32 diesters rapidly decreased (33% and −84% at t=35 min, respectively; Fig. 2B) and free PEG-32 rapidly appeared (+25% at t=35 min) indicating that PEG-32 monoesters were also hydrolyzed by pancreatic enzymes. Residual C8-C18 diacylglycerols also decreased linearly with time but at lower rate than in the case of gastric lipolysis step (26% of the initial amount at t=35 min). It is worth noticing that, the respective amounts of all of compounds did not significantly change after the first 5 min of the duodenal lipolysis step (t=35 min). Residual C8-C18 triacylglycerols were not hydrolyzed during the duodenal lipolysis step (6% at t= 90 min). All these results indicate that equilibrium is almost reached after 5 min due to the fast lipolysis rate.

Solubility Measurements of Piroxicam and Cinnarizine in the Biorelevant Medium

Piroxicam and cinnarizine solubility measurements were performed in the assay solution (NaCl 150 mM; NaTDC 4 mM; $CaCl_2$ 1.4 mM; Tris-HCl 1 mM) at pH 5.5 and at pH 6.25. The solubility of piroxicam after 24 h at 37°C under stirring was found to be equal to 46.651±0.427 mg/l and to 43.590±0.175 mg/l in the assay solution at pH 5.5 and pH 6.25, respectively. Piroxicam is a zwitterionic drug with a weakly acidic 4-hydroxy proton (pKa_2=5.46) and a weakly basic pyridyl nitrogen (pKa_1=1.86) (46). In both media pH is above the pKa_2 and thus piroxicam is in its negative form, explaining the similar solubility measured. The cinnarizine solubility in the assay solution was equal to 0.644±0.163 mg/l and 1.071±0.133 mg/l at pH 5.5 and at pH 6.25, respectively. Cinnarizine is a weak base with a pKa_1=1.95 and pKa_2=7.5 (47). In both media pH is comprised between its two pKa and cinnarizine is mainly in neutral form. However, the solubility of cinnarizine increases significantly in the duodenal medium as pH comes closer to its pKa_2.

The solubility of both piroxicam and cinnarizine in the two assay solutions after 1 week or 2 weeks at 37°C were similar to those measured after 24 h meaning that the equilibrium was reached.

Precipitation of Piroxicam and Cinnarizine in the Aqueous Phase in the Course of the Gastrointestinal Lipolysis of the Formulation

We studied the concentration of both piroxicam and cinnarizine dissolved in the aqueous phase, during *in vitro* gastrointestinal lipolysis of their formulations with either Labrasol® or Gelucire® 44/14. For each formulation, a blank experiment was performed without any enzyme, to evaluate the precipitation of the poorly water-soluble drugs only due to other parameters such as the assay solution, the dilution and the pH variation. We also performed experiments with inactivated enzymes in order to determine the precipitation observed upon lipolysis, was only due to enzyme activity. Indeed, it is possible to imagine that proteins or other compounds present in PPE induce the precipitation of the drug.

Fig. 3 shows the variation with time of piroxicam (Fig. 3A and B) and cinnarizine (Fig. 3C and D) dissolved in the aqueous phase during *in vitro* gastrointestinal lipolysis of the four formulations.

Whatever the formulation used, in absence or in presence of lipolytic enzymes, the percentage of piroxicam in solution remained always above 60%.

When piroxicam was formulated with Labrasol®, the results obtained with inactivated enzymes were close to those obtained in presence of active enzymes (i.e. lipolysis of Labrasol®) to reach at the end of the gastrointestinal simulation 69% and 74%, respectively. The percentage of piroxicam dissolved in the aqueous phase was higher during the blank experiment (90% at t=90 min; Fig. 3A). When no lipases were added, the precipitation of piroxicam mainly occurred when the gastric medium was diluted with the assay solution to mimic the dilution of the chyme by pancreatic and biliary secretions. When the enzymes were present, the precipitation also occurred when the gastric phase was diluted by pancreatic enzymatic solution but it was more pronounced which supposes that the precipitation was also due to the presence of pancreatic proteins.

When piroxicam was formulated with Gelucire® 44/14, the results obtained at the end of the experiments, in presence of inactivated or active lipases were also in the same order of magnitude (80% and 74% of piroxicam dissolved at t=90 min, respectively; Fig. 3B). As in the case of Labrasol®, the blank experiment performed without any enzymes, showed a higher concentration of piroxicam dissolved in the aqueous phase which corresponds to 80% of piroxicam initially present at t=90 min. In this case, the percentage of piroxicam dissolved in the aqueous phase decreased slowly with time and was not influenced by the dilution occurring between the gastric and the

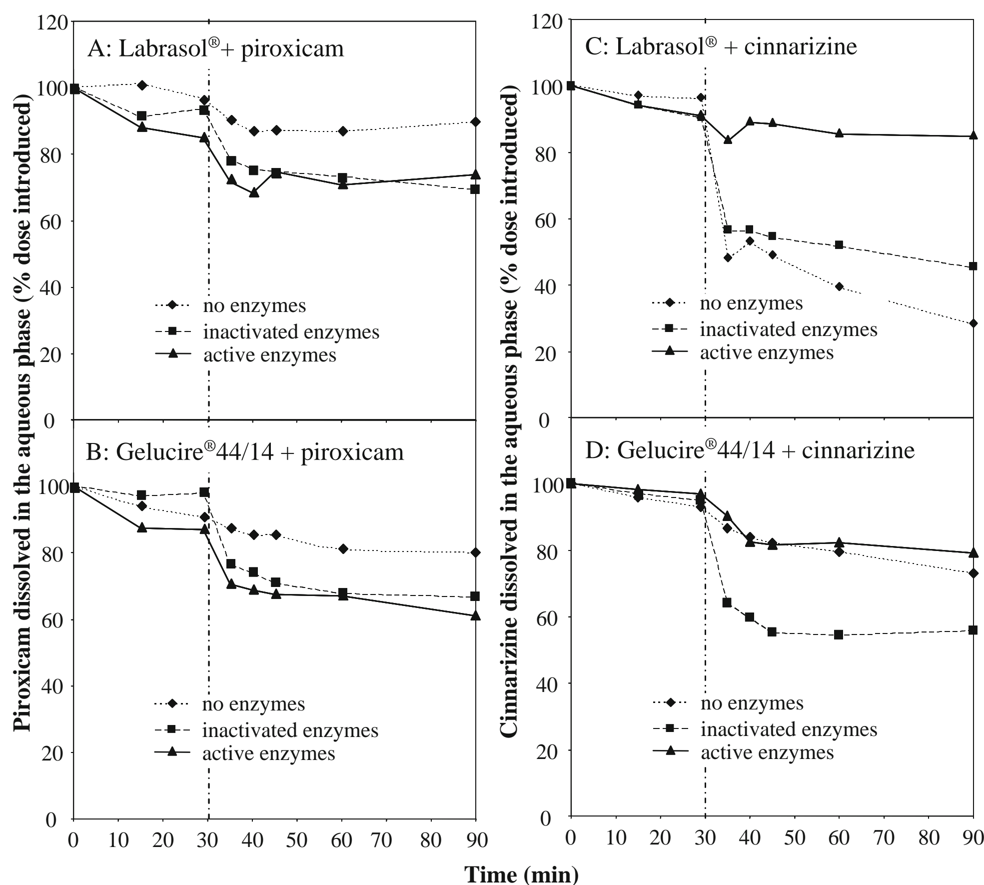

Fig. 3. Variation in the concentration of poorly water-soluble drugs dissolved in the aqueous phase, expressed in percent of the dose introduced, during the gastrointestinal lipolysis of a formulation using either Labrasol® or Gelucire® 44/14 by rDGL and PPE as a function of time. The vertical line at 30 min of elapsed time marks the end of the gastric phase and the beginning of the pancreatic phase. A and B: piroxicam dissolved in the aqueous phase during the gastrointestinal lipolysis by rDGL and PPE of Labrasol® and Gelucire® 44/14. C and D: cinnarizine dissolved in the aqueous phase during the gastrointestinal lipolysis by rDGL and PPE of Labrasol® and Gelucire® 44/14.

duodenal lipolysis steps. In presence of lipases, the precipitation of piroxicam occurred when the gastric medium was diluted by pancreatic enzymatic solution.

Whatever the excipient used, the precipitation of piroxicam was mainly due to the presence of porcine pancreatic enzymes. It is worth noticing that the precipitation of piroxicam was slightly more important during the gastric lipolysis step, when the lipolysis of both Labrasol® and Gelucire® 44/14 occurred, as compared to the results obtained with inactivated enzymes.

The results obtained with cinnarizine were different according to the formulation used (Fig. 3C and D). When cinnarizine was formulated with Labrasol®, the results obtained during the Labrasol® lipolysis, were significantly different from those obtained without enzymes or in presence of inactivated enzymes. When Labrasol® was not hydrolyzed, the dilution of the formulation induced a dramatic precipitation which carries on until the end of the experiment and consequently, the percentage of cinnarizine remaining in solution was low (28% in absence of enzymes and 45% in presence of inactivated enzymes at t=90 min; Fig. 3C). However, when Labrasol® is hydrolysed by the lipases , the overall cinnarizine precipitation was significantly much lower and the concentration of cinnarizine dissolved in the aqueous phase reached 85% at the end of the experiment. It is worth noticing that in this case, the cinnarizine precipitation was initiated during the gastric lipolysis step and was not affected by the dilution of the gastric medium by the pancreatic enzymatic solution.

When cinnarizine was formulated with Gelucire® 44/14 (Fig. 3D), no differences were observed in absence of enzymes or in presence of active enzymes. The precipitation only occurred when the gastric medium was diluted whatever the conditions. However, the cinnarizine precipitation was more important when the experiment was performed with inactivated enzymes. At the end of the duodenal phase, percentages of cinnarizine dissolved in the aqueous phase were equal to 73% in absence of enzymes, 79% when the lipolysis of Gelucire® 44/14 occurred, and 56% in presence of inactivated enzymes.

DISCUSSION

Simulation of Gastro-intestinal Lipolysis of Labrasol® and Gelucire® 44/14

Labrasol® and Gelucire® 44/14 are lipid-based excipients composed of acylglycerols (mono-, di-, and triacylgly-

cerols) (10% and 20% w/w, respectively) and of PEG esters (mono- and diesters) and free PEG which represent 90% in the case of Labrasol® and 80% in the case of Gelucire® 44/14. The acylglycerol fraction of these excipients is responsible for the solubilisation of lipophilic drugs (48), whereas the mono- and diesters of PEG are amphiphilic molecules which are responsible for the surfactant properties of Labrasol® and Gelucire® 44/14 and free PEG plays the role of co-solvent. *In vivo*, the hydrolysis of acylglycerols and PEG esters contained in both excipients might have a major influence on the fate of a poorly water-soluble drug in the gut.

In order to identify which compounds and/or lipolysis products of Labrasol® and Gelucire® 44/14 were responsible for the maintain of poorly water-soluble drugs in aqueous solution, we performed experiments that simulate gastrointestinal lipolysis, based on *in vivo* data recorded at 50% of gastric emptying of test meals (28,45). The gastric lipolysis was simulated with rDGL which is known to be a good model of human gastric lipase (HGL) (38,40,41). We also used bile salts (sodium taurodeoxycholate) which are normally not present in the stomach *in vivo*, to reduce the surface tension of the assay solution and consequently to reach a value close to the physiologic one (between 35 mN/m and 45 mN/m; (49)). The addition of surfactant is necessary in the absence of a meal. The use of synthetic surfactants like sodium lauryl sulphate (50) or Triton-X® 100 (51) can reduce the surface tension to physiological values but they also increase the solubilisation capacity of the assay solution (52) towards poorly water-soluble drugs and they can also inhibit digestive lipases. Moreover, Carrière *et al.* (27) showed that the lipolytic activity of human gastric lipase on a liquid test meal was not affected by the presence of bile salts at physiological concentrations.

To simulate the duodenal lipolysis, we used porcine pancreatic extracts as a model for human pancreatic juice. We previously showed that human pancreatic juice and porcine pancreatic extracts presented similar lipolytic activities on both Labrasol® and Gelucire® 44/14 (22,23) although their enzyme compositions are different. Pancreatic enzymatic solution was prepared by first mixing PPE and the assay solution containing bile salts in order to mimic *in vivo* process, where pancreatic and biliary secretions are mixed in the papilla of Vater before reaching the upper part of the small intestine. The mixture of pancreatic and biliary secretions may led, according to Lairon *et al.* (53), to the formation of a lipoprotein complex between pancreatic lipase, colipase and endogenous lipids, responsible for the lipolytic activity of dietary triacylglycerols. In order to respect a 1.7-fold dilution of the chyme by pancreatic and biliary secretions (45), the volume of pancreatic enzymatic solution added was adapted.

During the gastric lipolysis step, di- and triacylglycerols of both Labrasol® and Gelucire® 44/14 were well hydrolyzed by rDGL. Moreover, PEG-8 diesters of Labrasol® were also good substrates of rDGL whereas PEG-32 diesters of Gelucire® 44/14 were not hydrolyzed. One can assume that during the gastric phase, there was an accumulation of PEG-8 monoesters corresponding to PEG-8 monoesters initially present in Labrasol® and PEG-8 monoesters resulting from the hydrolysis of PEG-8 diesters. Concerning monoacylglycerols, C8-C10 monoacylglycerols were slightly hydrolyzed by rDGL whereas C8-C18 monoacylglycerols presented an important accumulation.

Consequently, at the end of gastric lipolysis phase the main hydrolysable compounds of Labrasol® were C8-C10 monoacylglycerols and PEG-8 monoesters. Concerning Gelucire® 44/14 the main hydrolysable compounds at the end of gastric lipolysis phase were C8-C18 monoacylglycerols and PEG-32 mono- and diesters. This finding suggests that *in vivo*, monoacylglycerols and PEG esters and particularly PEG monoesters will have a major role to maintain poorly water-soluble drugs in aqueous solution during the gastric digestion.

Concerning the duodenal lipolysis phase, the first 5 min were decisive. Monoacylglycerols were rapidly and, in the case of Labrasol®, totally hydrolyzed in less than 5 min after the addition of pancreatic enzymatic solution. PEG monoesters (free PEG released) and PEG-32 diesters were also significantly hydrolyzed during the beginning of duodenal lipolysis phase whereas their percentages remained constant after the first 5 min. Contrary to monoacylglycerols, remaining di- and triacylglycerols were not significantly hydrolyzed as well as PEG-8 diesters.

Although we were not able to follow the composition changes of PEG-8 and PEG-32 monoesters as precisely as other compounds, we estimated the percentage of PEG-8 and PEG-32 monoesters at the end of both gastric and duodenal phases. The percentage of PEG-8 and PEG-32 monoesters was 125% and 100% at the end of the gastric lipolysis phase, respectively and, at the end of duodenal lipolysis phase, the percentage of PEG-8 and PEG-32 monoesters were estimated to 85% and 90%, respectively.

These findings suggest that PEG monoesters probably play a crucial role to maintain the drug in solution. Concerning free PEG which corresponds to PEG initially present in these excipients and PEG released from the hydrolysis of PEG monoesters, it seems difficult that this co-solvent has a major role in the fate of the drug during the lipolysis. Indeed, water soluble co-solvents such as PEG-8 are used to increase the solvent capacity of the formulation but they loose rapidly their solvent capacity after the dispersion of the formulation in an aqueous phase (48). Moreover, 2-monoacylglycerols are not hydrolyzed *in vivo* because they are directly absorbed through the enterocytes. One can assume that the 2-monoacylglycerols and free fatty acids produced during the gastric digestion will help the drug transport next to the unstirred water layer.

Lipolysis of Either Labrasol® and Gelucire® 44/14 and its Effect on the Solubilisation of Piroxicam and Cinnarizine

For the first time to our knowledge, we studied in parallel the evolution of the composition of lipid-based excipients during their *in vitro* gastrointestinal lipolysis (Fig. 2) and the resulting precipitation of poorly water-soluble drugs (piroxicam and cinnarizine). The comparison of results presented in Figs. 2 and 3 allowed us to deduce some interesting observations.

In presence of active lipases, the precipitation of piroxicam formulated with either Labrasol® or Gelucire® 44/14 due to the lipolysis of the excipient was more important during the gastric step than during the duodenal step. Indeed, a more important precipitation of piroxicam was observed when the excipient was hydrolyzed as compared to the

experiments performed with inactivated enzymes. These findings suggest that *in vivo* the gastric digestion may have an influence on the fate of this poorly water-soluble drug and also underline the importance of gastric digestion step which was not taken into account in the *in vitro* bioavailability studies performed so far. Indeed, in these studies, the amounts of poorly water-soluble drugs dissolved in the aqueous phase might be underestimated. The piroxicam precipitation observed during the gastric lipolysis step of both Labrasol® and Gelucire® 44/14, corresponds to the significant lipolysis of C8-C10 di- and triacylglycerols and PEG-8 diesters in the case of Labrasol® and of C8-C18 di- and triacylglycerols in the case of Gelucire® 44/14. It seems quite clear that the lipolysis of these compounds affect the concentration of piroxicam dissolved in the aqueous phase whereas the concentration of cinnarizine dissolved in the aqueous phase was not affected by the lipolysis of di- and triacylglycerols and PEG-8 diesters in the case of Labrasol®. The changes in the composition of both excipients can led to a reorganisation of the microemulsion system. Indeed, we can assume that the particle size or organisation can be affected by the composition changes which can reduce or increase the particle size. Fatouros *et al.* demonstrated that the lipolysis of a SNEDDS immediately generated a lamellar phase which evolved as a function of lipolysis products into unilamellar vesicles and finally in mixed micelles (54,55).

In presence of active or inactivated enzymes, the main precipitation of piroxicam is due to the presence of pancreatic proteins, accentuated in the case of Labrasol® by the dilution that occurred between the gastric and the duodenal steps of lipolysis.

In absence of lipolysis, the precipitation of piroxicam was linear as a function of time in the case of Gelucire® 44/14 whereas it was induced by dilution in the case of Labrasol® (Fig. 3). This result can be explained by the fact that Labrasol® does not present the same organization (microemulsion *vs.* emulsion) as a function of its concentration. At concentration between 2 g/l and 31 g/l, Labrasol® forms an emulsion and a microemulsion at concentration above to 32 g/l. The dilution of the gastric medium by the assay solution corresponds to a change in Labrasol® concentration and organisation from a microemulsion to a fine emulsion. In the case of Gelucire® 44/14 no changes were observed in term of organization as a function of its concentration. These results indicate that piroxicam dispersion in the water phase is dependent on the surface of the dispersion (higher with a microemulsion), suggesting that piroxicam is an amphiphilic molecule with a high affinity for the oil-water interfaces.

As a conclusion, piroxicam slightly precipitated during the gastric lipolysis step due to the excipient lipolysis whereas the duodenal lipolysis step did not affect the concentration of piroxicam dissolved in the aqueous phase. The precipitation of piroxicam was also induced by the dilution and particularly by the presence of pancreatic proteins.

Both Labrasol® and Gelucire® 44/14 increased the solubility of piroxicam. Indeed, at the end of the experiments the percentage of piroxicam dissolved in the aqueous phase formulated with Labrasol® was equal to 90% (no enzymes), 74%, (inactivated enzymes) and 69% (active enzymes) which corresponds to a concentration of piroxicam in the aqueous phase equal to 328.944 mg/l, 299.480 mg/l, and 233.371 mg/l respectively. The solubility of piroxicam was 7.5 (no enzymes), 6.8 (inactivated enzymes), and 5.4 times higher than its solubility in the assay solution at pH 6.25. Concerning piroxicam formulated with Gelucire® 44/14, at the end of the experiments, the percentage of piroxicam dissolved in the aqueous phase corresponded to 80%, 67%, 61%, in absence of enzymes, in presence of inactivated enzymes, and in presence of active enzymes, respectively. These percentages corresponded to a concentration of piroxicam in the aqueous phase equal to 275.416 mg/l (no enzymes), 232.800 mg/l (inactivated enzymes), and 192.689 mg/l (active enzymes) which signifies that the solubility of the piroxicam in the assay solution was multiplied by 6.3, 5.3, and 4.4, respectively.

The cinnarizine precipitation strongly depended on the excipient used. When cinnarizine was formulated with Labrasol®, the lipolysis of Labrasol® significantly affected the fate of cinnarizine. No effect of the lipolysis was observed when using Gelucire® 44/14 as lipid-based vehicle.

During the gastrointestinal lipolysis of Labrasol®, the percentage of cinnarizine dissolved in the aqueous phase decreased linearly (Fig. 3C) and the PEG-8 monoesters were the only compounds which remained at a high level (100% at t= 0 min, 125% at t=29 min, and 85% at t=90 min). One can therefore assume that PEG-8 monoesters were involved in the solubilisation of cinnarizine during the gastrointestinal lipolysis. The precipitation of cinnarizine due to the dilution of the gastric medium did not occur when Labrasol® was hydrolyzed. This finding supposes that the changes in Labrasol® composition due to its lipolysis may not lead to the transformation of the microemulsion into a fine emulsion. Indeed during the lipolysis the ratio between the acylglycerol and PEG esters fractions was modified and consequently the organisation of Labrasol® is certainly affected by these changes.

The precipitation of cinnarizine formulated with Gelucire® 44/14 occurred when the gastric medium was diluted whatever the fate of the excipient (hydrolyzed or not). Since the percentage of PEG-32 monoesters remained high during the gastrointestinal lipolysis (100% at t=0 and 29 min, and 90% at t=90 min), PEG-32 monoesters might play a key role to maintain cinnarizine in aqueous solution, as in the case of Labrasol®. The precipitation of cinnarizine due to the dilution of the gastric medium might be attributable to a greater solubilisation of PEG-32 monoesters.

At the end of the experiment, the percentage of cinnarizine dissolved in the aqueous phase formulated with Labrasol® was equal to 28% (no enzymes), 45%, (inactivated enzymes) and 85% (active enzymes) which corresponds to a concentration of cinnarizine in the aqueous phase equal to 67.822 mg/l, 101.959 mg/l, and 151.577 mg/l respectively. The solubility of cinnarizine was 63.3 (no enzymes), 95.2 (inactivated enzymes), and 141.5 times higher than its solubility in the assay solution at pH 6.25, respectively.

When the cinnarizine is formulated with Gelucire® 44/14, the solubility of cinnarizine was increased as compared to the solubility of cinnarizine in the assay solution. Indeed, at the end of the experiments, the percentage of cinnarizine dissolved in the aqueous phase was equal to 73% (154.399 mg/l) in absence of enzymes, 56% (116.812 mg/l) in presence of inactivated enzymes, and 79% (141.986 mg/l) in presence of active enzymes. In this case, thanks to Gelucire® 44/14, the solubility of cinnarizine was multiplied by 144.2 (no enzymes), by 109.1 (inactivated enzymes), and by 132.6 (active enzymes).

General Conclusion

The study of the changes in term of composition of both Labrasol® and Gelucire® 44/14 during their *in vitro* gastrointestinal lipolysis provided useful information, particularly on the compounds which might be involved in the solubilisation of piroxicam and cinnarizine in the aqueous phase. We showed that monoacylglycerols and PEG monoesters were the main compounds at the end of gastric phase whereas at the end of duodenal phase, only PEG esters remained. These findings suggest an important role for these compounds which probably interact with free fatty acids released to form colloidal structures involved in the maintain of the poorly water-soluble drug in solution.

As shown by the piroxicam precipitation during the gastric lipolysis of both Labrasol® and Gelucire® 44/14, the gastric digestion step *in vivo* can strongly modify the composition of both Labrasol® and Gelucire® 44/14 and affect the precipitation of a poorly water soluble drug.

The main precipitation of cinnarizine is observed when the gastric medium was diluted to simulate the dilution of the chyme by pancreatic and biliary secretions, this underlines that *in vivo*, the dilution occurring in the small intestine may constitute a critical step to maintain the drug in solution.

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