International Journal of Pharmaceutics 417 (2011) 245–255

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

In vitro lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems

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ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 27 February 2011
Accepted 1 March 2011
Available online 8 March 2011

Keywords:
In vitro lipolysis
LSBDDS
Gastric lipase
Lipids
Bile acids
XRPD
Cryo-TEM
SAXS
PLM

ABSTRACT

With the increasing interest in lipid and surfactant based drug delivery systems (LSBDDS) for oral delivery of poorly soluble drugs, the need for efficient development tools is emerging. *In vitro* lipolysis models, simulating the digestion in the small intestine, is a promising tool in this regard. Several different *in vitro* lipolysis models have been used for characterization of LSBDDS, all using porcine pancreatin as lipase source, and primarily differing in the addition scheme of calcium and the kind of bile acids employed. Both calcium and bile influence the lipolysis. Calcium have been used both as fixed addition at the beginning of the experiment and with a continuous addition during lipolysis. Both pure bile acids and crude porcine bile extract have been used. Lipolysis of LSBDDS will generate mixed micelles, as well as lamellar and hexagonal phases. These have been characterized by dynamic light scattering, cryogenic transmission electron microscopy and small angle X-ray scattering. The faith of drug during *in vitro* digestion of a LSBDDS is often studied by ultracentrifugation and quantification of drug in the different phases formed. Further, drug precipitated during *in vitro* lipolysis has been characterized by X-ray powder diffraction and polarized light microscopy.

1. Introduction

With the purpose of increasing the oral bioavailability of poorly water soluble drug compounds, more advanced drug delivery systems have been developed (Gursoy and Benita, 2004; Fahr and Liu, 2007). One category of advanced drug delivery systems is lipid and surfactant-based drug delivery systems (LSBDDS), which refers to systems where the drug compound is in solution in surfactants or lipids or a mixture thereof. Since surfactants and lipids are part of a large group of compounds, LSBDDSs include many different drug delivery systems, e.g. oil solutions, emulsions, micro-emulsions, self-(nano)-emulsifying drug delivery systems (S(N)EDDS) and micellar systems (Mullertz et al., 2010). Vesicular systems for oral delivery, like liposomes and niosomes, can also be categorized as LSBDDS, however use of these systems is not very widespread at the present time. Especially S(N)EDDS have recently achieved a lot of attention both from industry and academia, due to their ability to administer drug in solution in a preconcentrate, which disperses to a nano-emulsion in the gastro-intestinal tract. Confusion about the difference between nano-emulsions and micro-emulsions exists in the pharmaceutical literature and therefore SNEDDS are often called self-micro-emulsifying drug delivery systems (SMEDDS) and vice versa (Anton and Vandamme, 2010). Micro-emulsions are thermodynamically stable systems, whereas nano-emulsions are not, however, nano-emulsions are kinetically stable and will typically be stable during the relevant timeframe: transit of the gastro-intestinal tract. In the following, the terms used by the respective authors will be employed.

The general hypothesis, which is applied to explain the advantage of LSBDDS, relates to their ability to keep the drug in solution throughout gastro-intestinal transit. This way LSBDDS circumvent the dissolution step in the gastro-intestinal tract, but instead many complex processes take place after ingestion of LSBDDS. These processes are not fully understood but involve digestion of excipients (Cuine et al., 2008; Fernandez et al., 2009; Larsen et al., 2008) and formation of different colloidal structures (Fatouros et al., 2007a; Kossena et al., 2003, 2005). The drug compound is distributed between these structures (Kossena et al., 2004) and is believed to partition into mixed micelles before it is absorbed. If the solubilization capacity of the generated colloid structures towards the drug is reduced, precipitation of the drug may occur (Sassene et al., 2010), which then will necessitate a dissolution step prior to absorption.

As a consequence of the increasing number of poorly soluble drug candidates in development in the pharmaceutical industry, increased interest have been directed towards development and use of LSBDDS, however a rational development scheme for LSBDDS has not yet been developed, mainly due to lack of understanding

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doi:10.1016/j.ijpharm.2011.03.002

of the important steps in drug release and absorption from LSBDDS and how to simulate it.

Due to the complexity of the events taking place after ingestion of LSBDDS, simple dissolution, or dispersion, tests are often not feasible for *in vitro* evaluation of LSBDDS, because these tests do not take into account that excipients can be prone to lipase catalysed hydrolysis in the gastrointestinal tract. *In vitro* digestion models, also known as *in vitro* lipolysis models, therefore play an important role in elucidating drug release from LSBDDSs. When some of these LSBDDSs are digested, the digestion phase is considered to be crucial for the release of the drug from the formulation (Pouton, 2006). Since LSBDDS includes such a diverse group of drug delivery systems, and the events taking place after ingestion are very complex, the development of LSBDDSs are conducted with the lack of standardized *in vitro* methods. A more rational approach to development of LSBDDSs is required, and an *in vitro* method that takes into account the gastrointestinal processes is crucial to achieve this. Solubilization of the drug in the different colloid structures formed during lipolysis of the formulations is of interest although it is not yet established how the compounds are absorbed from these phases. *In vitro* lipolysis models play an important role in elucidating the mechanisms behind drug compound movement between the different colloidal phases to an absorbable form.

This paper will review the current use of *in vitro* lipolysis models as a tool to develop and characterize LSBDDS. Existing *in vitro* lipolysis models used will be discussed, and the use of *in vitro* lipolysis models in the development of new LSBDDS will be treated. Furthermore, the use of *in vitro* lipolysis models to elucidate the events that occur after ingestion of LSBDDS is discussed. Increased knowledge of these events is a prerequisite for the development of optimal LSBDDS.

2. *In vitro* lipolysis models

In vivo lipid digestion encompasses an array of very complicated processes and is therefore difficult to imitate. However, in order to obtain useful information from *in vitro* lipolysis it is essential that the experimental conditions are physiologically representative (MacGregor et al., 1997). So far the main focus in developing *in vitro* lipolysis models has been on simulating lipolysis in the upper intestinal tract, however, lately the importance of gastric lipolysis, catalysed by gastric lipase, has been realised and *in vitro* digestion models encompassing both gastric and intestinal lipolysis have been developed (Fernandez et al., 2009; Larsen et al., 2010). However, in this review we have chosen to focus on intestinal lipolysis, since this is the area where most work has been done.

Different *in vitro* lipolysis models have been employed to characterize LSBDDS. Normally *in vitro* lipolysis is conducted in a medium containing bile acids and phospholipids in a thermostated vessel at a fixed stirring speed. During the lipolysis the hydrolysable ester bonds in the added LSBDDS are cleaved by a lipase source. Table 1 summarizes the experimental conditions for the three lipolysis models most extensively used for studying LSBDDS.

2.1. Pancreatic lipid digestion enzymes

As can be seen from Table 1, porcine pancreatin is used as source of lipases in all models. Porcine pancreatin contains a mixture of all enzymes secreted from the pancreas. The most important pancreatic enzymes for lipid digestion are; pancreatic triacylglyceride lipase, co-lipase, phospholipase A2, and cholesterol esterase. These enzymes will be described below.

2.1.1. Pancreatic triacylglyceride lipase

Pancreatic triacylglyceride lipase (pancreatic lipase) hydrolyses triacylglycerides at the *sn*-1 and *sn*-3 position forming one 2-monoglyceride and two fatty acids per triacylglyceride molecule. 2-Monoglyceride can spontaneously convert to 1- or 3-monoglyceride, which are substrates of pancreatic lipase. The affinity of pancreatic lipase is higher towards medium chain triglycerides (MCT) compared to long chain triglycerides (LCT). During *in vitro* lipolysis this results in a faster and more extensive lipolysis of MCT based formulations. It is generally recognised that the human pancreas produces lipase in greater amounts than required for digestion and for this reason basically all ingested lipid is digested and absorbed under normal physiological conditions (Carey et al., 1983). Pancreatic lipase can hydrolyse monomeric substrates; however it only attains its full catalytic activity in the presence of an oil/water interface (Embleton and Pouton, 1997). The enzyme is activated by interfacial binding and works at the interface. In the presence of bile acids and phospholipids, the pancreatic lipase requires colipase to achieve optimal activity. The colipase will facilitate the anchoring of the pancreatic lipase to the oil/water interface. It has been suggested that the colipase reorganises the lipid droplet interface, probably making the interface more susceptible to pancreatic lipase (Brockman, 2002). Calcium has been suggested to be of importance for the *in vivo* lipolysis by formation of a catalytically active lipase complex comprised of mixed micelles and calcium (Alvarez and Stella, 1989). In *in vitro* experiments the calcium dependency of pancreatic lipase is also due to the removal of free fatty acid by the formation of calcium soaps, since free fatty acids will inhibit the activity of pancreatic lipase by binding to the interface. The larger the interface is in the presence of excess lipase, the faster is the hydrolysis rate (Armand et al., 1992). Pancreatic lipase is pH dependent with an optimal activity between pH 6.5 and 8.0 (Armand et al., 1992).

2.1.2. Phospholipase A_2

Phospholipase A_2 is secreted as a proenzyme and requires activation by hydrolysis in the N-terminal chain. Hydrolysis of fatty acids from a variety of phospholipids at the *sn-2* position is catalysed by phospholipase A_2 and one lyso-phospholipid and one fatty acid are the products formed. The enzyme requires the presence of calcium ions to be active and the substrate has to be present in an aggregated form for the enzyme to hydrolyse the substrate (Carey et al., 1983). Phospholipase A_2 hydrolysis of phospholipids may be a mechanism for facilitating triglyceride hydrolysis by pancreatic lipase *in vivo* (Patton and Carey, 1981).

2.1.3. Cholesterol esterase

Cholesterol esterase has several names in the literature, e.g. it is also known as carboxyl ester lipase, carboxyl esterase, non-specific lipase, and bile salt-stimulated lipase. The cholesterol esterase is an enzyme with broad substrate specificity. Hydrolysis of water soluble carboxyl esters is catalysed by the enzyme, whereas insoluble carboxyl esters require presence of bile acids before the cholesterol esterase can catalyse the hydrolysis (Carey et al., 1983). The enzyme catalyses, e.g. hydrolysis of the lyso-phospholipids and 2-monoglycerides (Bernback et al., 1990) which are lipolysis products from phospholipid hydrolysis by phospholipase A_2 and triglyceride hydrolysis by pancreatic lipase, respectively.

2.2. Composition of the lipolysis medium

2.2.1. Bile acids and phospholipids

Bile acids and phospholipids are secreted from the gallbladder to the duodenal lumen and form mixed micelle, thus in order to resemble intestinal fluids the lipolysis media must contain bile acids and phospholipids. Furthermore bile acids and phospholipids impact the activity of the pancreatic lipase.

The concentration of bile acids and phospholipids in the intestinal fluids varies with the dietary state. Phospholipids originate both

Table 1
Experimental conditions of the three lipolysis models used in Copenhagen, Monash and Jerusalem.

Experimental condition	Copenhagen[a]	Monash[b]	Jerusalem[c]
Lipase source	Porcine Pancreatin (3× USP)	Porcine Pancreatin (8× USP)	Porcine Pancreatin (8× USP)
Lipase activity in the digestion medium	300–800 USP units/ml	1000 Tributyrin units/ml	1000 Tributyrin units /ml
Bile species in the digestion medium	Porcine Bile Extract containing various bile acids	Taurodeoxycholic acid	Taurocholic acid
Concentration of bile in digestion medium	5–30 mM	5–20 mM	5 mM
Phospholipid species in the digestion medium	Phosphatidylcholine	Lecithin (60% PC)	L-α-Phosphatidylcholine
Bile acid to phospholipid ratio	4	4	4
Calcium addition	Continuous addition of calcium at 0.045–0.181 mmol/min	Initial addition of 5 mM calcium	Initial addition of 5 mM calcium
pH (buffer)	6.5 (2 mM tris maleate)	7.5 (50 mM tris maleate)	6.8–7.4 (50 mM tris maleate)
Initial volume in the digestion medium	300 ml	10–40 ml	40 ml

[a] Christensen et al. (2004), Fatouros et al. (2007a, 2007b), Larsen et al. (2008), Zangenberg et al. (2001a, 2001b).
[b] Cuine et al. (2008), Kaukonen et al. (2004a, 2004b), Porter et al. (2004a, 2004b), Sek et al. (2002).
[c] Dahan and Hoffman (2006, 2007).

from the diet and from the bile secretions, while bile acids only originate from the bile, thus the ratio between bile acid and phospholipids can be highly variable, as has recently been reviewed (Kleberg et al., 2010b). The concentrations of bile acids in human intestinal fluids ranges from 2 to 5 mM (Brouwers et al., 2006; Kalantzi et al., 2006; Lindahl et al., 1997; Perez de la et al., 2006; Persson et al., 2005; Tangerman et al., 1986) in the fasted state and from 8 to 15 mM (Hernell et al., 1990; Kalantzi et al., 2006; Persson et al., 2005; Tangerman et al., 1986) in the fed state. The bile acid composition in human bile is shown in Table 2. The results vary between different studies; however a lipolysis media containing a mixture of different conjugated bile acids will imitate the *in vivo* conditions better. The lipolysis models employed in Monash and Jerusalem use pure bile acids taurodeoxycholate and taurocholate respectively, whereas in Copenhagen a crude porcine extract, containing a mixture of bile acids is used.

Bile acid mixtures are commercially available both from porcine and bovine origin. Literature is sparse in composition of bile acids from different animals however porcine and ox bile seems to be acceptable to use to resemble the composition of bile acids in human bile (Alvaro et al., 1986; Gillin et al., 1989). In the Copenhagen model the used bile acids are of porcine origin. Porcine bile contains mainly glycine conjugated bile acids (Alvaro et al., 1986) and therefore represents human bile well with respect to conjugation. However, the porcine bile extract contains hyocholic and hyodeoxycholic bile acids, which are not found in the human bile (Alvaro et al., 1986). Because bile extracts are natural products batch to batch variation is expected and therefore each batch has to be characterized. An alternative is to use a mixture of pure bile acids which more correctly resembles the content of the human bile, however this option is very expensive. It is known that mixed micelles of different bile acids and phospholipids can have different solubilization capacities towards drug compounds (Soderlind et al., 2010). In preliminary studies we have seen that the taurine conjugated bile acids are more prone to precipitate during lipolysis compared to the glycine conjugated bile acids. As a result, drug solubilization during *in vitro* lipolysis may vary because of the use of different bile acids.

The bile acid concentrations used in *in vitro* lipolysis models are typically ranging from 5 to 20 mM where the low levels simulate the fasted state and the higher level the fed state. The effect of increasing bile acid concentration at a fixed phospholipid level has been studied by MacGregor et al. (1997). They found that the extent of lipolysis was increased for both a medium chain triglyceride

Table 2
Bile acid composition in humans. Data is obtained from the literature and is presented as the mean in % ± SD.

	Fasted duodenum[a]	Fasted jejunum[b]	Fasted duodenum[c]	Fed jejunum[d]	Fasted duodenum[e]	Fasted jejunum[f]	Fed jejunum[g]	Hepatic bile[h]	Gall bladder bile[i]
Taurine-conjugated									
Taurocholic acid (TC)	41.9 ± 18.0	48.5 ± 19.4	30.1 ± 15.4	21	18.4	23	18	3.6	9.4 ± 0.8
Taurochenodeoxycholic acid (TCDC)	7.8 ± 2.3	8.0 ± 2.2	8.9 ± 3.2	13	8.5	21	17	4.7	11.6 ± 2.2
Taurodeoxycholic acid (TDC)	5.3 ± 2.7	4.0 ± 2.2	3.8 ± 3.3	10	5.6	3	8	0.3	5.0 ± 0.4
Total taurine conjugated	55	60.5	42.8	44	32.5	47	43	8.6	26
Glycine-conjugated									
Glycocholic acid (GC)	15.6 ± 4.6	19.0 ± 8.0	20.0 ± 5.5	27	33.5	37	26	61.3	26.1 ± 3.2
Glycochenodeoxycholic acid (GCDC)	15.6 ± 0.4	11.6 ± 9.0	20.3 ± 5.5	16	24.4	14	16	25.8	31.9 ± 3.4
Glycodeoxycholic acid (GDC)	12.4 ± 8.3	8.1 ± 5.9	15.9 ± 10.4	13	9.6	2	15	0.5	16 ± 2.2
Total glycine conjugated	43.6	38.7	56.2	56	67.5	53	57	87.6	74

[a] Perez de la et al. (2006).
[b] Perez de la et al. (2006).
[c] Brouwers et al. (2006).
[d] Persson et al. (2006).
[e] Bergman et al. (2006).
[f] Persson et al. (2005).
[g] Persson et al. (2005).
[h] Gillin et al. (1989).
[i] Alvaro et al. (1986).

and long chain triglyceride substrate as the bile acid concentration increased. However, a very high bile acid concentration (260 mM) was used to achieve 63% and >65% hydrolysis of long chain and medium chain triglycerides respectively. The effect was attributed to the bile acid micelles capacity to solubilize the lipolytic products and thereby avoiding inhibition of pancreatic lipase by the lipolytic products, especially free fatty acids, accumulating at the oil droplets surface.

In vivo the concentration of phospholipids ranges from 0.1 to 0.6 mM in the fasted state and from 0.1 to 4.8 mM in the fed state respectively (Kleberg et al., 2010b). A wide range of ratios between bile acids and phospholipids have been reported, from 1 to 38.9, but most studies report ratios ranging from 2 to 6 (Kleberg et al., 2010b). The ratio of 4 is usually employed in the lipolysis models. Phospholipids can inhibit the activity of pancreatic lipase–colipase complex in the presence of bile acids; in the presence of mixed micelles, containing bile acids and phosphatidylcholine, the pancreatic lipase can be displaced from the interface (Patton and Carey, 1981). Lipolysis of the lipid droplets is inhibited as a result. The inhibition is reversed in the presence of phospholipase A_2, most likely due to phosphatidylcholine being hydrolysed to lyso-phosphatidylcholine, which do not inhibit pancreatic lipase (Blackberg et al., 1979). The appearance of a lag time is observed before the hydrolysis reach steady state and the steady state hydrolysis rate that eventually is reached is lower in the presence of phospholipids and bile acids (Embleton and Pouton, 1997).

2.2.2. Lipase source for in vitro lipolysis models

As mentioned above the source of lipase commonly used for *in vitro* lipolysis models is Pancreatin of porcine origin. Pancreatin is a good source of duodenal enzymes to use for *in vitro* lipolysis, due to its natural origin it should contain all the relevant enzymes, e.g. pancreatic lipase, phospholipase A_2 and cholesterol esterase and possible also pancreatic lipase like protein 2, which represent a minor lipolytic activity in the pancreatic secretions. It is important to have a mixture of the enzymes present during *in vitro* lipolysis since LSBDDSs contains various potential substrates. Many surfactants are also substrates for one or several of the enzymes in pancreatin, e.g. Labrasol (Fernandez et al., 2009, 2007), Gelucire 44/14 (Fernandez et al., 2009), Labrafil M2125CS (Larsen et al., 2008) and Cremophor EL (Cuine et al., 2008). The lipolysis of the polyethylene glycol esters in Labrasol is mainly carried out by cholesterol esterase and pancreatic lipase related protein 2 (Fernandez et al., 2007).

The lipase activity of Pancreatin can be measured by different methods. The most commonly used method uses tributyrin as substrate and is expressed in TBU. Another method is given in the USP33/NF28 (USP33, 2010); in this case the substrate is olive oil emulsified with gum-arabic, which is a more relevant substrate for testing activity of lipase for digestion of LSBDDS, due to the widespread use of LCTs in the development of LSBDDS. Here the lipase activity is expressed as USP units; one USP unit of lipase liberating 1 μmol fatty acid per minute from the substrate. The μmol fatty acids liberated is measured by pH-stat titration at pH 9. The USP method requires the use of a USP pancreatin lipase reference standard and therefore this method is very robust and results obtained from different laboratories can be compared.

For *in vitro* lipolysis the lipase is usually added in excess as is the case in the *in vivo* situation. The lipase activity has been found to be of minor importance for the extent of lipolysis compared to other factors such as bile acids and calcium (Zangenberg et al., 2001a). MacGregor et al. reported that the percentage lipolysis increased by a factor 3 when the lipase activity was increased 50 times (MacGregor et al., 1997). The pancreatic lipase is inhibited by increasing levels of free fatty acids (Borel et al., 1994) and therefore the removal of fatty acids from the lipolysis medium is important for the extent of lipolysis. Both bile acids and calcium aids the removal of fatty acids from the substrate surface.

2.2.3. Calcium addition

Calcium is added to the *in vitro* lipolysis model for several reasons. As previously mentioned the presence of free fatty acids in the lipolysis medium inhibits the pancreatic lipase. *In vivo* the hydrolysed fatty acids are absorbed and thus inhibition of the pancreatic lipase by free fatty acids does not occur. However, during *in vitro* lipolysis, free fatty acids have to be removed to avoid inhibition of the lipase. MacGregor et al. achieved this by using very high level of bile salts that were not physiologically relevant; however they also showed that addition of calcium to the lipolysis media made the use of high bile salt concentration unnecessary (MacGregor et al., 1997). Wickham et al. confirmed this finding by showing that the pancreatic lipase activity increased with increasing bile salt and calcium concentrations (Wickham et al., 1998). Zangenberg et al. concluded that the lipolysis rate could be controlled by continuous addition of calcium (Zangenberg et al., 2001a). Calcium removes the fatty acids by formation of precipitating calcium soaps and thereby the absorption of fatty acids *in vivo* is mimicked. This may not be a physiological way to remove free fatty acids, but it has been proven useful, especially since removal of free fatty acids by bio-membranes or dialysis membranes have been shown to be very complicated.

It is common to observe a lag time before the achievement of steady state hydrolysis (Wickham et al., 1998). The lag time has been explained by a slow interfacial penetration of the lipase. The lag time is significantly decreased in the presence of increasing calcium concentrations *in vitro* (Armand et al., 1992). This is suggested to be due to reduced negative surface charge on the emulsion particle and thereby the repulsion of the enzyme. However in another study there was no correlation between the lag time and the zetapotential of a phospholipid emulsion in the presence of calcium and bile salts (Wickham et al., 1998). The electrostatic theory can therefore not fully account for the decreased lag time.

There are two different approaches to the addition of calcium to the lipolysis medium; continuous and fixed addition. The Copenhagen model, also known as the Dynamic *in vitro* lipolysis model, uses the continuous addition of calcium. Continuous addition of calcium results in a controlled lipolysis rate (Zangenberg et al., 2001a). By adding more or less calcium the lipolysis rate can be regulated. The Monash and Jerusalem model uses the fixed addition of calcium, 5 mM of calcium is added to the lipolysis medium prior to initiation of the lipolysis. With the fixed addition of calcium a very fast initial lipolysis is seen, almost all the lipolysis takes place within the first 5–10 min (Cuine et al., 2008; Zangenberg et al., 2001a).

2.2.4. pH in the lipolysis medium

The physiological relevant pH representing the milieu in the intestine during the fasted and fed state is between 6–7.5 and 5–6.6 respectively (Carriere et al., 1993; Kalantzi et al., 2006; Persson et al., 2005). The pancreatic lipase activity depends on the pH and an optimum activity is observed in the pH range 6.5 to 8.0 (Armand et al., 1992). In order for the fatty acids hydrolysed by lipases to be titrated with sodium hydroxide they need to be ionised. The pK_a-value of long chain fatty acids are >8 (Kanicky and Shah, 2002), but has been shown to decrease in the presence of bile salt and calcium (Patton and Carey, 1979) and in mixed bile salt micelles the pK_a is approx. 6.5 (Staggers et al., 1990). The pH chosen for the lipolysis medium is therefore a compromise; values between 6.5 and 8.5 have been used in *in vitro* lipolysis. However, since it is difficult to know exactly what the apparent pK_a values of the liberated fatty acids are, it is recommendable to conduct a back titration step at the end of the lipolysis experiment, in order to determine the total amount of free fatty acids generated. For a back titration the pH

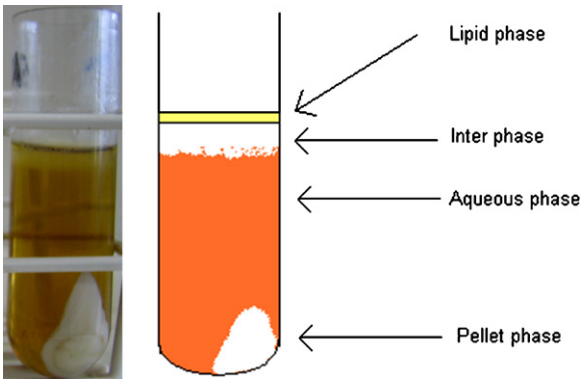

Fig. 1. The four phases present after ultracentrifugation of a lipolysis sample containing Labrafil M2125CS.

is raised to, e.g. 9, which titrates all the fatty acids generated during the lipolysis (Fernandez et al., 2007). Back titration is a phrase commonly used by the authors conducting these experiments, but in reality it is not a "real" back titration because no other reactant is added and titrated on, thus it is a normal titration performed at the end of the experiment. In the rest of this review we will refer to it as a back titration in accordance with the authors referred to. Another way to more precisely assess the level of lipolysis is to quantify the generated hydrolysis product by HPLC, this method enables a better understanding of the mechanism of lipolysis in terms of when, during lipolysis, different lipolysis products (free fatty acids, di- and mono-acylglycerides) are formed.

A buffer is usually added to the *in vitro* lipolysis medium, but the buffer capacity need to be low ensuring that fatty acid liberation causes a pH drop (MacGregor et al., 1997) in order to have an experimental evaluation parameter of the progress of the lipolysis.

2.2.5. Hydrodynamics and sampling

The lipolysis is conducted in a thermostated beaker with stirring. The stirring conditions are far from the hydrodynamic conditions in intestine. Stirring is however inevitable in order to be able to take out homogenous samples.

Immediately after sampling lipase activity in the samples are inhibited by addition of a lipase inhibitor (Zangenberg et al., 2001b). For the time zero sample it can be recommended to take out the sample before addition of pancreatin and then add inhibited pancreatin to the sample; that way lipolysis during mixing and handling is prevented. In order to isolate the different lipolysis digestion phases, the samples are ultracentrifuged (Larsen et al., 2008; Sassene et al., 2010; Zangenberg et al., 2001b) or filtered (Fernandez et al., 2009). Four phases are usually obtained upon ultracentrifugation: a pellet, a micellar aqueous phase, an inter phase, and an oil phase (Fig. 1). The formation of phases will be dependent on the type of formulations in use, e.g. SNEDDS will usually not form an oil phase and neither will surfactant systems. In addition the phases will change over time during the lipolysis; initially the oil phase can be rather large, but as lipid is being hydrolysed it tends to disappear, while the pellet phase is often very small in the beginning of lipolysis, but gets larger as lipolysis proceeds. The pellet is mainly comprised of calcium soaps of fatty acids and precipitated drug if any is present. The aqueous phase and the interphase contains different colloidal phases, e.g. mixed micelles and vesicles (Fatouros et al., 2007a; Fatouros et al., 2007b), and the oil phase contains the remaining lipids mainly triglycerides and diglycerides.

2.2.6. Practical considerations

In vitro lipolysis experiments should be performed taking into consideration the amount of substrate and the concentration of NaOH used. If the concentration of NaOH is low and the amount of substrate is high an unwanted dilution of the lipolysis media will take place. This becomes a problem if the solubilization of drug monitored becomes a function of a decreasing bile acid concentration instead of an effect of the lipolysis of the formulation tested. On the other hand it is also important not to have a too high NaOH concentration since this means that very small volumes are used and this can increase the experimental error and increase the risk of the titrator overshooting during the pH-stat titration.

When the amount of substrate in an experiment is low or if the substrate is poor it becomes very important that the lipolysis is initiated exactly at the specified pH. Otherwise, a very high standard deviation between lipolysis profiles is obtained due to this error.

As mentioned above it is necessary to have rather vigorously stirring in order to be able to take out representative samples. Precipitates of drug compound and calcium soaps will fall to the bottom and oil remaining from formulations tends to float. As a result, sampling from the lipolysis vessel is challenging, unless properly stirred.

It is important to keep in mind that not all of the NaOH used during the *in vitro* lipolysis experiment stems from the formulation tested. Lipolysis can arise from impurities in the bile extract and the crude pancreatic extract used and from the hydrolysis of phospholipids in the media to lyso-phospholipids. This background lipolysis is usually compensated for by conducting a lipolysis experiment on media without addition of formulation, and the obtained background profile is subtracted from the profiles obtained in experiments containing the formulation.

Additionally it is important to raise the pH as fast as possible during the back titration step in order to avoid false results due to base catalysed hydrolysis.

3. Characterization of *in vitro* lipolysis samples

As mentioned in the previous sections, samples taken out during the lipolysis can be treated and analysed in different ways. Usually samples are ultra-centrifuged in order to separate the 4 digestion phases; however when the generated colloid structures are to be characterized, the entire, non-centrifuged, sample can be used. In the following, methods to characterize the colloids phases are reviewed, followed by the relevant analysis on the different isolated digestion phases.

3.1. Analysis on non-centrifuged samples

3.1.1. Cryogenic transmission electron microscopy (Cryo-TEM)

Cryo-TEM is a way of microscopic imaging that makes it possible to take a snap-shot of a solution and visualize the different structures present with minimal disturbance, interference and rearrangement of the sample, due to the rapid cooling when preparing the samples (Bellare et al., 1988; Dubochet et al., 1988). These circumstances allow an image of the sample to be as close to the original solution as possible. It should be noted, however, that Cryo-TEM excludes structures that are larger than the grid upon which the sample is frozen. The thickness of the grid is around 150 nm and the distances between the sides of the grid can be up to 1 μm, further the thickness of the frozen sample is larger close to the grid, which can also lead to misinterpretations of the Cryo-TEM pictures. Cryo-TEM has been applied on various different solutions and has been used to obtain pictures of media simulating intestinal fluids and samples taken during lipolysis (Fatouros et al., 2007a; Kleberg et al., 2010a). Fatouros and colleagues used Cryo-TEM to analyse samples from *in vitro* lipolysis of a SNEDDS at different time points (Fatouros et al., 2007a). The SNEDDS consisted of sesame oil, Maisine 35-1, Cremophor RH 40 and ethanol (30:30:30:10%,

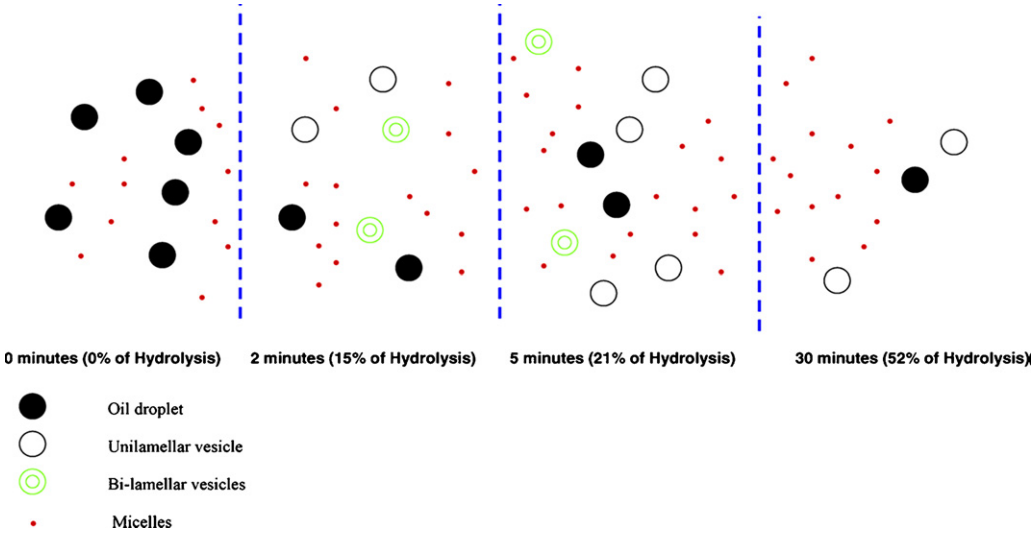

Fig. 2. Colloid phases generated during *in vitro* lipolysis of SNEDDS in fasted state, as assessed by Cryo-TEM. Note that the scheme does not take the actual size of the structures into consideration (with permission from Fatouros et al., 2007a).

w/w) and formed oil droplets of an average diameter of 50 nm. The Cryo-TEM studies allowed the identification of the morphology of different colloid structures present and the observation of how they changed during lipolysis. Prior to lipolysis, as expected, small bile acid/phospholipid micelles (approximately 10 nm) and oil droplets from undigested formulation were present. As lipolysis progressed the amount of oil droplets were reduced and formation of unilamellar and bilamellar vesicles occurred, while micelles were present throughout the lipolysis. After 30 min of lipolysis, only a few unilamellar vesicles and oil droplets were present and the bilamellar vesicles had vanished. The reduction in number of oil droplets was expected as the droplets were digested during the lipolysis. The events are schematically presented in Fig. 2. The information obtained from the use of Cryo-TEM on lipolysis samples provides an insight into the formation of colloidal phases and thus the possible colloidal structures that drug can partition into during digestion. Cryo-TEM can therefore be a useful tool in the development of LSBDDS (Fatouros et al., 2007a), but more studies are needed, with different LSBDDS before a full understanding of the impact of the formation of the different colloid structures can be obtained.

3.1.2. Small-angle X-ray scattering (SAXS)

SAXS has been used to study the formation of liquid crystalline phases during *in vitro* lipolysis of a SNEDDS using continuous addition of calcium (Fatouros et al., 2007b). The SNEDDS was the same as mentioned above for the Cryo-TEM analysis and was composed of; sesame oil, Maisine 35-1, Cremophor RH 40 and ethanol (30:30:30:10%, w/w). Samples from the digestion vessel were taken out and analysed by SAXS at $t = 0$, 5, 15, 30, 60 and 90 min. At time point zero, as expected, no liquid crystalline phases were present, however at $t = 15$ min lamellar phases were detected and these continued to be present alone until $t = 60$ min where a co-existence between the lamellar phase and a disperse inverse hexagonal phase entered and proceeded throughout the lipolysis. It was further determined that the lamellar phase was dominating up to approximately 60% digestion of the lipids present in the formulation. Hereafter the lamellar and hexagonal phase co-existed and towards the end of the lipolysis the hexagonal phase was dominating. Subsequently these findings were repeated using a flow-through lipolysis cell mounted directly on the SAXS (Roshan et al., 2006). This set-up enabled real-time monitoring of the evolution of liquid crystalline phases during *in vitro* lipid digestion. Recently Boyd and colleagues have confirmed the presence of both lamellar and hexagonal phases during digestion of the same SNEDDS using the lipolysis model with fixed addition of calcium. They also employed real-time monitoring, but by coupling lipid digestion directly to high intensity synchrotron SAXS. This methodology will make it possible to monitor the development of different liquid crystalline phases during *in vitro* lipolysis without having to take out samples and pretreating them, thus a better understanding of the relationship between formulation composition and colloid phases generated, as well the impact of different colloid phases on drug absorption.

3.2. Analysis of isolated digestion phases

After separation by ultra-centrifugation, the digestion phases can be characterized with respect to concentration of drug, bile acids and lipolysis products, as well as size of aggregates in the micellar aqueous phase. Since it is a prerequisite for absorption that the drug is dissolved in the aqueous phase, the drug concentration in the micellar aqueous phase is of particular interest.

3.2.1. Analysis of the aqueous phase

As mentioned previously the aqueous phase will contain drug solubilized in mixed micelles during lipolysis. Since the mixed micelles are expected to carry the drug to the unstirred water layer lining the intestine, release the drug and thereby facilitate the absorption, it is of great interest to determine the drug content in the aqueous phase. During lipolysis the composition of the aqueous phase will change due to the formation of lipolysis products that will interact with the mixed bile acid/phospholipid micelles that are initially present. Previous studies have shown that the average hydrodynamic radius of micelles present in the aqueous phase, as measured by dynamic light scattering, increases during lipolysis (Christensen et al., 2004).

Changes in the aqueous phase during *in vitro* lipolysis in terms of lipid and bile acid composition have also been assessed. Using soy bean oil as substrate in the Copenhagen lipolysis model Zangenberg et al. found that at 20:5 mM bile acid:phospholipid, the bile acid concentration remained constant until 75% of the oil had been hydrolysed, and then decreased. Sek et al. (2001), employing a constant calcium level of 5 mM and 20:5 mM taurodeoxycholate:phospholipid found no decrease in bile acid concentration, but only continued the lipolysis for 30 min at which point 75% lipolysis had not yet been reached.

It should also be noted that phospholipase A_2 is present in the Pancreatin and hydrolyse phospholipids to lyso-phospholipids (Sek et al., 2001). Lyso-phospholipids are a different type of surfactants compared to phospholipids, as recently have been reviewed (Mullertz et al., 2010), and therefore solubilization of drugs is expected to change as a function of the hydrolysis.

The formation of di- and monoacylglycerides as well as free fatty acids has also been monitored during lipolysis of triacylglycerides (Sek et al., 2001; Zangenberg et al., 2001b), with the conclusion that free fatty acid is the dominating species, followed by monoacylglycerols and diacylglycerols. More studies are needed, however, in order to understand the impact of the formation of the individual lipolysis product on the generated colloid phases and also their impact on drug solubilization and absorption.

3.2.2. Analysis of the pellet phase

In vivo precipitation of drug can be a concern, since precipitated drug needs to be re-dissolved prior to absorption from the intestine and when working with BCS class II or IV drugs the solubility of the drug compounds in the gastrointestinal fluids is limited and precipitation might decrease bioavailability (Dai et al., 2007; Mohsin et al., 2009; Porter and Charman, 2001; Pouton, 2000). Precipitation of a drug compound from a LSBDDS during passage of the gastrointestinal tract can be caused by numerous different factors. One of these factors is hydrolysis of excipients in the LSBDDS by enzymes present in the gastro-intestinal tract. Many excipients used in LSBDDS contain ester-bonds that are prone to hydrolysis by lipases or esterases present in the gastrointestinal tract (see section 2.2.2) (Cuine et al., 2008; Fernandez et al., 2009; Larsen et al., 2008). If the formed hydrolysis products have a lower solubilization capacity towards the drug, this can result in precipitation of drug (Porter et al., 2004a). In addition simple dilution of a LSBDDS by the gastrointestinal fluids can also cause the drug to precipitate; this is due to a loss of water soluble excipients, primarily co-solvents, from LSBDDS to the gastro-intestinal fluids. This also results in a lower solubility of the drug in the dispersed LSBDDS and thus causing it to precipitate (Pouton, 2006). Further, the degree of saturation of drug in a LSBDDS has an impact on precipitation as well, so the higher the concentration of drug, the less the solubility of the drug has to be reduced before initiation of precipitation.

The impact of drug precipitation on the bioavailability is believed to be dependent on the state of the precipitated drug and on the re-dissolution rate. Thus elucidation of the physical state of the precipitated drug is of great interest, when considering its influence on bioavailability. If the drug is polymorphous, the precipitated drug can have different crystalline structures with possible different dissolution rates. In addition, the drug can precipitate in a high energy amorphous state (Hancock and Parks, 2000). In order to characterize the solid state of the precipitated drug several tools can be used such as X-ray powder diffraction (XRPD) and polarized light microscopy (PLM) (Sassene et al., 2010; Seadeek et al., 2007; Wyttenbach et al., 2007).

In vivo assessment of drug precipitation is complicated and would require intubation studies in humans or animals. Therefore *in vitro* lipolysis models have been used to predict drug precipitation during digestion of LSBDDS. In recent studies, using cinnarizine and danazol as model drugs and a SMEDDS containing sesame oil, oleic acid, Cremophor RH40, Brij 97 and ethanol (20.6:15.4:45:9:10 w/w %), drug precipitation during *in vitro* lipolysis was assessed (Sassene et al., 2010; Sassene et al., in preparation). The Copenhagen *in vitro* lipolysis model was used at 5:1.25 mM bile acid:phosphatidylcholine. Drug content in the aqueous phase and in the pellet was determined at 8 time points, as depicted in Fig. 3, using cinnarizine as an example.

For both cinnarizine and danazol a continuous precipitation of drug was seen during the course of lipolysis. After 80 min the pellet was isolated and subjected to dissolution in a medium containing 5:1.25 mM bile acid:phosphatidylcholine. Fig. 4a shows that the precipitated cinnarizine has a much faster dissolution rate than the pellet spiked with crystalline cinnarizine (Sassene et al., 2010). On the contrary the precipitated danazol in Fig. 4b has similar dissolution rate as the pellet spiked with crystalline danazol (Sassene et al., in preparation).

In order to characterize the physical form of the precipitated model drugs, the pellet was analysed by XRPD and PLM. Fig. 5a and b shows diffractograms obtained using XRPD on pellet generated

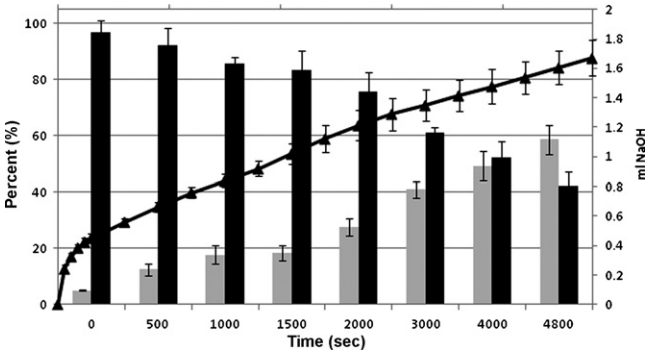

Fig. 3. Partition of cinnarizine during *in vitro* lipolysis in the duodenum step. The black bars are the percentage of cinnarizine in the aqueous phase and the grey bars show the percentage of cinnarizine precipitated in the pellet. The curve shows the addition of 1.0 M NaOH throughout the lipolysis (figure is reconstructed and modified from Sassene et al., 2010).

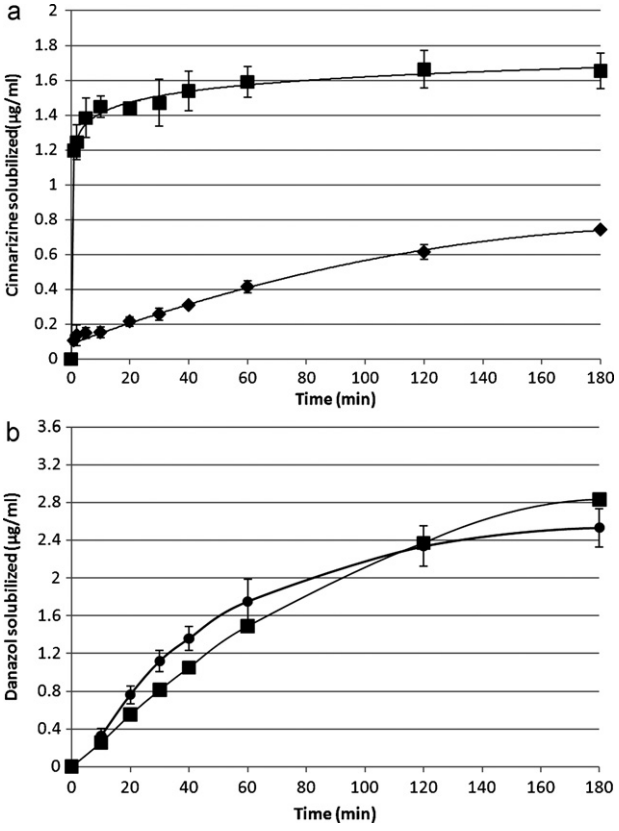

Fig. 4. (a) Dissolution rate of pellet with cinnarizine from *in vitro* lipolysis (●) and blank pellet spiked with crystalline cinnarizine (■). (n = 3) (figure is reproduced from Sassene et al., 2010). (b) Dissolution rate of pellet with danazol from *in vitro* lipolysis (■) and blank pellet spiked with crystalline danazol (●). (n = 3) (figure is reproduced from Sassene et al., in preparation).

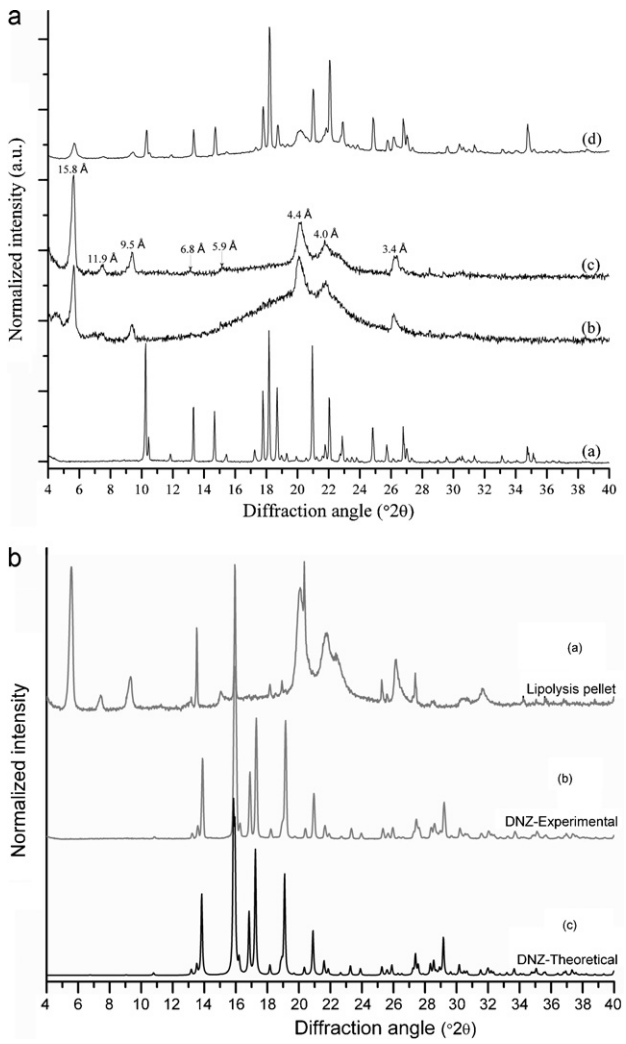

Fig. 5. (a) Diffractogram of cinnarizine obtained using XRPD. (a) Crystalline cinnarizine. (b) Pellet from *in vitro* lipolysis containing precipitated cinnarizine. (c) blank pellet. (d) Blank pellet spiked with crystalline cinnarizine (figure is reproduced from Sassene et al., 2010). (b) A diffractogram of danazol obtained using XRPD. (a) Pellet from end point of *in vitro* lipolysis containing precipitated danazol. (b) Crystalline danazol. (c) Danazol diffractogram from Cambridge structure database (CDS) (figure is reproduced from Sassene et al., 2010).

during *in vitro* lipolysis of a SMEDDS containing cinnarizine and danazol respectively.

From Fig. 5a it can be seen that cinnarizine precipitates amorphous or in a molecular dispersion during the *in vitro* lipolysis, which is indicated by the lack of peaks and presence of a large amorphous halo in sample (b) in the diffractogram, whereas danazol in Fig. 5b stays crystalline throughout the precipitation (Sassene et al., 2010, in preparation). This confirms that XRPD can be used to characterize the solid state of drug compounds precipitated during *in vitro* lipolysis of LSBDDS and differentiate between the different solid states the drug might have, without major interference from the complexity of the system.

PLM was performed on the same samples as used for XRPD and an example of PLM micrographs for the pellet containing cinnarizine is shown in Fig. 6, which also shows micrographs of crystalline cinnarizine (a), blank pellet (c) and spiked pellet (d) for comparison. As can be seen, both (a) and (d) contain rod like cinnarizine crystals whereas (b) and (c) do not. This is identical to the results obtained by XRPD diffractograms. Additionally in samples (b) and (c) birefringence caused by the calcium soaps formed during *in vitro* lipolysis was observed (Sassene et al., 2010).

These solid state characterizations were performed on the same amount of formulation (with 80% drug saturation) digested in the same dynamic *in vitro* lipolysis model with continuously addition of calcium. This indicates that the physico-chemical characteristics of a drug compound determines whether it precipitates in an amorphous or a crystalline form (Sassene et al., in preparation), however, various factors remains to be elucidated in order to understand which characteristics promote which behavior. Precipitation from the *in vitro* lipolysis model with continuous addition of calcium where the digestion of the lipids present is more controlled and linear (Zangenberg et al., 2001a) results in a linear precipitation (Fig. 3) (Sassene et al., 2010). This indicates that fast initial digestion of the formulation, which happens when calcium is present in the initiation of the lipolysis, will induce a fast precipitation of drug, which possibly could favour amorphous precipitation, as fast precipitation favours the amorphous state (Giron et al., 1997). Varying the amount of formulation added to the digestion vessel or varying the degree of saturation in the formulation might also play a role in the solid state characteristics of the precipitated drug but further studies are needed to elucidate these effects.

4. Predictivity of *in vitro* lipolysis models

A prerequisite for the usefulness of any *in vitro* model for development of drug delivery systems is that it is predictive towards the *in vivo* situation. Several studies have tried to correlate solubilization of drug in the aqueous phase during *in vitro* lipolysis to *in vivo* performance in pre-clinical trials; however, no clear cut recommendation of a single *in vitro* lipolysis model set-up to achieve *in vivo in vitro* correlation (IVIVC) has been developed so far. Most studies trying to correlate drug solubilization during *in vitro* lipolysis and *in vivo* bioavailability have not been attempting on achieving a level A correlation, but rather a rank order correlation by comparing AUC or C_{max} with amount of drug solubilized in the aqueous phase at a fixed time point (Dahan and Hoffman, 2006; Larsen et al., 2008; Porter et al., 2004a, 2004b).

Many studies have been focused on achieving a rank order correlation for LSBDDS containing either MCT or LCT. This has not always been successful (Reymond and Sucker, 1988), possibly due to the different substrate affinity of pancreatic lipase towards MCT and LCT, resulting in a faster hydrolysis of MCT and therefore a different extend of lipolysis at a fixed time point during *in vitro* lipolysis (Christensen et al., 2004; MacGregor et al., 1997). Under normal physiological conditions *in vivo*, lipids are expected to be completely hydrolysed, which complicates a direct comparison at fixed time points. These differences have been accommodated by reducing the lipid load, thereby enabling a 100% hydrolysis of LCT, at which point MCT will also reach 100% hydrolysis. This approach made it possible for Porter et al. (2004) to identify a rank order correlation between the AUC for halofantrine dosed in LCT or MCT to beagle dogs and the solubilization of halofantrine in the aqueous phase after 60 min of *in vitro* lipolysis with constant calcium level, when using 5 mg lipid pr. ml lipolysis media, but not when increasing the lipid load to 25 mg/ml. In contrast Dahan and Hoffman (2006) were not able to find a rank order correlation between the bioavailability in rats of vitamin D3 in MCT or LCT solutions and the solubilization of vitamin D3 in the aqueous phase after 30 min *in vitro* lipolysis using the same conditions as above and lipid loads of both 5 mg/ml and 25 mg/ml. This was explained by lymphatic transport of vitamin D3, but since halofantrine is also lymphaticaly transported, this cannot be the full explanation.

When LCT or MCT was incorporated into SMEDDS using Cremophor EL as surfactant, ethanol as co-solvent, and a mixture of long- or medium chain mono- and di-acylglycerides as co-surfactants, it was possible to identify a rank order correlation

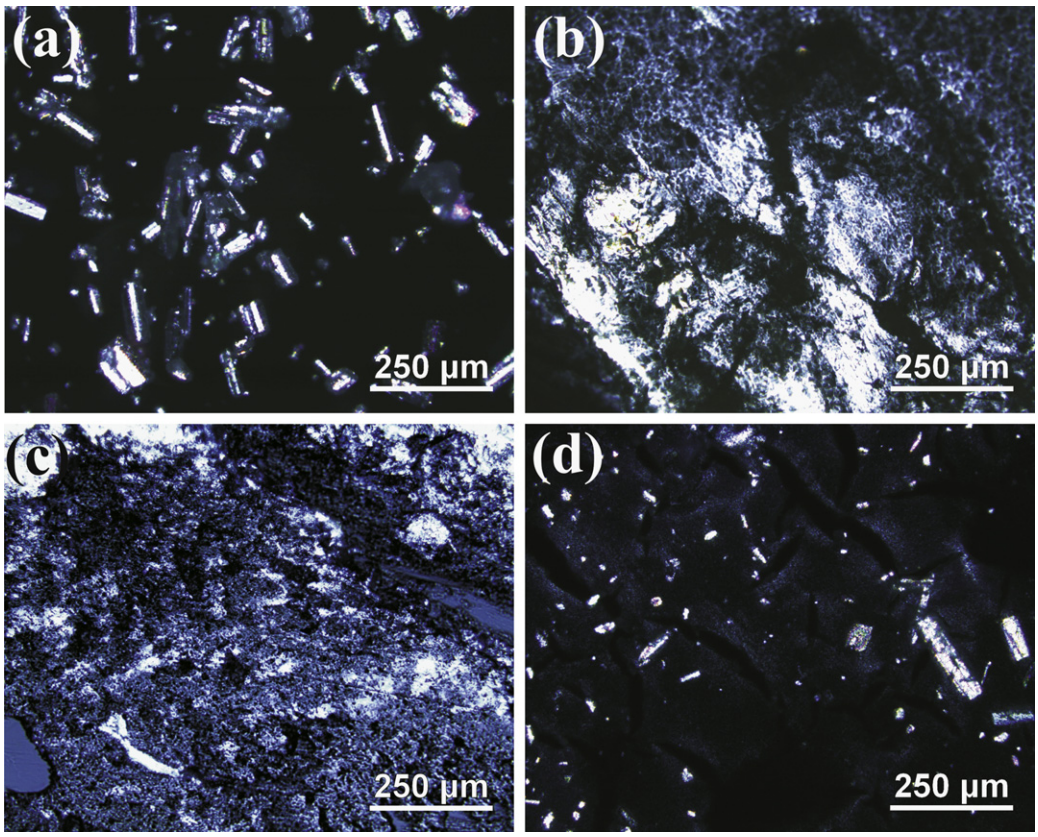

Fig. 6. PLM pictures of (a) crystalline cinnarizine, (b) pellet containing precipitated cinnarizine, (c) blank pellet, (d) blank pellet spiked with crystalline cinnarizine (figure is reproduced from Sassene et al., 2010).

between the AUC of the model drug, danazol, after dosing of the two SMEDDS to beagle dogs, and the amount of danazol solubilized in the aqueous phase after 30 min *in vitro* lipolysis with fixed calcium addition. No residual oil phase was apparent on top of the ultracentrifugation tubes, indicating 100% lipolysis of the lipid (Porter et al., 2004a).

The usefulness of the *in vitro* lipolysis model with initial addition of calcium to differentiate between two SMEDDS with either Cremophor EL and Cremophor RH40 used as surfactant, LCT as lipid and danazol as model drug, has also been studied. Cremophor EL is hydrolysed during *in vitro* lipolysis, while Cremophor RH40 is only hydrolysed to a small degree. This resulted in increased precipitation of drug during *in vitro* lipolysis when Cremophor EL was used, which corresponded to a lower AUC of danazol for the SMEDDS containing Cremophor EL, compared to the one with Cremophor RH40.

Using both solutions and suspensions of the model drug danazol in Labrafil® M2125CS the possibility of obtaining an IVIVC between bioavailability of danazol in a rat study and solubilization of danazol during lipolysis using the Dynamic model was examined. It was not possible to achieve a level A correlation, but a rank order was found at 70 min of lipolysis.

Very few studies comparing LSBDDS containing the same excipients at different ratios exists. Fatouros et al. (2008) used Neuro-Fuzzy Modelling to correlate the bioavailability of the model drug probucol with the solubilization during the lipolysis using the Dynamic *in vitro* lipolysis model. An oil solution and two self-emulsifying drug delivery systems were used, both composed of sesame oil, Maisine, Cremophor RH40 and ethanol, at slightly different ratios, resulting in particle sizes of either 45.0 ± 3.4 nm or 4.58 ± 0.84 μm. There were no differences in the bioavailability in mini-pigs of the two self-emulsifying systems, while they both were better than the oil solution. This could be predicted by the model (Fatouros et al., 2008).

Many attempts on achieving a rank order IVIVC between bioavailability and drug solubilization during lipolysis have been successful, however, a clear-cut recommendation of how to carry out the *in vitro* lipolysis study and how to use the models to identify the best LSBDDS has not yet been developed.

5. Conclusion and future perspectives

Several approaches exist towards simulating intestinal digestion, and many different analytical tools have been used to characterize the events taking place during *in vitro* lipolysis. However, the complexity of the events and the many different excipients used in LSBDDS, complicates a general understanding of the mechanisms behind and therefore also the use of these models for selection of optimal LSBDDS. Presently *in vitro* lipolysis models should be considered a tool for characterizing LSBDDS and not yet a development tool for ranking of different formulation approaches. Therefore more work is needed in order to understand and optimize intestinal *in vitro* lipolysis models. Especially development with focus on optimizing the models towards increased predictivity of *in vivo* performance of LSBDDS is needed.

It should also be considered that the gastric lipolysis is likely to be important for the digestion of some LSBDDS and therefore the development of predictive gastric lipolysis models should be prioritized, especially in combination with intestinal lipolysis models. However, this is currently hampered by the lack of availability of a suitable gastric lipase.

Currently the solubilization of drug in the aqueous phase is used as a predictor of drug absorption. In order to obtain a better evaluation of the ability of the micelles and other colloid structures

present in the aqueous phase to facilitate the absorption of drug, the *in vitro* lipolysis models can be combined with transport assays, for examples Caco-2 cells monolayers. Such a model is presently in development in our laboratory.

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