Review

Simulating the Digestion of Lipid-based Drug Delivery Systems (LBDDS): Overview of *In Vitro* Lipolysis Models

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Abstract

One of the greatest challenges in the pharmaceutical science is the improvement of oral bioavailability of poorly soluble drugs. Lately, one of the most attractive approaches has been formulation of lipid based drug delivery systems. However, the emerging popularity of these systems in the last decade has brought to light the need for efficient methods for their *in vitro* evaluation that would serve as their *in vivo* behaviour prediction tool. Because lipids are subject to lipid digestion and multiple absorption pathways *in vivo*, simple dissolution tests are not predictive enough when testing lipid based delivery systems. To assert these needs, the *in vitro* lipolysis model has been developed, utilizing pancreatic enzymes, bile and phospholipids in a temperature controlled chamber to simulate *in vivo* digestion. However, with very variable physiological conditions in gastrointestinal tract, this model has not been yet standardised and experiments vary among different laboratories. This review discusses *in vivo* events following oral application of lipid based delivery, *in vitro* lipolysis models to emulate them and their future perspectives.

Keywords: pH stat method, self-microemulsifying systems, lipid digestion, triglyceride, bioavailability prediction

1. Introduction

Efficient solubility of a drug moiety in gastrointestinal tract (GIT) fluids is often critical for its successful bioavailability following oral delivery. Due to modern drug discovery techniques, such as combinatorial chemistry and high-throughput analysis there seems to be an ever increasing number of poorly soluble drugs. Reportedly 90% of new molecular entities are considered to be poorly soluble, belonging to Biopharmaceutical Classification System (BCS) Class II (poorly soluble) and IV (poorly soluble and poorly permeable).[1] With solubility being the major differential factor between new molecular entities and drugs, pharmaceutical industry is more than ever focused on improvement of their solubility.[2] Several methods have been used to achieve solubility enhancement – formulations of amorphous solids, adjustment of pH, formation of salts, solid dispersions, nanosuspensions, cosolvent based formulations and lipid based formulations to name a few.[3,4,5,6]

Among them, lipid based delivery systems (LBDDS) have been rapidly gaining popularity in the last decade, with several successful formulations already on the market (Table 1).[7,8] LBDDS are typically composed of oil, surfactants or mixtures thereof to readily dissolve the drug prior to administration. Additionally, co-solvents can be added to the formulations.[9,10,11,12]

To distinguish between different LBDDS formulations, Lipid Formulation Classification System (LFCS) was proposed in 2000 and updated in 2006 (Table 1). Briefly, simple triglyceride, diglyceride and/or monoglyceride oil solutions are considered to be Type I formulations according to LCFS. The digestion of these formulations is considered vital for achievement of desirable bioavailability following oral administration. When these oil solutions are blended with lipophilic surfactants (HLB<12), they often gain the ability to self-emulsify, i.e. form oil-in-water emulsions upon contact with water. These Type II systems are therefore often defined as self-emulsifying drug delivery systems (SEDDS). Type IIIa

Table 1. Examples of LBDDS on the market.

Trade name	Active ingredient	Company
Sandimmune Neoral	Cyclosporine A	Novartis
Gengraf	Cyclosporine A	Abbott
Panimun Bioral	Cyclosporine	Panacea Biotec
Norvir	Ritonavir	Abbott
Fortovase	Saquinavir	Roche
Agenerase	Amprenavir	GlaxoSmithKline
Lipirex	Fenofibrate	Sanofi-Aventis
Convulex	Valproic acid	Pharmacia
Rocaltrol	Calcitriol	Roche
Targretin	Bexarotene	Novartis
Vesanoid	Tretinoin	Roche
Accutane	Isotretinoin	Roche
Kaletra	Lopinavir + Ritonavir	Abbott
Aptivus	Tipranavir	Boehringer Ingelheim

formulations are also considered as SEDDSs, but containing rather hydrophilic surfactants in comparison with Type II and additionally cosolvents. With even lesser oil content in the favour of hydrophilic surfactants and cosolvents, Type IIIb formulations typically form finer droplets, defining these systems as self-microemulsifying drug delivery systems (SMEDDS). Type IV formulations are considered to be surfactants and hydrophilic cosolvents systems entirely. While formulations with higher surfactant and hydrophilic cosolvent content have been shown to successfully incorporate a plethora of drugs, higher content of hydrophilic excipients can also be the culprit of higher precipitation risk (Figure 1).[13,14]

With lesser oil content in LBDDS formulations Type III and IV, the importance of their digestion for achievement of successful absorption is considered to dissipate. It should be noted, however, that some surfactants can also undergo digestion processes, as in the case of commonly used Labrasol, Cremophor RH40 and Labrafil M2125CS.[15,16,17]

2. *In Vivo* Digestion of Lipid Based Formulations

As shown by multiple studies, LBDDS can improve oral bioavailability of poorly soluble drugs; both BCS Class II and IV.[18,19,20,21] LBDDS are based on the concept of delivering the active pharmaceutical ingredient (API) to the GIT in a non-aqueous solution. By presenting the drug in the solubilized state, slow dissolution of drug from solid dosage forms, commonly associated with poorly water soluble drugs, is circumvented. Following gastric emptying, LBDDS are subject to GI processing, including dispersion, digestion and interaction with bile (Figure 2). This leads to transformation of LBDDS to colloidal structures such as vesicles, micelles, mixed micelles and microemulsions.[22] These structures can prevent precipitation of the drug during its passage through GIT. Additionally, lipids can prolong gastric residence time of the drug co-administered, resulting in improved dissolution at the absorption site.[23] Excipients in LBDDS can also increase

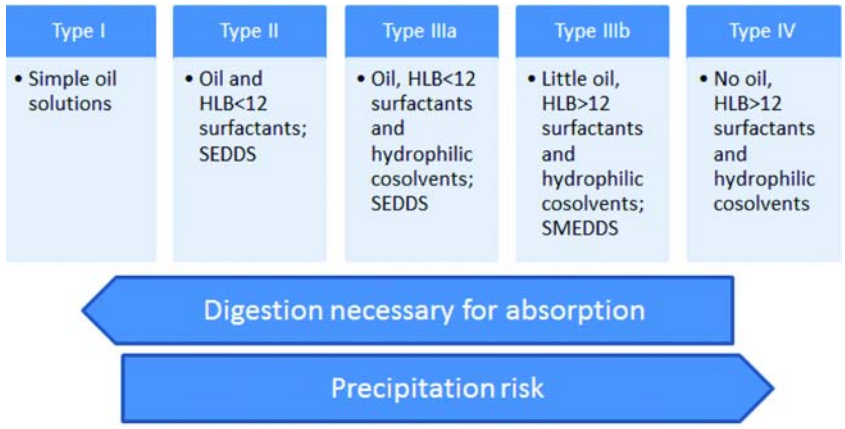

Figure 1. LCFS classification system according to Pouton.[14]

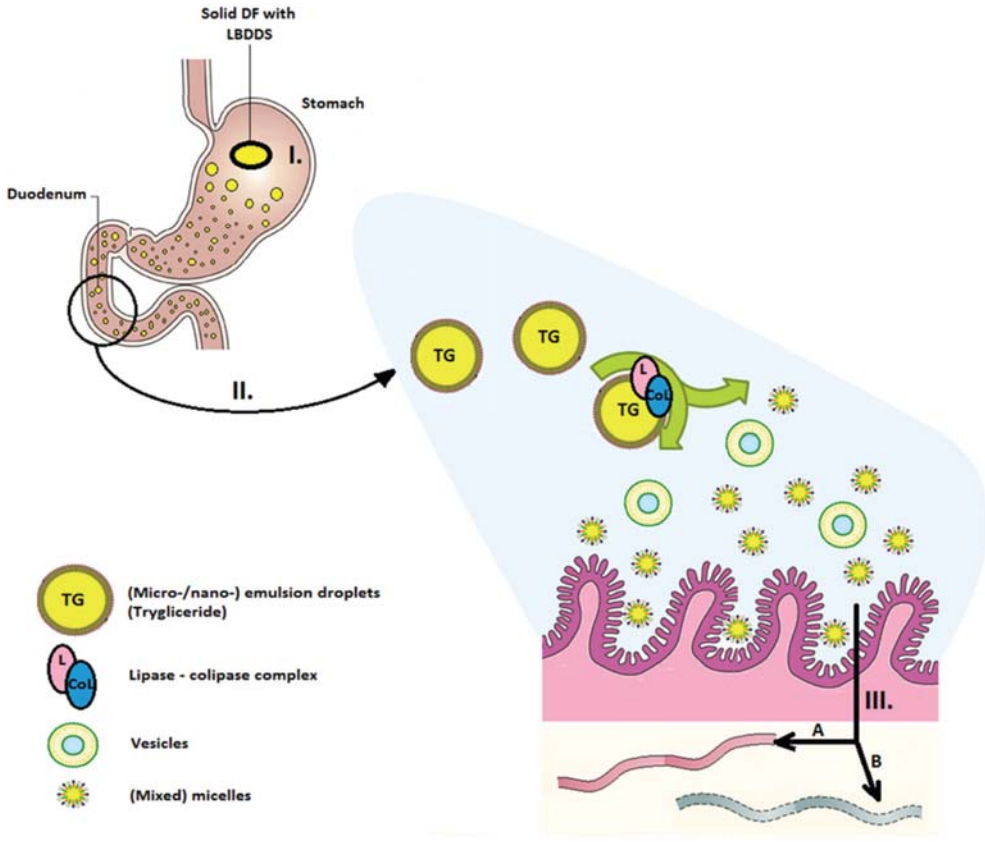

Figure 2. Schematic presentation of lipid digestion and drug solubilisation in the upper GIT. Upon ingestion, LBDDS or dietary products containing triglycerides (TG) are dispersed in the stomach, where lipids digestion is initiated by gastric lipase (I). Mechanical mixing in the stomach in combination with the amphiphilic moieties originating from the LBDDS or initial lipid digestion assists in emulsification of the lipids prior their entrance into the duodenum. The presence of exogenous lipids in the latter is the stimuli responsible for the secretion of bile fluid from the gall gladder and pancreatic fluid from the pancreas. Within the small intestine (II) the breakdown of ingested glycerides to di-glyceride, monoglyceride and fatty acid is completed by pancreatic lipase and its cofactor co-lipase. In the presence of raised bile salts concentrations, the lipid digestion products are subsequently incorporated into a series of colloidal structures (multi- and unilammelar vesicles, mixed micelles and micelles), within which co-administered drugs might residue during GI transit, thereby preventing precipitation and enhancing absorption of the drug. Upon uptake into enterocytes (III) drugs can enter either portal vein or intestinal lymphatic circulation, depending on their lipophilicity and the chemism of ingested lipids. Figure adapted from Ref. 12.

fluidity of lipid membrane, open tight junctions, interact with hydrophilic membrane domains, acting as absorption enhancers.[24] Increased intestinal permeability is of interest especially when dealing with poorly soluble drugs that are also poorly permeable, i.e. Class IV according to BCS. Furthermore, several common ingredients of LBDDS, such as Cremophors, d-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS), Labrasol and Polysorbates, can act also as inhibitors of intestinal eflux transporters, increasing the amount of the drug absorbed.[21,24,25,26] Through suppression of intestinal efflux transporter P-gp and its known interplay with CYP3A4, intra-enterocyte metabolism can be reduced.[27]

Predicting the drug absorption of the poorly soluble drug formulated in LBDDS across GIT is difficult, as it is a multi-pathway process. Once in GIT, drug is solubilized through various lipid/surfactant structures and can be subject of facilitated diffusion through the unstirred water layer to the gut wall. From there, the drug molecules can be absorbed either passively following transcellular and paracellular routes or actively with the aid of a facilitated transporter. Another possible pathway of absorption presents lymphatic transport, however significant only in the case of extremely lipophilic drugs (logP>5).[22,28] This option is considered attractive due to circumventing the portal vein, especially where pre-systemic metabolism is of concern. Indeed, bioavailability of several poorly soluble drugs loaded in LBDDS has been shown to improve due to their intestinal lymphatic absorption.[29,30] These lipophilic drugs are considered to form an intracellular association with lipid core of the chylomicron, lipoproteins created by the enterocytes. The drug-chylomicron associates are later entrapped in the Golgi apparatus and secreted from the enterocyte to the intracellular space, only to be later absorbed through porous mesenteric lymph vessels, finding their way along the lymphatic system draining to the systemic blood circulation. However, LBDDS formulation itself can also stimulate lymphatic transport of the

incorporated drug. In particular, long chain triglycerides were found to enhance lipid turnover through enterocyte and synthesis of the chylomicrons.[31,32,33]

Due to diversity of LBDDS and their complex effects that can be observed *in vivo*, simple dissolution tests do not have the predictability power to enable successful rational development of new LBDDS formulations, as they ignore lipolysis of the excipients taking place in GIT. The lipolysis of LBDDS can influence drug solubilisation and its distribution, creating the need for predictable *in vitro* models to reflect these conditions.[34] As a result, an *in vitro* lipolysis model has emerged in the last few years, mimicking *in vivo* physiological processes through use of digestive enzymes. pH stat method is probably most widely used *in vitro* analytical tool for simulation of LBDDS digestion in small intestine. This simple and rapid method is based on quantification of free fatty acid release from the sample containing lipids following lipase addition in a temperature controlled chamber.[35,36]

3. Theoretical Overview of *In Vitro* Lipolysis Model

Physical lipolysis models are based on the assumption that the amount of lipids digested in the sample equals the quantity of digested lipids, which would be absorbed by the cells in the epithelium.[37] Yet, with digestion of excipients in LBDDS, a loss in solubilisation capacity of the drug can occur. This can result in precipitation of the drug and its diminished bioavailability.[38]

In pH stat method simulating intestinal digestion, drug-loaded LBDDS is added to the temperature controlled chamber containing bile salts, phospholipids and buffer, with sodium hydroxide solution serving as a titrant (Figure 3). With the amount of bile salt and phospholipids used in the lipolysis buffer, fed or fasted state can be represented. Lipolysis is induced by addition of pancreatic enzymes, usually in the form of pancreatic extract, previously evaluated for lipase activity. Pancreatic lipase and other esterases present in lipolysis medium hydrolyse triacylglycerides and other LBDDS excipients, subsequently releasing free fatty acids. These reactions are reflected by a drop in the pH in the reaction chamber, which is immediately corrected by pH titrator unit through the addition of NaOH. Through known addition of NaOH, quantity of free fatty acids (FA) formed in reaction can be determined, as the stoichiometric ratio between them is 1:1. Addition of calcium to the lipolysis medium, either as a fixed amount prior to initiation of lipolysis or continuously through the experiment, ensures removal of liberated fatty acids from the droplet surface through formation of solid salts with FA.[36,39]

For development of successful *in vitro* lipolysis model, physiological representation of reaction volume is also important. Following oral administration, LBDDS will disperse in gastric fluid, given that the formulation does not entail of enteric coating. Gastric volume is considered to be merely 50 ml when fasted, with additional volume possibly coming from the liquid ingested with LBDDS (usually up to 250 mL). However, unlike solids, ingested liquids continuously pass through stomach.[40] The total volume of intestinal fluids, measured by magnetic resonance imaging, is considered to be 45–319 ml for fasted and 20–156 ml for fed state.[41] The initial volume of the digestion medium used in *in vitro* lipolysis experiments is

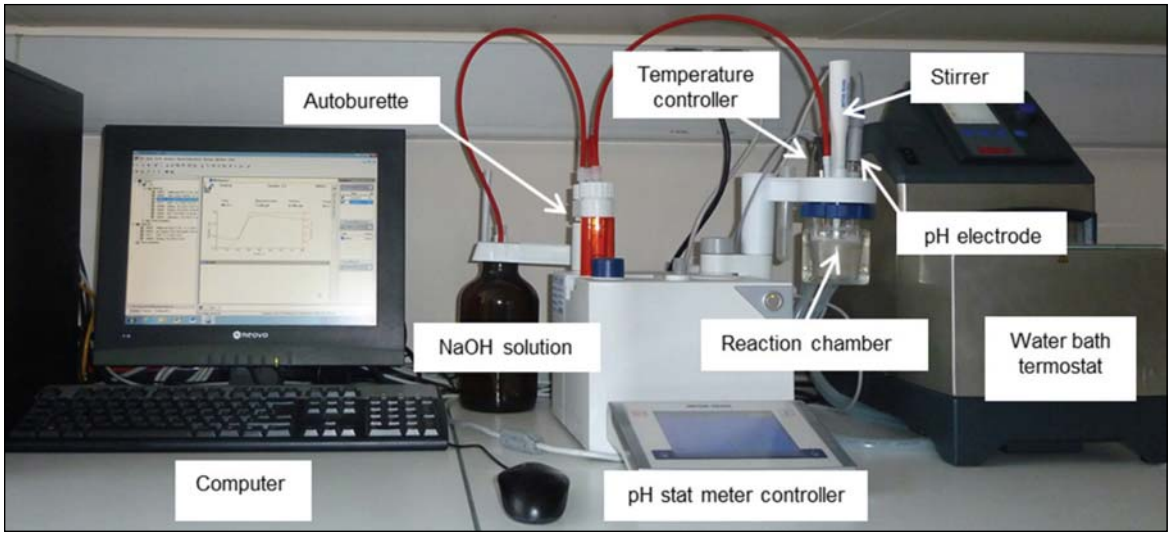

Figure 3. Example of an experimental pH stat setup. Lipid digestion is initiated by addition of pancreatic enzymes to the temperature controlled (37°C) vessel, containing bile salts and phospholipids to simulate intestinal fluid, stirred at a fixed speed. The process of lipid digestion causes liberation of fatty acids, resulting in decreased pH. The pH drop is quickly measured by pH-stat meter and titrated with NaOH solution. Through known addition of NaOH, the extent and kinetic of lipid hydrolysis can be determined.

commonly 10–40 mL for lipolysis with fixed addition of calcium, while in dynamic lipolysis experiments volumes up to 300 mL have been used.[40]

For a more complete representation of *in vitro* digestion, simulation of other GIT parts than the small intestine is needed, unless the final formulation includes enteric coat. While both gastric and intestinal *in vitro* digestion models have been successfully developed, the majority of the work has been done on the intestinal model, thereby our review will focus on the latter.

3. 1. Pancreatic Enzymes/Lipase

Human pancreatin is composed of several digestive enzymes, in particular lipase, protease and amylase, as well as other excretes of exocrine cells. The use of appropriate type and amount of pancreatic lipase in a pH stat model is of a great importance. Results of *in vitro* digestion simulation are influenced by source, isolation and purification process of pancreatin. While it is possible, yet more expensive, to use purified pancreatic lipase (of animal or human origin) in place of pancreatin to diminish some of the variation, other pancreatic enzymes should be added to prevent the inhibition of lipolysis (See chapter: Bile). As lipase is a sensitive material, its catalytic activity can decrease upon long storage or high temperatures. It is therefore recommendable that for each conducted experiment a fresh batch of lipase is prepared and assayed for catalytic activity. Lipase activity is usually determined through digestion of a fixed amount of standardized lipid, commonly triolein or tributyrin, under standardized conditions (temperature, pH, ionic strength, stirring speed). Crude porcine pancreatin with enzyme activity of 1000 tributyrine units (TBU)/ml is commonly used.[36,37] 1 TBU is equivalent to 1 μmol butyric acid released in 1 minute per 1 g of enzyme.[42] The lipase activity assay is also described by both European Pharmacopoeia (in "Pancreas powder" monograph) and American Pharmacopoeia (in "Pancrelipase" monograph).[43,44]

Pancreatic lipase hydrolyses one triglyceride molecule to one 2-monoglyceride and two fatty acids, with higher affinity to medium chain triglycerides vs. long chain triglycerides.[45] However, it is considered that in *in vivo* conditions, there is an abundance of lipase produced by human pancreas, essentially digesting all lipid ingested. To reflect that conditions, high levels of lipase are used in *in vitro* studies.[37]

3. 2. Bile

The presence of lipids in duodenum facilitates not only the secretion of pancreatic fluids, but also bile. As another important ingredient of small intestine aiding in emulsification and digestion of lipids, use of bile in simulated small intestinal fluid (SSIF) seems therefore logical and desirable.[46] Bile is composed of several ingredients, namely bile acids, bile lipids (endogenous phospholipids and cholesterol ester) and minerals, with concentrations dependent on dietary state and the results vary among studies. Four major bile acids represented are cholic, deoxycholic, chenodeoxycholic and litocholic acids including their corresponding glycine and taurine derivatives. Because of its inconsistent composition, use of bile extract in SSIF often yields variable results. It is also recommendable for the bile extract to be filtered prior to use, as potential insoluble matter particles present interference in the analysis of colloidal systems. Use of pure individual bile acids or their mixture in pH stat method is therefore common, although more expensive than bile extract and less representable of *in vivo* conditions.[36,37,47]

Another concern with use of bile salts in *in vitro* lipolysis is their ability to displace lipase from oil-water interface. As lipase's access to lipid droplet is hindered, lipid digestion process is also supressed. This can be prevented by addition of colipase, thereby using crude pancreatin extract as opposed to isolated porcine lipase.[48]

Phospholipids are important part of biliary excretions, although they can be ingested as food constituents, as well. The first are commonly termed as "endogenous phospholipids", while the later are known as "exogenous phospholipids". Their concentration in the GIT *in vivo* is therefore not only dependent on the fed/fasted state, but also on the type and the amount of food ingested. Because of their ability to form mixed micelles as lipophilic surfactants, they can aid in solublilisation of poorly soluble drugs.[36] Bile salts to phospholipids ratio can *in vivo* vary from as little as 1[49] to 16[50] in the fed state to 4.5[51] to 39[52] in the fasted state. As a compromise to these great physiological variations, ratio of 4 is usually used in *in vitro* lipolysis models. The reported amount of bile salts present in intestinal fluids itself also varies from 1.5 ± 1.8 mM in fasted conditions[53] to 16.19 ± 1.51 mM in fed conditions.[54]

Phospholipids, when present in the form of mixed micelles with bile salts, can also inhibit lipolysis through inhibition of pancreatic lipase-colipase complex. This can be reversed by addition of phospholipase A_2, enzyme present in pancreatin that can hydrolyse phosphatidylcholine to lyso-phosphatidylcholine.[55,56]

3. 3. Minerals

Physiologically, digestive juices of human small intestine contain approx. 5–30 mM calcium. While the presence of calcium in SSIF is considered crucial in enabling adequate activity of pancreatic lipase, its amount should be chosen carefully as it can both facilitate or supress lipid digestion rate. Probably the most widely discussed role of calcium in *in vitro* lipolysis is the formation of Ca-soaps with long chain FA on the surface of lipid droplets, essentially causing long chain FA to be removed from the droplets' surfaces and to precipitate. The process mimics ab-

sorption of FA that occurs *in vivo*. In this way, FA no longer obstructs access of the pancreatic lipase to emulsified lipid droplets. However, aforementioned insoluble calcium soaps have been shown also to reduce bioavailability of these long chain FA digestion products. In samples containing lipids and anionic surfactants, calcium could also promote droplet flocculation, shielding parts of the droplet surface. The chance of lipase coming in contact with droplet surface is more challenging, slowing the lipolysis process.[37]

So far, two different approaches for the addition of the calcium to the lipolysis medium were described. The Copenhagen model,[17,39,64–67] also named "dynamic *in vitro* lipolysis model", uses a continuous addition of calcium (0.045–0.181 mmol/min), through which the lipolysis rate could be simultaneously controlled.[36] Conversely, other models use a single fixed addition of calcium (5 mM) before the start of the process, as seen in models developed at Monash[16,57–61] and Jerusalem university,[62,63] among several others. This approach allows use of a single burette machine, as a second burette for calcium is not needed. However, the fixed addition of calcium results in rapid initial lipolysis, almost completed in the first 5–10 min. While the three models named all use the same lipase source (porcine pancreatin), concentration of bile in digestion medium (5 mM) and bile acid to phospoholipid ratio (4), other parameters vary (bile and phospholipid species, pH, initial volume), making comparisons between them difficult.[36]

Apart from calcium, digestive juices of human small intestine can also contain other mineral ions, such as sodium, potassium, sulfates, phosphate and bicarbonates. These minerals are considered of importance in digestion due to their role in electrostatic interactions. To mimic ionic strength of physiological digestive juices, a monovalent salt is usually used in SSIF (NaCl or KCl, approx. 150 mM).[10]

3. 4. pH

The pH value in the GIT spans from 1–3 in the stomach, increasing to 5.8–6.5 in duodenum, followed by pH 7–7.5 in small intestinal tract. The difference between intralumial pH values of fed and fasted state is slim and is usually not enforced in the *in vitro* lipolysis model.[47] The pH chosen for an *in vitro* digestion model is a compromise between the pH required for the optimal lipase activity and pKa of the free FA formed during lipolysis of the sample, as only ionised FA can be titrated by sodium hydroxide. It is considered that pancreatic lipase exhibits best activity at neutral pH values, with maximum at 8.5. While pKa values of long chain FA are above 8, they are expected to decrease when in contact with bile salts and calcium; their pKa in mixed bile salts is suggested to be 6.5. Therefore, pH values from 6.5 to 8.5 are usually used in *in vitro* lipolysis experiments simulating intestinal conditions.[36,37]

Assessment of the total amount of FA released during lipolysis experiment can be achieved either by performing the "back titration" at the end of the experiment or by quantification of FA in the sample with HPLC. The concentration of the titrant should also be carefully selected. Too low NaOH concentration relative to the substrate amount will dilute the media and subsequently bile acid present. This could affect the solubilisation of the drug. However, too high NaOH concentrations could negatively attribute to experimental error.[36]

3. 5. Stirring and Sampling

Since LBDDS are not always miscible with lipolysis media, reaction media is stirred at a constant speed to disperse the LBDDS and avoid formation of a two phase system. This enables homogenous samples to be taken out for further analysis with the purpose of determinating the amount of lipids already digested and the amount of solubilised vs. precipitated drug. As mentioned, at various time points, aliquots are withdrawn from the lipolysis media, followed by immediate addition of a lipase inhibitor. 4-bromobenzeneboronic acid[64] or orlistat[68] has been used to terminate lipolysis in the samples. This treatment is, of course, not considered to be necessary for samples taken after the lipolysis endpoint.

Since the digested mixture is rather complex, containing buffer, enzymes, lipids, surfactants and drug, samples are usually ultracentrifuged to differentiate between different structures or phases formed in the digested mixture,[16,64,69] tough filtration has also been used.[70] Several phases are observed upon ultracentrifugation: pellet, micelle phase and oil phase,[36] with some authors additionally dividing micelle phase on inter phase and aqueous micellar phase (Figure 4).[37] The formation of various phases and their ratio is dependent on composition of studied LBDDS and the time point of obtaining the sample during the lipolysis. For instance, not all LBDDS will form oil phase, especially in the case of systems containing surfac-

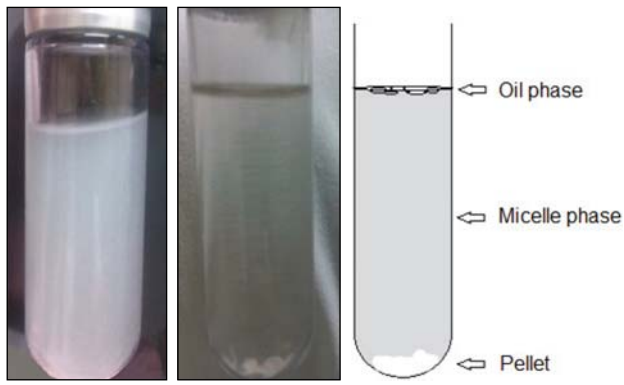

Figure 4. Lipolysis sample upon digestion (left), sample following centrifugation (middle), schematic illustration of phases in the digested mixture (right).

tants.[37] Even with oil phase initially present, its volume will reduce in time with on-going lipolysis. Conversely, the pellet phase, containing calcium soaps of FA and possibly precipitated drug, continues to grow in time. Micelle phase essentially contains colloidal structures, such as mixed micelles, unilamelar and bi-lamelar vesicles (with solubilised drug, if present).[65]

To detect background lipolysis, blank lipolysis experiments can also be carried out by conducting a lipolysis experiment with no tested formulation. Doing so is recommended, as phospholipids in lipolysis media can be hydrolysed during the experiment to lyso-pospholipides. Impurities in the bile and crude pancreatic extracts can also be the subject to lipolysis. By performing the mentioned blank experiments detection of falsely higher NaOH volumes used to titrate the liberated fatty acids can be omitted.[37]

4. Further Evaluation of Lipolysis Samples

Following centrifugation, the digestion phases of LBDDS lipolysis samples can be further characterised using different techniques. Researchers typically focus on concentration of the drug incorporated in the LBDDS, bile acids, lipolysis products and the size of the aggregates found in the micelle phase.

Since dissolution of the drug in the aqueous phase is considered critical in successful absorption, assessment of its concentration in the aqueous digestion phase is of particular interest. Aqueous phase concentration of bile acids, phospholipids, and different lipid species through the lipolysis process has also been monitored, as well as micellar radius. Christensen discovered that hydrodynamic radius of micelles increases as a function of time, as lipolysis products in aqueous phase interact with micelles composed of bile and phospholipids initially present.[66] Thereby obtained mixed micelles are expected to facilitate drug absorption due to their ability to incorporate drug molecules and carry them from the intestinal lumen through unstirred water layer to apical enterocyte membrane, where absorption takes place. The formation of the various lipid species such as fatty acids, mono- and ditriglycerides has also been monitored during the lipolysis, but more knowledge about their impact on colloidal formations is needed.[64,69] Not only lipid composition can change during lipolysis, though phospholipase A_2 present in pancreatic extract hydrolyses phospholipids to lysophospholipids also. Since their surfactant properties are not identical, this can also affect solubilisation of the drug during lipolysis.[40]

The sediment of lipid digestion sample, also known as pellet phase, can also provide valuable information. The presence of the precipitated drug is of a particular interest. The drug can precipitate from LBDDS *in vivo* due to several reasons. For instance, some LBDDS excipients can be subject of the hydrolysis, such as those containing ester bonds. If the drug is less soluble in these hydrolysis products, precipitation can occur. The dilution of the LBDDS in GIT itself can be the reason of precipitation, with the loss of hydrophilic excipients, primarily co-solvents, being the underlying reason. In this case, precipitated drug is the result of its lesser solubility in dispersed LBDDS. Moreover, the concentration of the drug in LBDDS is also important factor. The closer it is to the saturation point, the higher is the susceptibility of the drug to precipitation. Avoiding *in vivo* precipitation of the drug is desired especially because re-dissolving poorly soluble drug, usually loaded in LBDDS, can be proven difficult, resulting in diminished bioavailability. This is hardly surprising, as it is also the reason for the incorporation of the drugs BCS Class II and IV into LBDDS in the first place.[13,14,71] However, the solid state of the precipitated drug and the size of formed particles should also be taken into consideration. If the drug precipitates in a form of nanometer sized particles, the dissolution and absorption of drug may not be considerably decreased as the formation of nanosuspensions is one of the approaches used to enhance the solubility of drugs that are poorly soluble in water as well as lipid media.[4,72] The drug could also precipitate in larger particles of amorphous (preferred for fastest re-solubilisation) or crystalline state, with some drugs exhibiting different polymorphous crystalline states with different dissolution profiles.[73,74] The solid state of drugs can be assessed using different methods, such as X-ray powder diffraction (XRPD) and polarized light microscopy (PLM)[75] and differential scanning calorimetry (DSC).[76]

Although lipolysis samples are usually ultra-centrifuged prior to their further evaluation, as mentioned previously, they can be also characterised without separating them into phases. Whole samples are especially used in identification of the colloidal structures formed during lipolysis, namely with Cryogenic transmission electron microscopy (Cryo-TEM) and Small-angle X-ray scattering (SAXS) techniques.

Microscopic imaging with Cryo-TEM offers the ability to visualise structures present in lipolysis samples through snapshots taken following rapid cooling/freezing of the samples on the grid. Hence, only structures smaller than the grid can be observed – ca. 150 nm in thickness, with up to 1μm distance between the sides. Nevertheless, Cryo-TEM has been proven useful in morphological characterization of the colloidal structures present before, during and after the lipolysis process. With the progress of lipolysis experiment, reduced number of oil droplets in lipolysis samples of SNEDDS was observed, correlating with the expected digestion of the oil droplets during the lipolysis. With progression of lipolysis experiment, unilamellar vesicles and bi-lamellar vesicles also occurred. After 30 min, however, only a few oil droplets and unilamel-

lar vesicles, but no bi-lamellar vesicles were observed. Micelles, on the other hand, were present throughout the experiment.[65] The comparison of different LBDDS lipolysis samples under Cryo-TEM could therefore provide us with information about possible colloidal phases that occur upon digestion. These colloidal phases are of high importance for increased solubilisation capacity of colloidal aqueous phase for poorly water soluble drugs administered with LBDDS.

SAXS as a method enables the characterization of liquid crystalline phases formed during lipolysis experiment. It has been employed to study LBDDS lipolysis both with continuous and fixed addition of calcium. During SNEDDS digestion, initial lamellar phase is transformed to hexagonal phase due to hydrolysis of the LBDDS. To avoid disturbances during sampling, a real time monitoring can be performed, either by mounting flow-through lipolysis cell on top of SAXS or by coupling lipid digestion directly to high intensity synchrotron SAXS.[36,67,77]

5. *In Vivo* Predictability And Future Perspectives

When studying novel LBDDS by means of *in vitro* lipolysis, researchers commonly search for the highest aqueous micellar solubilisation and the lowest sediment recovery among the tested formulations following ultracentrifugation of the samples in the hopes of the better *in vivo* bioavailability. This could be misleading, as the model takes into the account only pre-enterocyte processes, overlooking other possible *in vivo* events, such as active transport through the gut wall, saturable enzyme degradation, efflux transporters, lymphatic transport, and hepatic first pass metabolism. It is therefore not surprising, that no clear-cut recommendation has been yet developed on how to conduct *in vitro* lipolysis model in order to achieve *in vivo – in vitro* correlation (IVIC). Moreover, instead of level A IVIC correlation, researchers usually attempt to achieve rank order correlation. This is done by comparing *in vivo* AUC or C_{max} with content of the solubilised drug in the aqueous phase at pre-determined time points.[36]

But even achievement of rank order correlation is not always successful. For LBDDS composed of either MCT or LCT, greater affinity of pancreatic lipase for MCT might result in greater extent of MCT hydrolysed at pre-determined time point compared to LCT. Porter managed to achieve rank-order correlation in the case of halofantrine by reducing the lipid load per volume of lipolysis media. In a similar experiment though, using *in vitro* lipolysis under the same conditions but comparing the results with rat model instead of beagle dogs, Dahan and Hoffman could not achieve a rank order correlation for vitamin D3. Since both halofantrine and vitamin D3 undergo lymphatic transport, there must be other reasons for this difference.[59,62]

Ideally, *in vitro* lipolysis model would allow for rational development of LBDDS, differentiating between formulations with different ratios of excipients. This has not been much studied yet, although Fatouros did manage to predict bioavailability of probucol in two self-emulsifying systems with same ingredients but different ratios and simple oil solution. Using neuro-fuzzy networks to correlate between models, bioavailability in mini-pigs of probucol from SMEDDS was comparable to SNEDDS, but for both better than in simple oil solution. This was predicted by dynamic *in vitro* lipolysis model.[78]

In the cases of formulations that undergo lymphatic transport pathway following administration in a significant portion, cultured intestinal epithelial cells can provide valuable information on intestinal lymphatic transport. CaCo–2 cell line, commonly used for permeability predicting, can also be used to assess influence of LBDDS on likelihood of the drug to be incorporated in lipoproteins; coupling with *in vitro* lipolysis is therefore of great interest, expected to be further researched in the future.[79–81]

While the pH-stat method is increasingly being used to monitor lipid digestion, several experimental factors can impact the rate and extent of lipid digestion in a great manner, such as lipase concentration, bile extract concentration, $CaCl_2$ concentration, and droplet size. To enable more straightforward comparison of the results from different studies, standardisation of the conditions for pH stat testing has been proposed.[82]

Lastly, does *in vitro* lipolysis really outperform conventional dissolution studies generally accepted for biopharmaceutical characterization of solid dosage forms? As the active ingredient in LBDDS is already presented in solution, equipment for dissolution testing can be used for the preparation of dispersions for further characterisation, rather than monitoring the dissolution itself. Although Ali et al found that classical dissolution studies are better suited for predicament of the investigated drug performance in the digestion media over *in vitro* lipolysis, other authors argue that the strength of *in vitro* lipolysis lays particularly in its ability to simulate colloidal structures following lipid digestion that affect drug distribution and consequently its absorption.[83,84] This is especially important when evaluating LBDDS systems that can undergo digestion before they deliver the active ingredient dissolved within.

6. Conclusion

Lipid based drug delivery systems offer effective bioavailability enhancement of poorly water-soluble drugs. Due to complexity of the events taking place following oral administration of such systems, successful development of *in vivo* predictive *in vitro* digestion models is challenging, although it would be of a high value. Further research of these models is much needed to achieve *in vi-*

tro lipolysis model that would allow for rational development of LBDDS systems, as the latter is now focused predominantly on drug solubilisation efficiency of the systems. In the meantime, characterization of colloidal phases obtained through *in vitro* lipolysis models can still offer helpful insight on *in vivo* fate of the drug loaded in LBDDS.

7. References

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Povzetek

Enega največjih izzivov na področju farmacevtskih znanosti predstavlja izboljšanje biološke uporabnosti peroralno vnesenih učinkovin, ki so slabo vodotopne. Z namenom zagotavljanja tega cilja raziskovalci v zadnjem času veliko pozornosti namenjajo razvoju na lipidih osnovanih dostavnih sistemov. Z uveljavljanjem teh sistemov in širjenjem njihove uporabe v zadnjih dvajsetih letih se je razkrila tudi nujnost razvoja novih *in vitro* metod, ki bi omogočile učinkovito napovedovanje njihovega obnašanja *in vivo*. Slednje otežuje predvsem dejstvo, da se na lipidih osnovani dostavni sistemi v prebavnem traktu vključujejo v proces prebave, ki lahko spremeni njihove lastnosti. Z vgradnjo v na lipidih osnovani sistem lahko vplivamo tudi na pot absorpcije učinkovine iz prebavnega trakta. Ker enostavni testi sproščanja učinkovine *in vitro* nimajo ustrezne napovedne moči za *in vivo* obnašanje teh dostavnih sistemov, se razvijajo novi modeli *in vitro* lipolize. Slednji posnemajo fiziološke pogoje prebave z izpostavitvijo testiranega vzorca pankreatičnim encimom, žolču in fosfolipidom v termostatirani reakcijski posodi. Zaradi spremenljivosti pogojev v prebavnem traktu model *in vitro* lipolize še ni standardiziran, kar se odraža v veliki variabilnosti rezltatov, pridobljenih med različnimi laboratoriji. V preglednem članku smo se osredotočili predvsem na predstavitev fizioloških procesov, ki sledijo peroralnemu vnosu na lipidih osnovanih sistemov ter pregledu in perspektivnosti obstoječih modelov *in vitro* lipolize.