In vitro lipolysis assay as a prognostic tool for the development of lipid based drug delivery systems

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Frau Sandra Klein
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Gutachter

1. Prof. Dr. rer. nat. Karsten Mäder

2. Prof. Dr. rer. nat. Markus Pietzsch

3. Prof. Dr. rer. nat. Thomas Rades

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Content

List of abbreviations

API	active pharmaceutical ingredient
BCS	biopharmaceutical classification system
BA	bile acid
BS	bile salt
CEH	carboxyl ester hydrolase
CMC	critical micellar concentration
DAG(s)	diacylglyceride(s)
DG(s)	diglyceride(s)
ER	endoplasmatic reticulum
EQ.	equation
FA(s)	fatty acid(s)
FaSSIF	fasted state simulated intestinal fluid
FaSSGF	fasted state simulated gastric fluid
FeSSIF	fed state simulated intestinal fluid
FeSSGF	fed state simulated gastric fluid
GC	gas chromatography
GI	gastrointestinal
GRAS	generally regarded as safe
HGL	human gastric lipase
HLB	hydrophilic-lipophilic balance
HPL	human pancreatic lipase
HTS	high throughput screening
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
LC	long chain
LCS	lipid classification system
LCT	long chain triglyceride
LD	laser diffraction
LFCS	lipid formulation classification system
LP	lipoprotein
MAG(s)	monoacylglyceride(s)
MC	medium chain
MCT	medium chain triglyceride
MG(s)	monoglyceride(s)
PCS	photon correlation spectroscopy

PDI	polydispersity index
PEG	polyethylene glycol
P-gp	P-glycoprotein
PLRP2	pancreatic lipase-related protein 2
PL	phospholipids
PPL	porcine pancreatic lipase
PSD	particle size distribution
rDGL	recombinant dog gastric lipase
SD	standard derivation
SE	sucrose ester
SEDDS	self-emulsifying drug delivery systems
SIF	simulated intestinal fluid
SLS	sodium lauryl sulphate
SEDDS	self-emulsifying drug delivery systems
SMEDDS	self-microemulsifying drug delivery systems
SMEDDS	self-nanoemulsifying drug delivery systems
TAG(s)	triacylglyceride(s)
TBU	tributyrin unit
TIM	TNO intestinal model
THL	Tetrahydrolipstatin (Orlistat)
TG(s)	triglyceride(s)
USP	United States Pharmacopeia

I Introduction

The advances in automated synthesis, combinatorial chemistry and innovative high-throughput screening have led to an increasing number of drug candidates which are characterised by high molecular weight, unchanged H-bonding properties, higher lipophilicity and, hence, poor aqueous solubility[1]. The proportion of new chemical entities (NCEs) with poor solubility in water is estimated to be more than 40% as these drug candidates exert their pharmacological action in or at biological membranes or membrane associated proteins[2]. This challenges drug delivery institutions in industry or academia to develop carrier systems for the optimal oral and parenteral administration of these drugs.

According to the Biopharmaceutical Classification system (BCS), drugs are classified into four categories based on their aqueous solubility and ability to permeate biological membranes (Figure 1)[3]. A drug is considered as 'highly soluble' when the highest dose strength is soluble in < 250 ml water over a pH range of 1-7.5. A compound with 90% oral bioavailability is considered as 'highly permeable'. Drugs with reasonable membrane permeability but poor aqueous solubility belong to class II of the BCS. For these drugs, the dissolution is often the rate-limiting step of absorption. The bioavailability from conventional drug delivery systems like tablets might be unacceptable but an appropriate formulation design can help to improve the dissolution step and, thereby, enhance their oral bioavailability. For drugs with poor solubility and poor permeability (belonging to class IV of the BCS), the best option to improve their bioavailability might be to go back to the lead optimisation phase of drug discovery and to modify their structures in order to obtain appropriate physicochemical properties[4]. A suitable formulation may improve the bioavailability of class IV drugs, however they are likely to be compromised by their poor membrane permeability. Nevertheless, several APIs belonging to class IV of the BCS are available on the market.

Proper formulation is of key importance to establish a successful product for the oral administration of a poorly soluble compound. If the bioavailability of the drug is recognised to be formulation-dependent at an early stage it is desirable to have a strategy for maximising absorption as soon as possible. The use of poor formulations in early animal efficacy studies can lead to an overestimation of the likely human dose possibly compromising future development of the candidate drug. If poor formulations are used in early toxicity studies, the toxicity might be underestimated due to limited exposure resulting from low bioavailability[5,6]. The need to reduce the attrition rates has resulted in increased efforts of drug formulation and especially formulation characterisation in the early development stage. *In vitro* tests are necessary that predict the *in vivo* performance of a formulation in order to have a rational guidance during the screening phase.

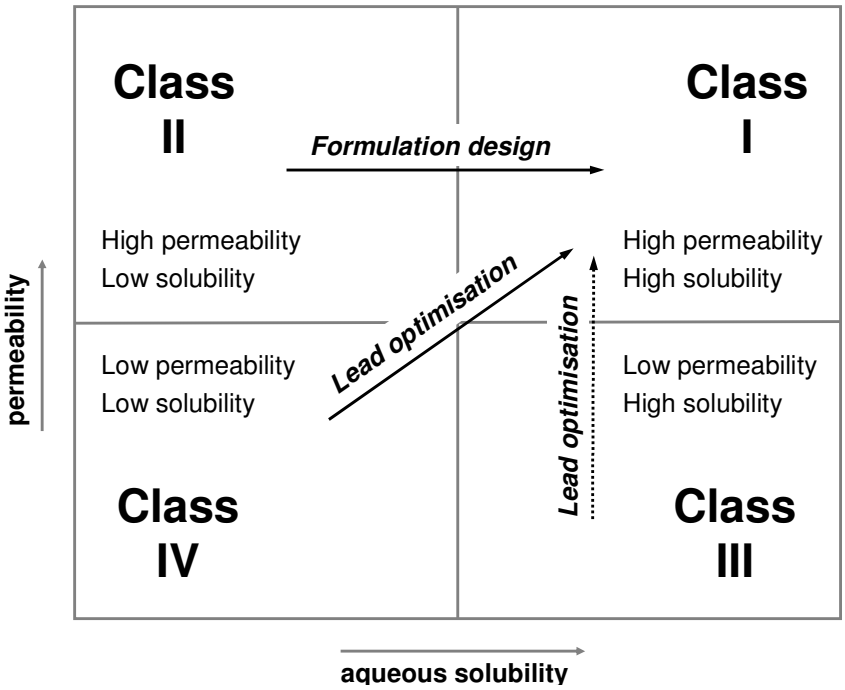


Figure 1 *Biopharmaceutical classification system (BCS) of drugs (modified from Pouton et al [5]). If a class II drug can be maintained in a solubilised state in the gut lumen one can achieve an absorption profile comparable to that of a Class I compound. The best solution to improve the bioavailability of BCS Class IV and III is to go back to the lead optimisation phase of drug discovery to modify their chemical structures and select a candidate with more appropriate physicochemical properties.*

The present thesis is focused on lipid based drug delivery systems as one formulation strategy to increase the oral bioavailability of poorly soluble compounds. These formulations provide the ability to present the drug in solution in the GI tract, thereby circumventing the dissolution step. Although there has been an increasing interest in the utilisation of lipid formulations, their development is largely empirical. This can be attributed to a lack of understanding of the interactions between API, lipidic compounds and gastrointestinal fluids.

The most significant issue to consider when formulating poorly water-soluble drugs is the threat of drug precipitation in the lumen of the GI tract. Drug precipitation can be caused by a loss of solubilisation capacity due to dilution in GI fluids or digestion of the lipid vehicle.

Since most of the excipients which are used in lipid formulations are potential substrates for enzymatic digestion, it is evident that fundamental knowledge about the fate of the lipid formulation and the administered API in the GI tract is needed.

The process of digestion can increase or decrease the capacity of drug solubilisation. In this regard, the effect of digestion on drug solubilisation is not easy to predict. Nevertheless, several studies indicate that the use of long chain glycerides instead of middle chain glycerides in a formulation might minimise the loss of solubilisation capacity on digestion, particularly for highly lipophilic drugs[7-9].

A further issue that has gained importance is the variability of oral bioavailability which is associated with different bile and enzyme levels. This ‘food effect’ is especially critical for compounds with a narrow relation between efficacy and toxicity.
Within this work, lipid formulations differing in their lipid composition and physicochemical properties were investigated in terms of drug loading capacity and their ability to maintain the solubilisation capacity upon dilution and simulated lipolysis. Moreover, the impact of the triglyceride source and the polarity on the performance of the formulation was examined.

In the recent years, the development of more polar self-emulsifying drug delivery systems comprising large amounts of surfactant has gained increasing interest. Upon the beginning of this work, little was known about the impact of surfactant digestion on the solubilisation capacity of a formulation. For that purpose, the fate of a range of pharmaceutical relevant surfactants during pancreatic enzyme-mediated digestion was the focus of the second part of this work. The studies were expanded on sucrose esters as interesting excipients with various capabilities in the field of oral drug delivery systems.

The gastric step of lipid digestion has been mainly neglected during formulation development so far. Upon the beginning of the experimental work, there was a lack of knowledge in this field. In order to close this gap, an *in vitro* gastric digestion assay was developed.

The overall aim of this work was to contribute to a better understanding of the mechanisms that occur upon ingestion of lipid formulations. This finally leads to a more rational development of more ‘biorobust’ drug delivery systems with increased bioavailability and decreased variability. Thereby, predictive *in vitro* tests are capable to decrease costs and attrition rates during the development phase.

II Scientific background

1 Formulation strategies for poorly soluble drugs

In the recent years, many efforts have been made to develop appropriate drug delivery systems for the oral administration of poorly soluble drugs. The main principles of these formulation strategies involve an increase of the dissolution rate by reduction of the particle size of the drug or formulation of the API in solution as amorphous system or lipid formulation.

However, there exists no golden standard formulation strategy for each new compound emerging from the chemical laboratories. Things are even more complicated since poorly soluble molecules are often different in their characteristics. The group of Bergström and co-workers have sub-classified poorly soluble drugs into two types of molecules: 'grease ball' and 'brick dust' compounds[10]. Grease ball molecules represent highly lipophilic compounds with a high log P (> 4) and a low melting point (< 200 °C). These compounds cannot form bonds with water molecules, thus, their solubility is limited by the solvation process. By contrast, brick dust molecules are usually compounds with a high melting point (> 200 °C) and a low log P (< 2). The solubility of the compounds in water is restricted due to strong intermolecular bonds within the crystal structure.

The oral bioavailability of 'grease ball' molecules can be increased if appropriate formulation strategies are used to overcome or improve the solvation process. Appropriate technologies could involve the inclusion into cyclodextrines, application of micelles and lipid-based formulations. By contrast, brick dust molecules are not only poorly soluble in water, but also in oils. For that reason, the formulation as lipid-based drug delivery system is an inappropriate approach due to low API loading. For brick dust molecules, the development of nanosuspensions and amorphous systems has shown to be successful formulation concepts which allows improved drug dissolution and absorption[10,11].

The following chapter summarises the most important galenical approaches that were applied in order to improve oral bioavailability of poorly soluble compounds in the recent years.

1.1 Salt formation

Salt formation is a simple way to modify the properties of a drug with ionisable functional groups to overcome undesirable features of the parent drug[12]. Today, automated salt and polymorph screening has become standard in early drug discovery stage.

Salts can be formed with drugs that are weakly acidic or weakly basic. Scientists have to choose the most appropriate salt with care regarding the formation of hydrates, hygroscopicity, polymorphism and chemical stability. But even when stable salts can be prepared, formulators often face some challenges: The inactive counter ions often represent

about 20-50% of the weight of the drug substance[13]. Thereby, formulations require a higher drug loading. In addition, salts may precipitate out into their free acid and base forms in the GI fluid after oral administration. The precipitates might not re-dissolve rapidly due to their very low aqueous solubility. In some cases pH-modifiers in solid dosage forms are used to minimize the conversion of salts to their respective acid or base upon storage and due to pH effects in the GI fluid[14,15].

1.2 Inclusion in cyclodextrins

Cyclodextrins are cyclic oligosaccharides which are produced by enzymatic conversion of starch. The molecular structure of these glucose derivatives generates a hydrophilic exterior surface and a non-polar cavity interior. Cyclodextrins can interact with molecules of appropriate size by forming inclusion complexes. These non-covalent complexes offer a variety of advantages: They increase the aqueous solubility, stability and bioavailability of drugs. Furthermore, labile compounds can be protected against enzymatic or chemical degradation and undesirable taste and odour can be masked effectively. These benefits have led to a number of marketed products with cyclodextrins for parenteral, oral, ophthalmic, and nasal drug delivery.

The outcome of a cyclodextrin formulation is highly dependent on the physicochemical properties of the drug to be formulated. The structure of the guest molecule has to fit inside the cyclodextrin cavity and the complexation conditions have to be optimised[16-18]. Therefore, the use of cyclodextrins is restricted to a limited number of compounds.

1.3 Micronisation

A simple approach to increase the dissolution of poorly soluble drugs is the reduction of its particle size. According to the laws of Noyes-Whitney and Nernst-Brunner, the dissolution rate is directly proportional to the surface area of the drug (eq. 1)[19]:

$$\frac{dx_D}{dt} = \frac{A \cdot D}{\delta}\left(c_s - \frac{x_D}{V}\right) \qquad \textit{eq. 1}$$

A is the effective surface area of the solid drug, D is the diffusion coefficient of the drug, δ is the effective diffusion boundary layer thickness adjacent to the dissolving surface, c_s is the saturation solubility of the drug under luminal conditions, x_D is the amount of drug which is already dissolved and V is the volume of the dissolution medium Small particles dissolve faster than large ones because the surface area is increased. Micronisation using colloid or air-jet mills has been used for many years to obtain particles in the range of 2-5 µm[20]. Micronisation of poorly soluble drugs increases the dissolution rate of the compound due to the increase in surface area, but does not change the saturation solubility. At very low saturation solubilities, the achieved increase in dissolution rate cannot lead to a sufficiently high bioavailabilities[2].

1.4 Nanosuspensions

Nanosuspensions for pharmaceutical application are very finely dispersed solid drug particles in an aqueous vehicle. The particle size distribution of the solid particles in nanosuspensions is usually < 1 µm, with an average particle size range of 200-600 nm [2]. Drug crystals reduced in size from 10 microns to 100 nm particles generate a 100-fold increase in the surface area to volume ratio. The relation between saturation solubility and particle size is described in the Ostwald-Freundlich equation (also known as the Gibbs-Thomson and as the Kelvin equation)[21]. According to the equation, a tremendous decrease of the particle size (below the µm range) leads to an increase of the saturation solubility. Hence, downsizing of a drug particle, particularly to the submicron level, has a profound impact on the bioavailability of the molecule[22] due to simultaneous enhancement of both the saturation solubility and the dissolution rate.
Meanwhile, many manufacturing processes are described in the literature.

1.4.1 High pressure homogenisation

The process of high-pressure homogenisation includes three steps in general: Firstly, drug powders are dispersed in a stabiliser solution to form a pre-suspension. Secondly, the pre-suspension is homogenised by the high pressure homogeniser at low pressure as a kind of pre-milling. Finally, the main homogenisation is performed at high pressure for 10-15 cycles until the desired particle size is achieved. During homogenisation, drug particles are fractured by high shear force, the collision of the particles against each other and cavitation,[11,23].
This approach is applicable for a wide range of APIs. Furthermore, an aseptic production of the nanosuspensions is easily possible.
One approved product is Triglide® (Fenofibrate, Skye Pharma): By means of high pressure homogenisation, the solubility of Fenofibrate could be increased resulting in pharmacokinetics that are now less dependent from food effects (comparable absorption under fed and fasting conditions). This allows patients to take the drug at any time, thereby improving compliance and simplicity for the patients[24,25].

1.4.2 Milling method

Another technique is the patented milling procedure by Elan: Using this technique, drug and aqueous surfactant solution are milled in special ball pearl or jet mills until the desired particle size of typically less than 2 µm is achieved[26]. The NanoCrystal® particles of the drug are stabilised against agglomeration by surface adsorption of selected stabilisers. The result is an aqueous dispersion of the drug substance that behaves like a solution. In many cases, a secondary process such as spray drying is necessary to prepare the product for the final manufacturing of a solid dosage form. The first approved product incorporating the NanoCrystal® technology was the re-developed immunosuppressant Rapamune® (Sirolimus)

by Wyeth. The old formulation was an oral solution in bottles or sachets which required refrigeration storage and mixing with water or juice prior to administration. The new tablet developed using NanoCrystal® technology provides patients with more convenient administration and storage in comparison to the Rapamune® oral solution[22,26,27].

1.4.3 Microprecipitation or controlled precipitation

Utilising the precipitation approach, the API is firstly dissolved in a solvent. Then the solution is mixed with a miscible antisolvent (usually water) in the presence of surfactants. Rapid addition of the drug solution into the anti-solvent leads to sudden supersaturation of the drug in the mixed solution and generation of an ultrafine crystalline or amorphous drug suspension. A drawback of this method is the toxicity of non-aqueous solvents used[11].

1.4.4 Emulsion and microemulsion method

In general, there are three methods to fabricate API nanosuspensions by the emulsion method:

a) particle precipitation by evaporating low-medium boiling point solvents with negligible water solubility (such as chloroform, acetone, methylene chloride)
b) by a quenching technique using partially water miscible solvents such as ethyl acetate or benzyl alcohol
c) by an extracting technique using supercritical CO_2 as extraction agent.

The main drawback of the described methods is the high concentration of undesired surfactants and residual solvents[11].

1.4.5 Microprecipitation-High Pressure Homogenisation (Nanoedge®)

The Nanoedge® process developed by Baxter Healthcare Corporation is a combination of microprecipitation and high-pressure homogenisation. At first, the drug is dissolved in an organic solution. This drug solution is then added to a second solvent, in which the API is not soluble, containing stabilisers under high speed agitation. During diffusion of the miscible solvent to the second solvent microparticles precipitate. At the same time, the surfactants absorb to the freshly prepared microparticles. Then this pre-suspension is homogenised under high pressure for several cycles[11,23].

1.4.6 Melt emulsification method

The melt emulsification method was traditionally used for the preparation of solid lipid nanoparticles. As a first step, the drug is added to an aqueous dispersion containing solubilisers. Then the suspension is heated up above the melting point of the API and homogenised to form an emulsion with the melted liquid drug. This emulsion is further homogenised in a high pressure homogeniser at temperatures above the melting point of the API. Finally, the emulsion is cooled down and the drug particles precipitate. However, the main drawback of this technique is that only few APIs are suitable for this method[11].

1.5 Solid dispersions

Solid dispersions are systems in which the drug is dispersed in solid water-soluble matrices either molecularly or as fine particles. Various manufacturing methods for solid dispersions have been reported in the literature e.g. solvent-based techniques like spray drying or lyophilisation and melt extrusion. The most commonly used carriers for solid dispersions are polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), hydroxy-propyl methyl cellulose (HPMC), hydroxypropyl cellulose (HPC), hydroxypropyl methyl cellulose phthalate (HPMCP), polyacrylates and polymethyl acrylates, urea and organic acid derivatives[28,29].

Basically, solid dispersion systems can be divided into six different categories: Eutectics, solid solutions, amorphous precipitations in a crystalline carrier, complex formations, glass solutions and glass suspensions[28-30]. The dissolution rate of a poorly water soluble drug in a solid dispersion is increased by

a) increasing the surface area as a result of a reduction in drug particle size up to the molecular level and the impediment of aggregation,
b) improving wettability by appropriate selection of the carrier system,
c) enhancing the solubility of the drug by the formation of a supersaturated solution.

Moreover, transformation of the crystalline drug to the amorphous state upon solid dispersion formulation increases the dissolution rate since no lattice structure has to be disrupted for dissolution. Amorphous drugs are, however, thermodynamically unstable and tend to re-crystallise in time. In contrast to the popularity and the promising results of the solid dispersion strategy, only few marketed products rely on this concept e.g. Kaletra® (Lopinavir /Ritonavir, Abbott), or Gris-PEG® (Griseofulvin, Novartis). The reasons for this discrepancy are difficulties in incorporating the amorphous drug into the final dosage form, scale-up of manufacturing process, stability of the drug and vehicle and costs[28-30].

1.6 Lipid formulations

It is known that the oral bioavailability of poorly soluble drugs with 'grease ball' characteristics can be improved if co-administered with high fat meals[31-33]. Hence, formulators began to utilise lipids as vehicles for drug candidates to enhance drug solubilisation in the GI tract. Early approaches to formulate poorly soluble drugs included the use of simple solutions and suspensions of the drug in either single digestible lipids or blend of lipids. Another option was to use oil-in-water emulsions after incorporation of the API in the oily phase. In the recent years, more complex systems like self-emulsifying and self-microemulsifying drug delivery systems (SEDDS/SMEDDS) have entered the stage[34-48].

The main advantage of lipid formulations is that they are able to present and maintain the drug in dissolution throughout its way through the GI tract.

1.6.1 Classification of lipid formulations

Nowadays, the term lipid formulation includes a variety of systems with different physicochemical properties. D.Small has classified lipids based on their interaction with bulk water and their behaviour in the air-water surface[49]. In 2000, Pouton introduced the Lipid Formulation Classification System (LFCS) as a framework in order to identify critical performance parameters[50]. In 2006, a further formulation type was added[5]. The LFCS divides lipid formulations into four classes according to their composition and the possible effect of dilution and digestion on their ability to prevent drug precipitation (Table 1).

Type I formulations represent simple mixtures of triglycerides or mixed mono- and diglycerides. These are safe food substances, classified as GRAS by the regulatory agencies and do not represent a toxicological risk to the formulators[50]. The liquid formulations can be easily filled into gelatine capsules. Upon ingestion, poor initial dispersion can be expected. These systems need to be digested *in vivo* in order to generate more amphiphilic lipid digestion products and to promote drug transfer into the colloidal aqueous phase[47].

However, oils often have a low solvent capacity, hence, drugs with high potency or high solubility in oil are the main target for type I formulations.

Type II formulations or SEDDS represent isotropic mixtures of oil (TG or mixed MG and DG) and lipophilic surfactants (HLB < 12). The inclusion of the surfactant enables an improvement of the solvent capacity, thus promoting emulsification. Upon contact with gastrointestinal fluids, the formulations form polydisperse o/w-emulsions. This type of formulation is likely to retain its solvent capacity after dispersion. Digestion of the oily components will lead to an intercalation of the lipolytic products into endogenous colloidal structures.

Type III formulations or SMEDDS represent mixtures of oils, hydrophilic surfactants (HLB > 12) and co-solvents, such as ethanol, propylene glycol or polyethylene glycol. Type III formulations are further divided into subtypes A and B representing high (up to 80%) and low (< 20%) oil content, respectively. Upon contact with aqueous fluids, they form very fine dispersions (typically < 250 nm), commonly referred to as microemulsions. Microemulsions are characterised by their spontaneous formation and thermodynamical stability.

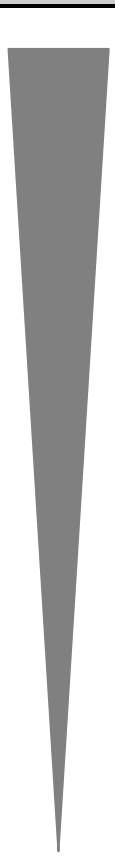
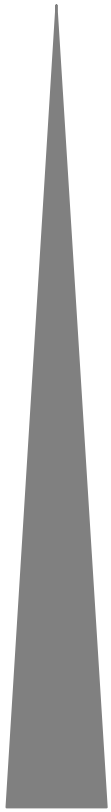
The water soluble components of the formulation will tend to part from the oil during dispersion and become dissolved in the aqueous phase. This might result in a loss of solvent capacity leading to drug precipitation. These formulations have the advantage to form colloidal structures by themselves and are, therefore, much less dependent from endogenous factors like bile flow or fat digestion.

SMEDDS formulations have shown to improve oral bioavailability for several APIs. The most famous marketed SMEDDS formulation is the re-formulated immunosuppressant Cyclosporin A Sandimmun® Optoral or Neoral® (Novartis). This formulation comprises a mixture of MG, DG and TG as lipid phase, Cremophor® RH 40 as hydrophilic surfactant, propylene glycol and ethanol as co-solvents and tocopherol as antioxidant[51]. When introduced to aqueous media, it spontaneously forms a transparent, thermodynamically stable dispersion with particle sizes of below 100 nm. The old Sandimmun® formulation formed a coarse emulsion after dispersion in water. The bioavailability of Cyclosporin A from this formulation was highly dependent from endogenous factors like fat digestion and bile flow[52]. The improved dispersion characteristics of Sandimmun® Neoral have been suggested to be responsible for the increased absorption and reduction in inter- and intrapatient variability in bioavailability[53,54].

The distinction between SEDDS and SMEDDS is commonly based on the composition and the appearance of the resulting dispersions. SEDDS formulations typically provide opaque dispersions with particle sizes > 250 nm, whereas SMEDDS form optically clear or opalescent dispersions with particle sizes of typically < 250 nm. However, a rigorous evaluation of the presence of true microemulsions rather than emulsions with very small particle sizes is rarely attempted[47].

Type IV formulations are oil-free systems based on surfactants and co-solvents. The co-solvent facilitates the dispersion of the surfactant. They often provide good solvent capacity for many drugs and disperse into micellar solutions. In analogy to type III formulations, dilution often leads to a loss of solvent capacity. Type IV formulations are useful for drugs that are hydrophobic but not lipophilic. The high content of surfactants may cause local irritations in the GI tract especially if the drug has to be administered on a chronic basis. An example of a type IV formulation is the formulation of the HIV protease inhibitor Amprenavir (Argenase®, GSK) which contains TPGS, PEG 400 and propylene glycol[55].

Table 1 *The lipid formulation classification system according to Pouton including their respective characteristics*

Type	*Composition*	*Characteristics*	lipophilicity	Significance of dilution	Significance of digestion
I	100% oils (Mono-, Di- and Triglycerides)	Non-dispersing oils			
II	40 – 80% oils 20 – 60% surfactants (HLB < 12)	SEDDS without water soluble components			
III-A	40 – 80% oils 20 – 40% surfactants (HLB > 12) 0 – 40% co-solvents	SEDDS/SMEDDS with water soluble components			
III-B	< 20% oils 20 – 50% surfactants (HLB > 12) 20 – 50% co-solvents	SMEDDS with water soluble components & low oil content			
IV	0 – 20% surfactants (HLB < 12) 30 – 80% surfactants (HLB > 12) 0 – 50% co-solvents	Oil-free formulations based on surfactants & cosolvents			

1.6.2 Excipients used in lipid formulations

As broad as the range of lipid formulations is, as broad is the group of excipients which can be used in the design of an adequate lipid based drug delivery system.

Lipids are a diverse range of compounds for which no agreed definition exists. According to Christie, lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds[56].

D. Small has developed a physicochemical-based system to classify lipids (including surfactants) based on their interaction with bulk water and their behaviour at the air-water interface. According to this system, one distinguishes between polar and non-polar lipids. Lipids belonging to the class of non-polar lipids are insoluble in bulk water and do not spread to form a monolayer on the surface. Waxes and sterol esters of long-chain fatty acids are typical members of this class.

Polar lipids have a certain surface activity and form stable or unstable monolayer. There are, in general, three classes: Polar lipids of class I are insoluble in bulk water but will spread at the interface to form stable monolayer. Triglycerides, non-ionised long-chain fatty acids and cholesterol are members of this class. Polar lipids of class II are insoluble in water but they

undergo lyotropic mesomorphism to form swollen liquid crystalline structures. Typical representatives of this class are 2-monoacylglycerides and phospholipids. The group of class IIIA lipids contains soluble amphiphiles forming micelles in aqueous systems and unstable monolayer at the interface. At low water concentration, they form liquid crystalline structures, whereas at high water concentrations, above their respective critical micelle concentration, they form micelles. Examples of this class are lysophospholipids, fatty acid soaps and aliphatic surfactants.

Conjungated or free bile salts, saponines and other water soluble compounds with bulky aromatic ring systems belong to class IIIB lipids. Members of this class form micelles but they do not form liquid crystals.[49,57]

The main problem is that many pharmaceutical excipients are multi-component mixtures of different classes of the classification system by Small, thus complicating the classification of the excipient.

2 Lipid digestion and its impact on drug absorption

2.1 Biochemistry of fat digestion

Lipids play an important role in determining the desirability and palatability of many food products, as well as being an important source of energy and other nutritive substances (e.g. vitamins A, D, E and K, ω-3 fatty acids, phytosterols and carotinoids)[58]. The fats in the human diet constitute about 40% of the energy intake in the western world. The quantitatively most important lipids are triacylglycerides which may amount to 100 g per day or more[59]. Dietary fats are characterised by very poor aqueous solubility which impedes their direct absorption. Hence, degradation of the fats by enzymes of the gastrointestinal tract is necessary. The healthy human digestive system is able to digest TG and absorb them with an efficacy of 95%[59,60].

In the mouth, food is mixed with saliva and physically broken down to smaller pieces by chewing. Thus, the surface area is increased and a food bolus is formed. The impact of lingual lipases secreted by von Ebner's and lingual serous glands within the mouth has been discussed a lot in the literature, but it is now in general agreement that lingual lipases are more important in rodents and human infants than in healthy human adults[58,61,62].

After the food bolus is swallowed, it rapidly passes down the oesophagus into the stomach where it is mixed with acidic digestive juice. In the stomach, lipids are crudely emulsified by shear forces (propulsion, grinding, retropulsion) and emulsifying properties of other nutrient ingredients (e.g. partially hydrolysed proteins)[63]. The presence of lipids in the stomach leads to the secretion of gastric lipase from the chief cells lining the gastric mucosa. This enzyme hydrolyses exclusively triacylglycerides (TAGs) with prevalence on the sn-3 position and has no activity on phospholipids or cholesteryl esters[61,64]. In contrast to other digestive lipases,

gastric lipase requires no cofactors or bile salts for its lipolytic activity[61,64,65]. Furthermore, it is resistant to the proteolytic action of pepsin[65]. In an acidic milieu (pH-optimum: 4.5-5.5[66]), gastric lipase initially degrades up to 30% of the TAG to diacylglycerides (DAGs) and fatty acids (FAs) as main products[67,68]. These lipolytic products further stabilise the oil droplets. The initial fat digestion in the stomach is important for an efficient lipolysis in the intestine. The release of FAs in the stomach stimulates the release of cholecystokinin which in return stimulates the secretion of pancreatic lipase and bile in the duodenum[69]. Furthermore, FAs facilitate the binding of pancreatic lipase-colipase binding to the substrate[70]. Thus, for healthy adults, the primary role of gastric fat digestion is to facilitate efficient pancreatic lipolysis by promotion of emulsification and activation of pancreatic lipases. Remarkably, for patients with pathologic pancreatic insufficiency, lipolysis by pre-duodenal enzymes can compensate the lipolytic activity permitting digestion and absorption of 50-70% of the dietary fat[71].

The lipolysis is completed in the upper intestine. The pre-homogenised chyme is released to the duodenum and subsequently mixed with bile and pancreatic juice which are already mixed at the level of the Vater ampulla before they enter the duodenum. Pancreatic juice contains further digestive enzymes including trypsinogen, chymotrypsinogen, elastase, carboxypeptidase, α-amylase, phospho-lipase A, pancreatic lipase, and colipase[72]. Gall bladder bile consists of conjungated bile acids (70-150 mmol/l), phopsholipids (21-45 mmol/l), cholesterol (7-15 mmol/l) and other components such as bile pigments and inorganic salts[73,74]. Bile acids are natural biosurfactants present in the gut lumen and play an essential role in both, the digestion and the absorption of lipids. Bile salts have a flat planar structure. The common steroidal nucleus forms the hydrophobic side and the hydroxyl groups and the ionic head group (glycine or taurine) forms the hydrophilic side. About 26% of the total bile acids are conjungated with taurine and 74% are conjungated with glycine[75].
In the upper small intestine the dietary fat is further emulsified by a complex cocktail of amphiphilic species including bile salts, phospholipids, lipolytic and other digestive products from the stomach[76]. The emulsification is important because human pancreatic lipase (HPL) can only act at the oil-water interface. However, bile salts can also inhibit pancreatic lipolysis: *In vitro* experiments have shown that high concentrations of bile salts restricts the adsorption of pancreatic lipase to emulsion interfaces[77]. In order to activate HPL, a co-factor called co-lipase is necessary. Co-lipase is a non-enzymatic protein co-factor which is secreted by the pancreas as a pro-co-lipase. Trypsin cleavage after the five position creates the active form[78,79]. Co-lipase facilitates the action of human pancreatic lipase in two ways: Firstly, co-lipase binds to an oil-water-interface and acts like an anchor for the pancreatic lipase which could otherwise be easily desorbed by bile salts[80]. Pancreatic lipase binds to its cofactor and has now access to its substrate. The catalytic site of the enzyme contains a serine-histidine-aspartic acid triad[81] which is imprisoned by an overlaying polypeptide chain. This ‘lid’ is rearranged by conformational changes induced by binding at oil-water interfaces[82]. Co-lipase

stabilises the lid in an open configuration via hydrogen bonding with the alpha-helical surface loop[78,79].

Pancreatic lipase-colipase hydrolyses triacylglycerides (TAGs) and diacylglycerides (DAG) with prevalence on the sn-1 and sn-3 ester bonds. Thus, major products of the lipolysis are 2-monoacylglycerides (MAG) and fatty acids (FA). However, it is worth noting that 2-MAGs undergo slow and non-enzymatic isomerisation at alkaline pH values resulting in 1-MAGs which are in turn available for hydrolysis[76].

In addition to gastric and pancreatic lipases, a number of other lipolytic enzymes operate within the GI-tract. This includes phospholipase A_2 which cleaves the ester bond at the 2-position of phospholipids and carboxylester hydrolase which cleaves cholesteryl ester to free cholesterol[72].

Undigested, non-polar lipids like TAG and DAG form the virtual core of the emulsion particle, whereas polar lipolytic products (MAG and FA) accumulate at the surface (Figure 2). The removal of the lipolytic products is crucial for the efficacy of fat digestion. The mechanism includes complex changes in the phase behaviour that occur as lipolytic products are diluted or incorporated in intestinal fluids. Patton and Carey as well as MacGregor et al. described the production of swollen, multilamellar liquid crystalline structures formed by MAG and FA[83,84]. Lamellar structures were evident in the presence of medium chain digestion products whereas more viscous cubic phases were identified in long chain lipid containing systems. In the presence of low bile salt concentration, the lamellar phases produced at the surface of the oily droplet separate to form large, multilamellar and unilamellar vesicles. In the presence of sufficient amounts of BS and PL, lipolytic products are abstracted from the oil-water interface and incorporated into unsaturated micellar structures. On further dilution with intestinal fluids, the presence of mixed micelles and unilamellar vesicles with intercalated lipolytic products is dominant[9,85-87]. These colloidal species serve as a kind of transporter to the apical brush border membrane of the enterocytes.

The brush-border membrane of enterocytes is separated from the bulk aqueous phase in the intestinal lumen by an unstirred fluid layer. This unstirred water layer (UWL) is poorly mixed with the bulk phase in the intestinal lumen and represents the intestinal diffusion barrier. Solute molecules have to diffuse across this barrier to gain access to the brush border membrane. The solubility of FAs in aqueous media is extremely low, hence, only few molecules would pass this layer. In contrast, micellar solubilisation greatly enhances the number of molecules that are available for uptake by the enterocytes[72,88-90] .

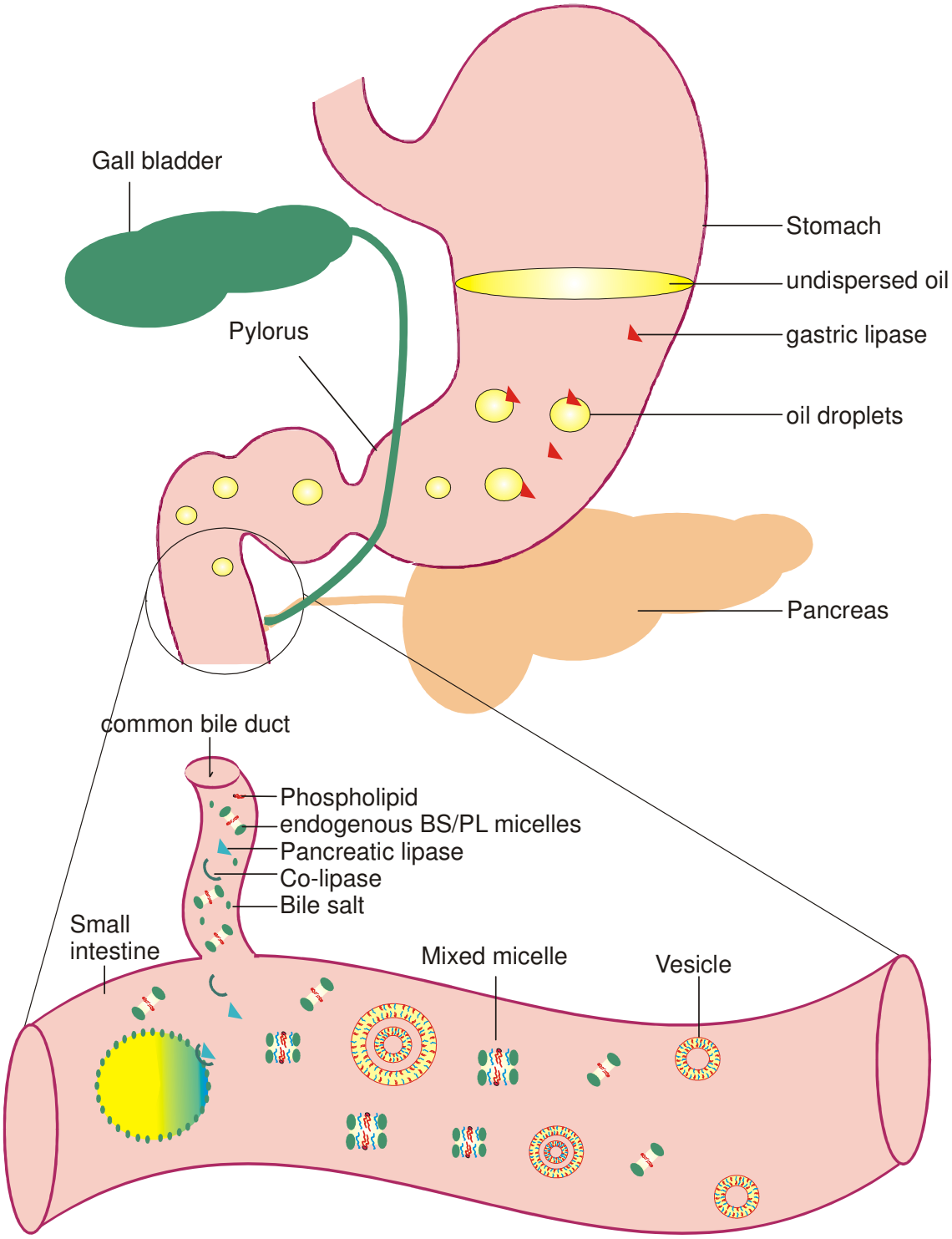


Figure 2 *Schematic representation of lipid digestion in the stomach and in the small intestine. Modified from the work by Porter et al.* [9] *In the stomach, initial lipid digestion is catalyzed by gastric lipase. Furthermore, oil is dispersed by shear forces and emulsifying properties of other nutritional components. In the upper small intestine, lipids are further emulsified by bile. Pancreatic lipase-colipase binds at the o/w interface and hydrolyses TAG to MAG and FA. During digestion, a variety of liquid-crystalline structures emerge (visualised by blue and green colour at the surface of the oil droplet). The lipolysis products are subsequently incorporated into colloidal structures including mixed micelles, multilamellar and unilamellar vesicles and transported to the enterocyte membranes where absorption takes place.*

Until today, it is not completely understood how lipolytic products are absorbed by the enterocytes. However, micelles are not believed to be absorbed intact across the brush border membrane[9,90,91]. It has been suggested that the acidic microclimate of the UWL facilitates micellar dissociation[92]. FAs and MAGs can then be absorbed across the apical membrane by simple passive diffusion or carrier mediated transport. By now, multiple potential apical transporters have been identified including FA binding protein (FABP), CD36, FA transporter (FAT), FA transport protein 4 (FATP4), scavenger receptor BI, GP330 and caveolin[9,89]. Carrier-mediated uptake is believed to be dominant if lipid concentrations are low, while passive transport takes place at higher FA concentrations.
Alternatively, transfer from micelles to the brush border membrane may occur directly by a collisional mechanism or micelles might undergo vesicular-mediated uptake[9].
In addition to transport proteins facilitating the passage of compounds across the apical membrane, several transporters have been identified that efflux material back to the intestinal lumen following absorption. Most of these efflux transporters belong to the ATP binding cassette (ABC) superfamily of proteins. Perhaps the most famous of this family is P-glycoprotein (P-gp). P-gp is able to bind and transport a broad range of drug substances and thereby affects the bioavailability of these compounds. Lipidic excipients, especially ethoxylated lipids and surfactants, have been shown to inhibit drug efflux by P-gp at least *in vitro* and potentially enhance oral bioavailability *in vivo*[93-101].

Following the uptake into enterocytes, lipid digestion products can cross the enterocytes by diffusion across the cytosol alone or by association with intracellular lipid-binding proteins[102]. MAG and FA can be re-synthesised to triglycerides via two different pathways: The 2-monoglyceride (2-MG) pathway associated with the smooth endoplasmatic reticulum (ER) or the α–glycerol-3-phosphate pathway (G3P) associated with the rough endoplasmatic reticulum. The lysolecithin absorbed by the enterocytes can be re-esterified in the smooth ER to form lecithin. Cholesterol can be transported out of the enterocytes as free cholesterol or as esterified cholesterol. The enzyme responsible for the esterification of cholesterol to form cholesterol ester is acyl-CoA cholesterol acyltransferase. The re-assembled triglycerides, lecithin, cholesterol, and cholesterol esters are then packaged into lipoproteins (LP). The intestine produces two major classes of lipoproteins: chylomicrons and very low density lipoproteins (VLDLs). The LPs are exported from the enterocytes via exocytosis and enter the lamina propria containing capillaries and central lacteal (lymph vessel). LPs preferentially cross the highly permeable lymphatic endothelium. Drug molecules can also enter the systemic circulation either through uptake into the portal vein or via the mesenteric lymphatic system (Figure 3).

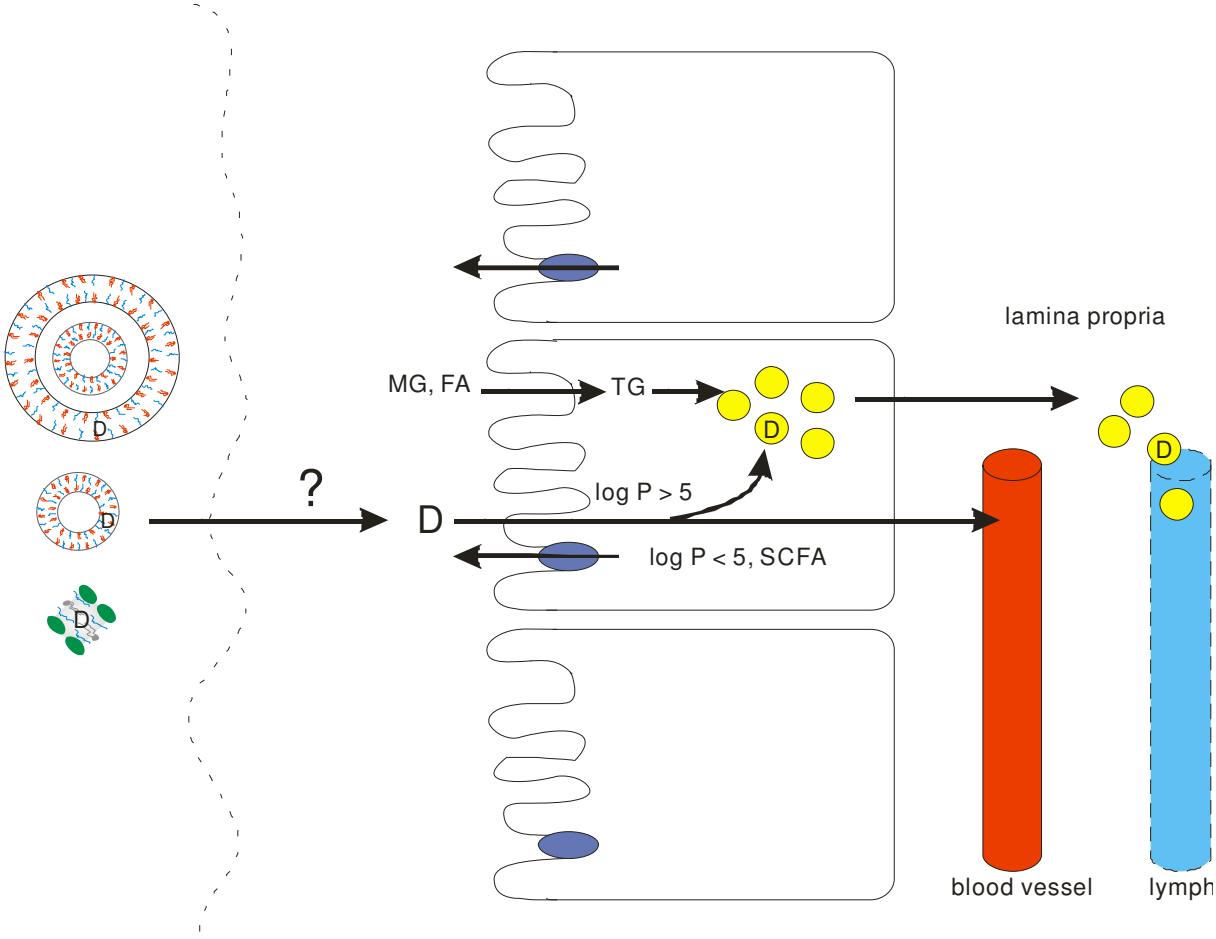


Figure 3 *Schematic diagram of the mechanisms of intestinal lipid and drug transport (modified from Porter et. al and O´Driscoll et.al [9,103]). Following digestion, lipolytic products and lipophilic drugs are solubilised by amphiphilic species (e.g. BS, vesicles). In order to get access to the enterocyte they have to cross the unstirred water layer (UWL). Until today, the mechanism of absorption is not completely understood. Free FA or drugs might also be effluxed back into the intestinal lumen by an efflux transporter (depicted as blue ovals).*
After absorption into the enterocyte, MG and FA can be re-synthesised to TG. Packed into Lipoproteins (represented as yellow circles) they reach the systematic circulation via the mesenteric lymphatic route. Highly lipophilic drug molecules enter the lymphatic pathway in association with the TG core of the chylomicrons. More polar drugs or short chain FA pass directly through the enterocyte cells and enter the portal vein where they are subsequently transported to the systemic system via the liver.

Highly lipophilic drugs (log P > 5 and solubility in LCT > 50 mg) are supposed to partition into the developing LPs in the enterocyte and thereby get access to the intestinal lymph[9]. They enter the systemic circulation bypassing the first pass metabolism. Drug molecules of low or moderate lipophilicity (log P < 5) preferentially enter the systemic circulation via the portal vein.

Fatty acids can also travel in the blood bound to albumin. While the most of the long-chain fatty acids are transported from the small intestine as triglycerides packaged in chylomicrons and VLDLs, some are transported in the portal blood bound to serum albumin. In analogy to moderate lipophilic drugs, most of the medium chain (8 to 12 carbons) and all of the short-chain (< 8 carbons) fatty acids tend to pass directly through the enterocyte cells and enter the portal vein where they are subsequently transported to the systemic system via the liver[72,88].

2.2 Influence of fat digestion on drug absorption

Lipophilic APIs, which are co-administered with fatty meals or incorporated in lipid-based delivery systems, often show an increased oral bioavailability[41,42,44,47,83,104-108]. Lipids can affect drug absorption in different ways. The presence of lipids in the GI tract stimulates the secretion of bile into the small intestine from the gall bladder. The components of bile provide a good vehicle for the solubilisation of poorly soluble dugs. Moreover, lipid digestion generates a wide range of amphiphilic colloidal species (e.g. vesicles, micelles and mixed micelles) that further increase the solubilisation capacity of the intestinal milieu. Drug molecules that are solubilised in colloidal species benefit from a higher mass transport across the unstirred water layer in comparison to solute drug molecules which diffuse slowly due to their poor aqueous solubility.

The uptake of the APIs through the apical brush border membrane is dominated by passive diffusive diffusion or carrier-mediated transport. Lipids and lipidic excipients influence the enterocyte-based transport processes. These mechanisms include an increase in membrane fluidity facilitating transcellular absorption, opening of the tight junction to allow paracellular transport, and inhibition of P-gp and/or CYP450 to increase intracellular concentration and residence time[9,103].

Moreover, the co-administration of exogenous lipids (derived from food or lipid formulations) stimulates the formation of lipoproteins in the duodenal cells which in turn might trigger the absorption of lipophilic drugs via the lymphatic pathway. This alternative pathway reduces the first pass metabolism of APIs as the intestinal lymph travels directly to the systemic circulation bypassing the liver. Thereby, an enhanced oral bioavailability of lipophilic drugs can be achieved.

3 Assessment of lipid formulations

The grown interest in lipid formulations is reflected in the large number of publications which describe the clinical and preclinical bioavailability studies for lipophilic compounds in a variety of lipid vehicles. However, the development pathway seems to be largely empirical. One reason for this has been the absence of discriminating *in vitro* tests which are necessary to screen potential lipid formulations. Such tests can be useful in guidance of formulation development prior to cost and time-consuming *in vivo* evaluation of early prototypes. If poor formulations are used in early animal efficacy studies, the prediction of the likely human dose can be overestimated. In addition, poor formulations in early toxicity studies might lead to an underestimation of the toxicity due to limited exposure resulting from low bioavailability[5].
Furthermore, *in vitro* tests can provide information regarding the batch-to-batch consistency and ensure that the formulation performance is maintained throughout the shelf-life.

3.1 Dispersion testing

Simple dissolution testing using pharmacopeial dissolution apparatus is a basic tool in the evaluation of conventional oral solid dosage forms. Simple buffer solutions or diluted acids serve as media to reflect the pH gradient of the GI environment. In contrast to conventional dosage forms like tablets, lipid-based formulations contain the drug in solution in an anhydrous formulation. Consequently, the focus of these tests should be on dispersion properties, e.g. dispersion time or influence of different pH-value on dispersion, and possible drug precipitation rather than on dissolution. Dispersion testing is especially important for Type III and IV formulations which might loose their solvent capacity due to migration of water soluble compounds into the bulk aqueous phase.
Furthermore, particle size analyses of the resulting dispersions conducting laser diffraction measurements or photon correlation spectroscopy can be used for further characterisation of the formulations.
In recent years, there have been many approaches in order to find adequate *in vitro* tests that are able to predict *in vivo* performance of active pharmaceutical ingredients. There are many aspects that have to be taken into consideration for the set up of a predictive *in vitro* assay, e.g. BCS category of a drug, digestible compounds in a formulation and physiological parameters. Since the solubility of drugs belonging to class II of the BCS is the rate limiting step to absorption, the choice of the dissolution media plays a very important role. In the recent years, Dressman and co-workers have developed more biorelevant dissolution media which reflect the endogenous factors like bile salts and phospholipids, osmolarity and pH value in fasted or fed stomach and small intestine[109-111].

3.2 Simulating fat digestion: *In vitro* lipolysis assay

As demonstrated in the previous chapter, digestion will have a significant impact on the performance of lipid-based drug delivery systems. Hence, *in vitro* tests were needed in order to gain predictive information about the physicochemical changes that will occur *in vivo*. Although lipolysis experiments have been used for many years by biochemists, the application of the technique by pharmaceutical scientists has been slow. In 1988, Reymond et al. published the first *in vitro* lipid digestion model in order to simulate the patterns of solubilisation of a lipophilic drug in the GI milieu and this technique has been used more commonly in the recent years[83,112-117]. Unfortunately, there exists no standard protocol for *in vitro* lipolysis; hence parameters may vary from laboratory to laboratory. Table 2 summarises the experimental conditions for three lipolysis models which are most extensively used for studying lipid formulations.

Table 2 *Experimental conditions of the most established lipolysis models for the characterisation of lipid formulations. The table is modified from Larsen et al[118].*

Experimental condition	**Müllertz group[112,113]**	**Charman/Porter group[114,115,119]**	**Carrière/Jannin group[120-122]**
Enzyme source	porcine pancreatin (3 x USP)	porcine pancreatin (8 x USP)	porcine pancreatic extract (PPE); rHPL; rHPLRP2, CEH (bovine)
Enzyme activity	300 [a] /800USP U/ml	1000 TBU U/ml	250 µg/ml for PPE
Source of bile acids	porcine bile extract	purified Tauro-deoxycholic acid	purified Tauro-deoxycholic acid
Concentration of bile acids	5[a]/30[b] mM	5 [a] /20[b] mM	4 mM
Phospolipid concentration	1.25 [a] /7.5 [b] mM	1.25 [a] /5[b] mM	-
Calcium concentration	45[a] -181[b] µmol/min (continuous addition)	5 mM (initial)	1.4 mM (initial)
Sodium concentration	150 mM	150 mM	150 mM
pH (buffer)	6.5 (2 mM tris maleate)	7.5 (50 mM tris maleate)	6.25 (1 mM Tris HCl)
Initial volume	300 ml	10-40 ml	15 ml

[a]…simulating fasted intestinal conditions

[b]…simulating fed intestinal conditions

The models are designed to mimic gastric or intestinal conditions in terms of pH value, ion concentration and endogenous factors like BS, PL and digestive enzymes (predominantly pancreatic lipase). Bile acids and phospholipids form mixed micelles and are needed for anchoring the lipase at the oil-water interface and for removing the lipolytic products evolving during digestion. The concentration of bile acids in human intestinal fluids ranges from 2-

5 mM in the fasted state and 8-15 mM in the fed state[86,118,123,124]. Bile extract is a complex mixture containing taurine and glycine conjungated bile acids and may therefore more accurately reflect in vivo conditions than individual bile acids. Individual bile acids can be purchased in pure form, which often facilitates the design and interpretation of experimental digestion measurements. On the other hand, the use of purified bile acids may be less representative of the complex composition of actual small intestinal fluids[125].

The addition of calcium is mainly done in order to remove the FAs from the oil-water surface by formation of precipitating calcium soaps. Thereby, the rate and extent of digestion is maintained during the *in vitro* experiment. There are two different approaches of adding calcium: a continuous or a fixed addition. With the fixed calcium addition, a very fast initial lipolysis can be observed, whereas the continuous addition of calcium can be used to control the lipolysis rate[118].

The choice of the pH value of the lipolysis medium is influenced by the physiological relevant pH representing the intestine during fasted (pH 6-7.5[123,124]) and fed state (pH 5-6.6[123,124]) and the optimum activity profile of the lipase (pH 6.5-8[126]). The pH value chosen by researchers who monitor the lipolysis experiments by pH-stat titration is often a compromise between the physiological parameters and the ionisation of the FAs.

Pancreatic enzymes are the key components in any *in vitro* model designed to simulate lipid digestion within the small intestine. Consequently, it is important to use an appropriate type and concentration of the enzymes. Most research groups use pancreatin of porcine origin, a dry extract of porcine pancreas which should contain all relevant enzymes, e.g. pancreatic lipase-colipase, phospholipase A_2 and cholesterol esterase and possibly pancreatic lipase related protein 2 (PLRP2). Pancreatin preparations are normally standardised with respect to the activity of pancreatic lipase. The lipase activity can be measured by different methods which mainly differ in the choice of the substrate. Hence, two expressions of lipase activities can be found in the literature: TBU, using tributyrin as substrate as well as USP units, using an olive oil emulsion stabilised with gum arabic.

Purified pancreatic enzymes that have been isolated from various animal and human sources are also commercially available. However, this is more expensive than using pancreatin. In addition, it is important to use an appropriate amount of colipase with pancreatic lipase to ensure its optimum performance. The research group of Carrière and Jannin have worked with several isolated or recombinant enzymes in order to identify the enzymes which have the main lipolytic activity towards lipid excipients[120-122].

The concentration of human pancreatic lipase underlies high intra- and interindividual variability: Basal values of 265±209 µg/ml HPL and 328±260 µg/ml following a test meal have been reported[127].

Most *in vitro* digestion models have a static experimental design; that means a change of the test parameters, especially pH value as well as concentrations of ions or endogenous factors, which would be associated with GI transit is not foreseen.

The common protocols include dispersion of the lipid formulation in biorelevant media containing BS, PL and calcium ions. The lipolysis is initiated by the addition of digestive enzymes and is allowed to proceed for a fixed time.

During the *in vitro* lipolysis experiments, samples are taken and subjected to further analyses. Non-centrifuged samples can be analysed by cryogenic transmission electron microscopy (Cryo-TEM). Cryo-TEM is a method of microscopic imaging that enables the visualization of the colloidal structures of a frozen sample. However, it should be noted that Cryo-TEM excludes structures that are larger than the grid upon which the sample is frozen. The typical thickness of the grid is around 150 nm with distances between the grids of up to 1 µm[118]. Fatouros et al. successfully applied Cryo-TEM imaging to lipolysis samples providing an insight into the formation and changes of colloidal structures during *in vitro* digestion of a SNEDDS formulation[128]. In addition, small-angle X-ray scattering (SAXS) has been used to investigate the formation of liquid crystalline phases during simulated lipolysis[129]. Rübe and Abdalla et al. utilised a different approach: electron paramagnetic resonance spectroscopy (EPR) was used in order to characterise the microenvironment of dispersed lipid formulations during lipolysis[130,131]. All these methods shall contribute to a better understanding of the relationship between formulation composition, the colloidal phases which are generated during digestion and its impact on drug solubilisation and absorption.

In general, samples from the lipolysis assays are further introduced to ultracentrifugation separating the samples into up to four digestion phases (Figure 4).

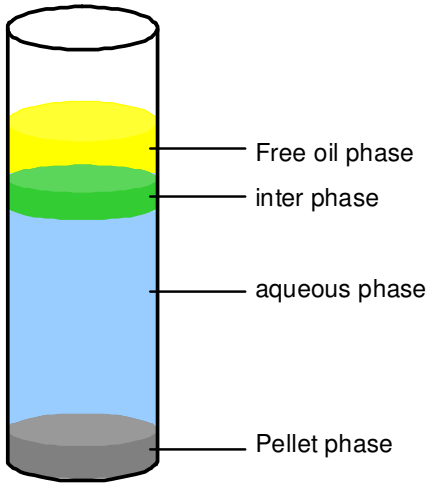


Figure 4 *Schematic representation of the four phases typically present after ultracentrifugation of a lipolysis sample containing LCT. The upper layer consists of undigested oil. The interphase is dominated by lamellar crystalline phases. The aqueous phase consists mainly of micelles and vesicles. The pellet phase is dominated by calcium soaps of fatty acids but liquid crystalline phases and precipitated drug may also be present[86]. The occurrence of the different digestion phases is dependent on the nature of the lipid vehicle which undergoes lipolysis.*

The samples are characterised with respect to drug content usually by HPLC measurements. The drug concentration in the aqueous phase is of particular interest as it is a prerequisite for absorption that the drug is in a dissolved or solubilised state. Nevertheless, further analysing of the pellet phase with respect to drug content and physical form of the precipitated drug (e.g. by x-ray powder diffraction or polarised light microscopy) can provide further information about the impact of digestion on the absorption of a poorly soluble compound[132].

Different techniques have been described to monitor the progress of lipid digestion. In most laboratories, the pH-stat technique is used. The pH stat method is a simple and rapid technique in which a measured volume of NaOH is titrated to maintain a previously defined pH value of the reaction mixture. The onset of lipid digestion results in the liberation of FA, which in turn causes a drop of the pH value. The number of moles of neutralising hydroxyl ions is equal to the moles of FA liberated during lipolysis. In order to decrease the pH value, FAs liberated by digestion have to be ionised. That means the pH value used for the assay has to be near or higher than the pka value of the fatty acids. Unfortunately, the pka value is strongly dependent on the polarity of the fatty acid. Short chain carboxylic acids with a good solubility in water and a pka value of about 4.8 are less problematic. However, the apparent pka value of middle and especially long chain fatty acids underlies high variations due to their interaction with water and the presence of other compounds like proteins or bile salts[133-135]. This subject will be discussed more detailed in the results section.
However, with the background of this issue it is advisable to conduct the test as 'back titration'. That means, after an incubation period the digestion is stopped by the addition of a lipase inhibitor and the pH is abruptly increased to pH 9. The pH value of 9 is chosen according to the USP's monograph 'assay for lipase activity'. At this pH, even long chain FAs are ionised to a great extent which allows the detection of all species of FAs. Blank experiments without substrate are performed according to the same protocol in order to define the amount of NaOH needed for the adjustment of the pH. In fact, the term 'back titration' is not correct because there is no other reactant added and titrated. However, most authors conducting the before mentioned procedure refer to this term[118].
More detailed data about the physicochemical changes of the lipid formulation itself can be obtained by other techniques of lipid analyses, for instance high performance thin-layer chromatography (HPTLC), HPLC or gas chromatography (GC). These methods provide important information about the lipolytic products that emerge during digestion and, thereby, help to explain the solubilisation behaviour or drug precipitation from a lipid formulation.
Since intestinal lipolysis is supposed to have the dominating impact on the performance of lipid based drug delivery systems, most laboratories have focused on the simulation of intestinal digestion, whereas the gastric lipolysis is neglected.

More detailed information about the fate of a drug delivery system after oral administration can be obtained by the computer-controlled dynamic model of the human GI tract (TIM model) by TNO. The TIM-1 model consists of different compartments that mimic the stomach, duodenum, jejunum and ileum. Utilising this model, the following parameters can be varied: the body temperature, the flow of saliva, the gastric- and pancreatic juice including digestive enzymes and bile, the peristalsis for mixing, gastrointestinal transit times, as well as gastric and intestinal pH. The continuous removal of digested lipophilic and hydrophilic compounds using hollow fibre membranes for further analysis is possible[136,137]. However, this approach is associated with enormous costs.

III Materials and Methods

1 Materials

All chemicals used within this work are listed in Table 3.

Table 3 *List of all chemicals used in this thesis*

Substance	Supplier	Remarks
Materials for digestion and dispersion media		
pancreatin powder, porcine (P 7545, 8xUSP specification activity)	Sigma chemicals, Germany	Lipase activity according to Sigma chemicals: 51.3 (Lot: 064K1451), 47.6 (Lot: 055K0688) and 29.6 (Lot: 018K0691) USP U/mg respectively
bile extract, porcine (B 8631)	Sigma chemicals, Germany	Bile salt concentration as determined by Ecoline S+: 1.08 (Lot: 075K0064) and 0.986 (Lot 113K0695) µmol/mg
phosholipon 90 G	Lipoid, Germany	92-98% phosphatidyl choline
sodium chloride	Grüssing, Germany	
calcium chloride $Na_2HPO_4x2H_2O$ KH_2PO_4	C. Roth, Germany	Analytical grade
Materials for simulated gastric medium		
citric acid sodium chloride hydrochloric acid sodium hydroxide potassium chloride calcium chloride sodium carbonate acetic acid sodium acetate $Na_2HPO_4x2H_2O$ KH_2PO_4	C. Roth, Germany	Analytical grade
Organic solvents		
acetonitrile ethyl acetate hexane chloroform methanol	VWR, Germany	HPLC grade
ethanol	Merck, Germany	

Substance	Supplier	Remarks
Reference material		
triolein, 1.2, 1.3-diolein 2-monoolein oleic acid 12-hydroxystearic acid ricinoleic acid stearic acid	Sigma chemicals, Germany	Assay ≥ 99%
APIs		
Probucol	Wako, Japan	-
Ibuprofen	Sigma chemicals, Germany	
miscellaneous		
4-bromophenylboronic acid	Sigma-Aldrich, Germany	Lipase inhibitor
copper sulphate	C. Roth, Germany	Analytical grade
phosphoric acid (85%)	C. Roth, Germany	Analytical grade
Lipofundin 10% N	B. Braun Melsungen AG, Germany	One litre of the emulsion contains 100 g of soybean oil, 25 g of glycerol 8 g of egg lecithin, 12 g of sodium oleate, α-tocopherol and water for injections.

2 Methods

2.1 Dispersion experiments

In order to investigate the submicron structures of lipid formulations dispersion experiments were performed. For that purpose, 50 mg of the respective formulation was weighed into a closable glass tube. Subsequently, 5 ml of the dispersion media was added. The tubes were closed and incubated at 37°C at gentle agitation. As dispersion media either 0.1 N HCl, Sørensen's phosphate buffer pH 6.8 or digestion buffer without the addition of pancreatin was used.

Unless stated otherwise, an end-over-end apparatus was used for incubation of the samples. The end-over-end apparatus was constructed by the engineering group of the chemical department of the Martin-Luther-University of Halle/Wittenberg. It was a temperature controlled chamber in which the reaction vessels rotate at constant speed (Figure 5). Unless stated otherwise, a standard operating speed of 10 rotations per minute was used. The temperature was maintained at 37°C and a sample volume of 5 ml was used in all experiments.

The dispersions were investigated in terms of appearance and particle size distribution. Furthermore, the dispersions resulting from lipid formulations which were loaded with API were investigated in terms of the amount of solubilised drug. For that purpose, samples of the dispersions were taken and centrifuged at 12,100 g (F-45-12-11, Minispin®, Eppendorf, Germany). An aliquot of the supernatant was diluted with acetonitrile and centrifuged again for five minutes at 1073 g. The organic supernatant was filled into a vial for HPLC analysis.

2.2 *In vitro* digestion experiments

2.2.1 Preparation of the digestion buffer

The *in vitro* digestion experiments were conducted using similar methods and conditions to those described in the literature[112-114,119]. Furthermore, the conditions used in this assay have been evaluated earlier[138].

Sørensen's Phosphate Buffer pH 6.8

4.845 g (35.6 mmol) of potassium dehydrogenate phosphate and 5.531 g (31.1 mmol) of disodium monohydrate phosphate dehydrate were dissolved in 1 l deionised water. The pH-value of the solution was checked and adjusted to 6.8 using 0.1 M solutions of sodium hydroxide or hydrogen chloride.

Bile Salt – Phospholipid Preconcentrate 30 / 7.5 mM

Porcine bile extract served as heterogenic source of bile salts. The concentration of 3-hydroxy bile salts was determined by means of the enzymatic test kit Ecoline S^+ (Diagnostic Systems, Germany) with Glycochenodeoxy cholate as reference. Phospholipon® 90G was used as source for phospholipids. It contains 92-98% purified phosphatidylcholine from soybean lecithin. The concentration of lecithin was calculated referring to the average molecule mass of palmityl - oleyl - phosphatidyl choline (759 g/mol).
An amount of Phospholipon® 90G corresponding to 7.5 mM was dissolved in deionised water at 50°C under high shear stress. Upon dispersion of the lecithin, the bile extract corresponding to 30 mM was added under agitation until a clear, brownish solution was obtained.

Sodium Chloride Preconcentrate (4.5 M) / Calcium Chloride Preconcentrate (0.15 M)

The solutions were prepared by dissolving 26.3 g of sodium chloride and 1.66 g of calcium chloride in 100 ml of deionised water, respectively.

Pancreatin

Pancreatin powder (8xUSP specification) was used as received. Upon delivery, the batch was split to different subportions which were stored at -20 °C in order to avoid stress by

freeze/thaw cycles. Pancreatin powder equivalent to 450 U was weighed and added to the digestion buffer to start the reaction.

Preparation of the in vitro digestion buffer

For the preparation of the digestion buffer, Sørensen's phosphate buffer, the BS/PL preconcentrate, sodium chloride and calcium chloride solution were added to receive the composition as given in Table 4. The solution was heated to 37°C and the pH value was corrected to 6.8 using 1 N NaOH if necessary.

Table 4 *Composition of the digestion media used in this study. The addition of Pancreatin powder was optional.*

Ingredient	Simulated fasted state intestinal conditions	Simulated fed state intestinal conditions
Bile salts [mM]	5	15
Phospholipids [mM]	1.25	3.75
Sodium chloride [mM]	150	150
Calcium chloride [mM]	5	5
Buffer system	Na_2HPO_4/ KH_2PO_4 31.1/ 35.6 mM, pH 6.8	Na_2HPO_4/ KH_2PO_4 31.1/ 35.6 mM, pH 6.8
Pancreatic lipase activity [U/ml]	450	450

2.2.2 *In vitro* digestion experiments

The lipid formulations were accurately weighed into closable glass tubes. Then the freshly prepared digestion buffer was added. The final concentration of the dispersion was 10 mg lipid formulation per ml digestion medium (1% (w/v)). In all cases, formulations were allowed to disperse prior to initiation of digestion. Unless stated otherwise, an end-over-end apparatus was used for incubation of the digestion samples. The temperature was maintained at 37°C and a sample volume of 5 ml was used in all experiments.

The digestion was initiated by the addition of pancreatin powder equivalent to 450 U/ml of pancreatic lipase activity. The pH value of the samples had to be adapted to 6.8 by manual addition of 1 N NaOH. The correction of the pH was done prior to sampling.

During the digestion experiments, samples were taken at the start of the experiments, at 5, 15, 30, 60, 120 and 240 min. The lipolysis inhibitor (0.5 M 4-BPB in methanol, 9 ml/ml digestion medium) was added immediately to each sample to prevent further lipolysis. The samples were either assayed for API content or for lipid composition. If the sample was assayed for API content, it was centrifuged at 12,100 g to separate the micellar phase from precipitated drug, calcium salts of fatty acids, and precipitated parts of pancreatin. An aliquot of the supernatant was diluted with acetonitrile and centrifuged again for 5 minutes at 1,073 g. The organic supernatant was filled into a vial for HPLC analysis.
The uncentrifuged samples were used for lipid analysis. The samples were immediately diluted with chloroform/methanol 1:1 (v/v). The ratio of sample to organic solvent was 1:10 (v/v). Upon contact with organic solution, the enzyme precipitated. The diluted samples were centrifuged at 1,073 g for 5 min in order to separate the precipitate. The supernatant was further diluted with chloroform/methanol if necessary. The organic solutions were subsequently subjected to HPTLC analysis as described later.
In all cases, blank digestion experiments without any lipid formulation were performed.

2.3 pH-stat titration

In order to involve an alternative technique to monitor *in vitro* lipolysis, pH-stat titration was conducted. For that purpose, the sample containing the digestion buffer and the respective formulation as described in the previous chapter was placed in a water bath. The temperature was maintained at 37°C which was monitored during the experiment using a thermocouple. Agitation was provided by a magnetic stirrer. A suitable pH electrode was placed in the sample. The electrode was connected with the titration unit (Mettler DL 21, Mettler-Toledo, Germany) which automatically added 0.1 N NaOH (Titrisol®, Merck, Germany) via an auto burette to the sample if the desired pH value dropped. The amount of NaOH in ml was recorded via Microsoft HyperTerminal. The principle experimental set-up is presented in Figure 5. The moles of hydroxide ions were equivalent to the moles of FA liberated during digestion.
Unless stated otherwise, a standard pH value of 6.8 and a sample volume of 15 ml were used in all experiments.

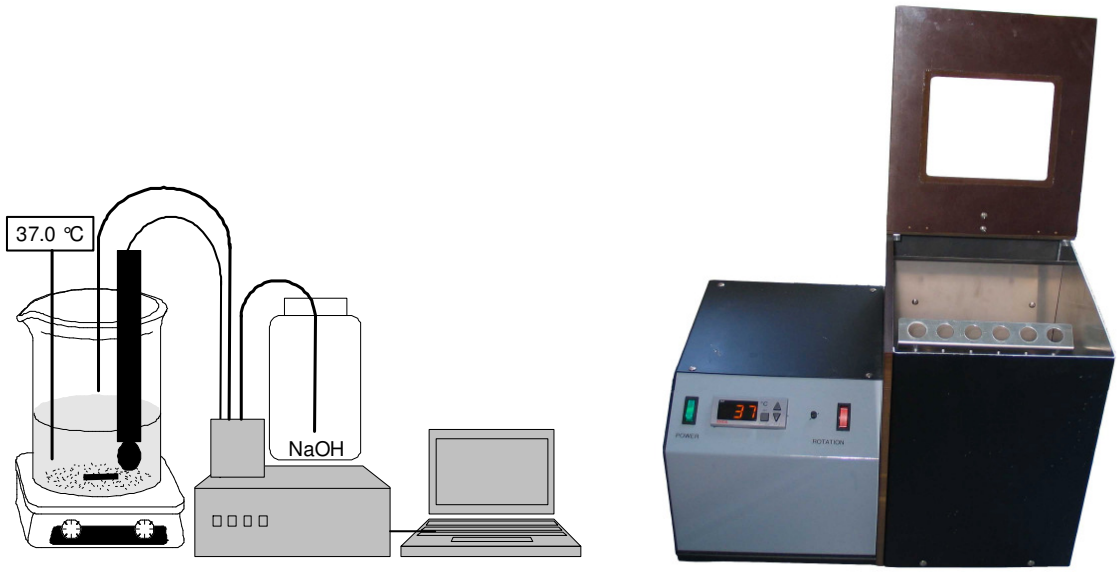


Figure 5 ***Left:*** *Schematic illustration of the experimental set-up of in vitro digestion experiments using pH stat titration. The fatty acids produced during lipolysis were titrated with NaOH.*

Right: *End-over-end apparatus: Reaction vessels are rotating in a temperature controlled chamber.*

2.4 Lipid analyses by high performance thin layer chromatography (HPTLC)

In order to investigate the change in chemical compositions of the lipid drug delivery systems during digestion, HPTLC and spectrodensitometric measurements were performed.

Preparation of the lipid mixtures and application onto HPTLC plates

Samples of the digest were diluted with chloroform/ methanol (1:1 v/v). Standard lipid mixtures containing 50-450 µg/ml of each class of long chain lipid (oleic acid, 2-monoolein, 1,2-diolein and triolein) were prepared in chloroform/methanol (2:1, v/v). Furthermore, standard solutions of 12-hydroxy stearic acid, ricinoleic acid and stearic acid were produced in the same manner. All standard lipid solutions were stored in glass vials with teflon lined caps at -20°C.

The organic solutions were plotted on a silica gel 60 HPTLC plate (Merck, Germany) using an Automatic TLC Sampler 4 (CAMAG, Switzerland).

Separation of the lipids

The automated multiple development chamber (AMD 2, CAMAG, Switzerland) allows the multiple development of each HPTLC plate with different mobile phases of decreasing polarity.

Table 5 *HPTLC gradients used for the separation of long chain glycerides (left) or sucrose esters (right)*

	AMD gradient used for separation of long chain lipids		
step	**migration distance [mm]**	**hexane [%V/V]**	**ethylacetate [%V/V]**
1	14.0	70	30
2	19.1	73	27
3	24.2	76	24
4	29.3	79	21
5	34.4	82	18
6	39.5	85	15
7	44.6	88	12
8	49.7	91	9
9	54.8	94	6
10	59.9	97	3
11	65.0	100	0

AMD gradient used for separation of sucrose ester		
migration distance [mm]	**methanol/water (94+6) [% (v/v)]**	**chloroform [% (v/v)]**
60.0	30	70
65.0	20	80
70.0	10	90
80.0	0	100

The mobile phase composition and migration distance of each development step used for the separation of long chain lipids is given in Table 5. Furthermore, a gradient for the separation of sucrose esters was developed.

Post-chromatographic derivatisation and densitometry

After the chromatographic steps were finished, the plates were stained with an aqueous copper sulphate (10% $CuSO_4$, 8% H_3PO_4 and 5% methanol in water, all w/w) solution for 20 s. Subsequently, the plates were heated in an oven to 150 °C for 20 min. After post-chromatographic derivatisation, the lipids were quantified by *in situ* spectrodensitometric measurements using a TLC Scanner 3 at 675 nm (CAMAG, Switzerland). The optical densities were converted into masses using standard curves obtained from the respective standard solutions which were separated simultaneously on each plate to avoid inter-assay variations. The masses of lipids obtained from scanning densitometric measurements were converted into concentrations using the molar masses of the standards relative to the volume of the samples[131,138]. In case of the long chain glycerides, all calculations were based on the oleic acid reference materials but in the further discussions the terms ‘monoglycerides’, ‘diglycerides’, ‘fatty acids’ and ‘triglycerides’ instead of monoolein, diolein, oleic acid and triolein were used.

Since the total lipid mass of a TG substrate is conserved throughout the *in vitro* digestion experiment, mass balance calculations were performed using equation 2.

$$\text{Mass balance}\,[\%] = \frac{FA_{formed} + MG_{formed} + (2\ DG_{formed}) + (3\ TG_{remaining})}{(3\ TG_{initial})} \text{ x } 100 \qquad \textit{eq. 2}$$

The total FA equivalents present in the initial TG are equal to the FA equivalents derived from the remaining TG and FAs, MGs, and DGs formed during the lipolysis.

Materials and methods which were used beyond the ones described above are depicted in the sections, respectively.

IV Results and discussion

1 Influence of digestion on lipid based formulations: Impact of formulation type and triglyceride source

1.1 Introduction

In recent years, lipid based drug delivery systems have been developed in order to increase the oral bioavailability of compounds belonging to class II and IV of the BCS[34-48]. The mechanisms behind this improvement have been attributed to a number of factors:

a) presentation of the drug in solution to the GI tract
b) increase bile flow caused by the presence of lipids
c) enhanced mass transport of APIs across the UWL by colloidal species derived from lipid digestion
d) stimulation of lymphatic transport
e) modulation of enterocyte-based transport systems

However, the development of these delivery systems is not trivial and requires fundamental understanding of the physicochemical nature of the compound as well as the excipients and their interaction upon contact with the gastrointestinal environment including fat digestion. The crucial parameters for the performance of lipid formulations are predominantly the impacts of dilution and digestion on the solubilisation capacity of these systems.

Figure 7 schematically summarises alterations of the submicron structures that could occur upon dilution and digestion of the different classes of the LFCS. Formulations belonging to class I of the LFCS are supposed to be resistant towards dilution. Co-administered APIs will remain solubilised within the oil droplets. Upon contact with digestive enzymes, lipid digestion will generate various colloidal species including vesicles, micelles and mixed micelles which promote drug transport across the intestinal membrane. Similarly, class II formulations are supposed to remain their solvent capacity upon dilution, although surfactants may desorb from the oil-water interface to form micelles or monomeric solutions in water. In analogy to class I formulations, digestion is likely to occur.

Type III formulations are supposed to be more sensitive towards dilution. Drug precipitation is often reported as a result of diffusion of the polar components into the aqueous outer phase[5,50,139]. However, gastrointestinal digestion may influence the solvent capacity of type III formulations since surfactants have been demonstrated to be susceptible towards lipolysis as well[35,36,122,140]. The same applies to type IV formulations which are solely composed of polar components.

A successful lipid formulation is capable of solubilising a drug in the lumen of the GI-tract after dispersion and during digestion of the system. It is well known that food lipids are

digested and absorbed with high efficacy. However, little is known about the relationship between the type of lipid formulation, as defined by the LFCS, and the fate of APIs formulated in lipid systems for oral delivery.

In this study, lipid formulations which meet the characteristics of the different types of the LFCS were produced and loaded with the lipophilic model drug Probucol. Probucol (Figure 6) is a non-ionisable drug which is used in the treatment of hyperlipidemia. It is a challenging compound with an aqueous solubility of 2-5 ng/ml[141] and a calculated octanol/water partition coefficient (clogP) of 11 (ChemDraw Ultra, version 8.0.3).

For a better comparison of the different formulations, a limited number of excipients was used. Furthermore, formulations based on long chain (LC-derivatives) and medium chain glycerides (MC-derivatives) were investigated. The focus of this study was to evaluate the susceptibility of these formulations towards dilution and *in vitro* fat digestion. The results of these investigations should lead to a better understanding of the mechanisms that occur upon ingestion of lipid formulations. This finally leads to a rational development of more 'biorobust' lipid formulations; which means that oral bioavailability shall be less dependent on food effects and interindividual variability (e.g. bile flow and bile composition).

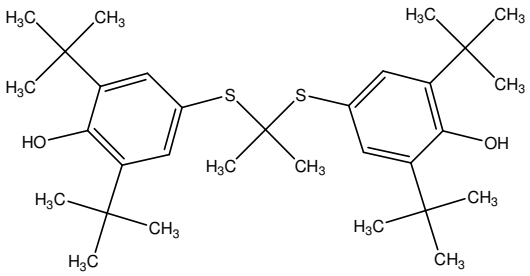


Figure 6 *Chemical structure of Probucol*

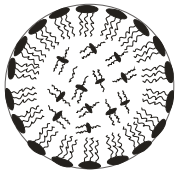
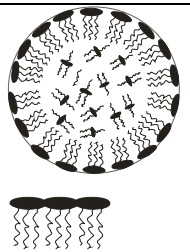
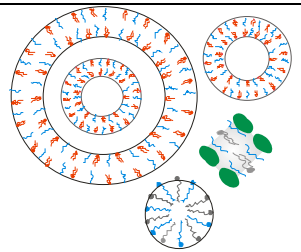
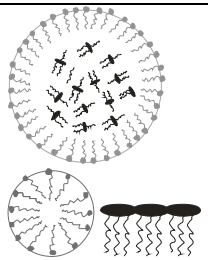
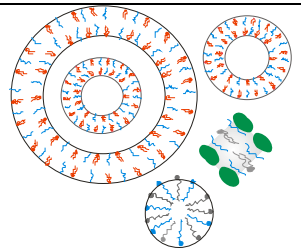
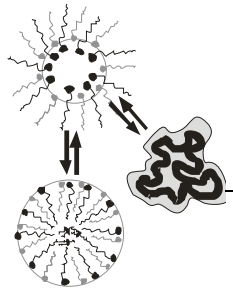
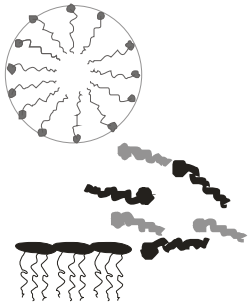
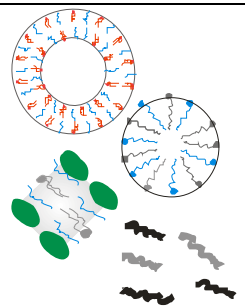
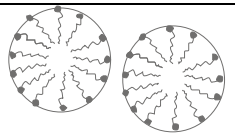
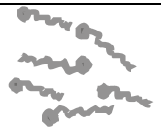
LFCS	Structures of dispersed formulations	Potential structures after dilution	Potential structures after digestion
I	 Crude emulsion Oil droplets diameter > 2 µm	 resistant to dilution or phase separation	 Vesicles with incorporated lipolytic products mixed bile salt micelles micelles
II	 SEDDS Oil droplet stabilised with surfactant diameter ~ 0.25-2 µm	 resistant to dilution or phase separation formation of surfactant micelles	 Vesicles with incorporated lipolytic products mixed bile salt micelles micelles
IIIA	 SMEDDS Oil > 40% diameter ~250-100 nm	 formation of surfactant micelles dissolution to surfactant monomer phase separation → loss of solvent capacity	 Vesicles with incorporated lipolytic products mixed bile salt micelles micelles hydrolysed surfactant → loss of solvent capacity
IIIB	SMEDDS Oil < 20% diameter 100-50 nm		
IV	 Micellar solutions diameter < 50 nm	 monomer formation → loss of solvent capacity	 hydrolysed surfactant mixed micelles → loss of solvent capacity

Figure 7 *Schematic representation of the submicron structures that potentially result upon dilution and digestion of different lipid formulations. For more details refer to the text.*

1.2 Materials and methods

Materials for the preparation of the lipid formulations

Soybean oil and Miglyol® 812 (MCT) were purchased from Caelo, Germany. Soybean oil is a natural dietary vegetable fat. It is extracted from the beans of *Glycine soja* and *Glycine hispida* and has a high amount of unsaturated long chain fatty acids bound in triglycerides. The major fractions of unsaturated fatty acids are linoleic acid (48-58%), oleic acid (17-30%) and linolenic acid (5-11%). Miglyol® 812 is a mixture of semisynthetic and liquid triesters of glycerol and fatty acids. The major fractions of the saturated fatty acids are caprylic acid (50-65%) and capric acid (30-45%).

Maisine® 35-1 complies with the EP monograph of glycerol monolinoleate. It is obtained by partial alcoholysis of maize oil. Despite the EP nomenclature, Maisine® 35-1 contains a mixture of 32-52% of monoacylglycerides (MG), 40-55% of diacylglycerides (DG), and 5-20% triacylglycerides (TG). 30-39% of the monoglycerides represent 1-monoglyceride isomers. The dominating fatty acid fraction is linoleic acid with more than 50% but up to 35% of the derivatives can be oleic acid. Batch 120348 which was used for this study consisted of 33.5% MG, 50.9% DG, and 14.7% TG. Maisine® 35-1 is a good solvent for lipophilic drugs and is mainly used as oily vehicle for use in self-emulsifying lipid formulations to obtain a coarse dispersions or emulsions. Its HLB value is four.

Imwitor® 742 (Sasol, Germany) is a blend of mono-, di- and triglycerides of caprylic and capric acid (C_{8-10}). Its monoglyceride content is 45-55% (51% as certified for batch 703789).

Tween 85 (polyoxyethylene-(20)-sorbitan trioleate, HLB 11) was kindly donated by Croda, England. Solutol® HS 15 (macrogol-15-hydroxystearate, HLB ~ 15) was supplied by BASF, Germany. Ethanol was purchased from Merck, Germany.

Formulation preparation

Different formulations were prepared which meet the characteristics of the four classes of the LFCS (Table 6). Preparation included weighing of appropriate amounts of oil, surfactant and co-solvent into a glass vial and subsequent mixing at 50°C.

As oily component, a 1:1 mixture of Soybean oil and Maisine® 35-1 was used for long-chain glyceride based formulations. For medium chain glyceride based formulations a mixture of MCT and Imwitor® 742 (2:1) was used. The different ratios between the TG (soybean oil and Miglyol) and the partial glycerides (Maisine® and Imwitor®) were chosen in order to achieve a similar polarity of the formulations.

The water-insoluble surfactant was Tween 85. Solutol® HS 15 served as water-soluble surfactant and Ethanol was used as co-solvent.

Table 6 *Composition of different lipid formulations used in this study in% (w/w)*

	Soybean oil/ Maisine® 35-1 (1:1)	***MCT/ Imwitor® 742 (2:1)***	***Tween 85***	***Solutol® HS 15***	***Ethanol***
I-LC	100	-	-	-	-
I-MC	-	100	-	-	-
II-LC	60	-	40	-	-
II-MC	-	60	40	-	-
IIIA-LC	50	-	-	50	-
IIIA-MC	-	50	-	50	-
IIIB-LC	15	-	-	75	10
IIIB-MC	-	15	-	75	10
IV	-	-	-	80	20

Determination of saturation solubility

The thermodynamic saturation solubility of Probucol in the different excipients and formulations was determined. For that purpose, Probucol was added to each sample in excess and allowed to equilibrate for 24 h at 37 °C under gentle rotation. After that period, samples were centrifuged at 12,100 g to separate precipitated drug molecules. Samples were diluted with acetonitrile and introduced to HPLC analysis. All experiments were performed in triplicate.

Probucol was incorporated at 70% of its saturation solubility in the formulations in order to maintain constant thermodynamic activity across all formulations. The components were mixed at 50 °C to facilitate drug solubilisation. The mixtures were cooled down to room temperature and allowed to equilibrate for 24 h prior to analysis.

Dispersion experiments

The formulations were evaluated for dispersion properties and particle size after dilution 1% (w/v) in phosphate buffer pH 6.8. Particle sizes of the resulting dispersions of formulation IIIA, IIIB and IV were determined by means of photon correlation spectroscopy (PCS) using a Malvern HPPS instrument (Malvern, UK). Measurements were performed at 37 °C. Due to the larger droplet size of the emulsions formed upon dilution of formulations I and II, particle size determination was performed by microscopically investigation (ZeissAxiolab RE, Zeiss Microimaging GmbH, Germany) or by using laser diffraction measurements with a Mastersizer 2000 instrument (Malvern, UK). All results are mean values of at least 5 measurements.

In order to investigate drug precipitation upon dilution, Probucol loaded formulations were dispersed in phosphate buffer and simulated intestinal medium without enzymes. The dispersions were introduced to an end-over-end apparatus providing gentle agitation at 37°C. In regular time intervals, samples were withdrawn and centrifuged at 12,100 g. An aliquot of the supernatant was diluted and subsequently assayed for Probucol content by HPLC. Due to the high intrinsic variability of sampling from coarse dispersions formed by formulations type I and II, the complete dispersion with the exception of the precipitate was used for subsequent analyses. Nevertheless, the ratio between acetonitrile and the aqueous sample was kept constant.

Digestion experiments

In vitro digestion experiments were performed as described earlier utilising pH-stat titration and lipid analyses by HPTLC. Moreover, the concentration of Probucol in the aqueous digestion medium was investigated.

HPLC analyses

Probucol was analysed by HPLC using a modified method as previously described by Nourooz-Zadeh et al[142]. The HPLC system consisted of a Merck Hitachi system equipped with an AS 4000A auto sampler, a L 6200A programmable pump, a L 4250 UV-Vis detector and a column oven. Probucol was analysed using a 125-4 mm LiChroCART C18 (5 µm) column fitted with a C18 guard column (LiChroCART 4-4, Merck, Germany). The mobile phase was 90/10 (v/v) acetonitrile/ water and a flow of 1.5 ml/min led to a retention time of 4.5 min. The lowest concentration of Probucol which could be quantified with acceptable accuracy and precision (±20%) was 2 µg/ml (n=5).
Statistical data analysis was performed using the Student's t-test with a minimal level of significance of 5% assuming that data distribution was normal.

1.3 Results and Discussion

Saturation solubility studies in anhydrous formulations

The maximum solubility of a drug in its formulation determines the maximum drug load. Figure 8 shows the solubility of Probucol in the single excipients and final formulations. The solubility in MCT was significantly higher than in long-chain glycerides. This phenomenon is known for many drugs and is most likely attributed to the higher number of ester groups per mass unit[42,143]. A lower solubility was observed for the partial glycerides Imwitor® and Maisine®. Surprisingly, the solubility in the surfactants Tween 85 and Solutol® was similar, which was on the other hand advantageous in terms of modelling different types of lipid formulations whilst maintaining similar solvent capacities for Probucol. The highest solubility was determined in ethanol in which Probucol is freely soluble.

The solubility of Probucol in buffer could not be determined due to the lower limit of quantification of 2 µg/ml. The solubility of Probucol in the simulated intestinal fluid without enzyme at 37°C as used in this study was 4.62 ± 0.64 µg/ml.

The solubility in the single excipients dictates the solubility in the final formulations: For MC-based formulations a higher amount of Probucol could be dissolved compared to LC-based systems. However, with presence of ethanol this difference was compensated. These data were used to calculate Probucol concentrations equivalent to 70% solubility for the use in dispersion and digestion experiments.

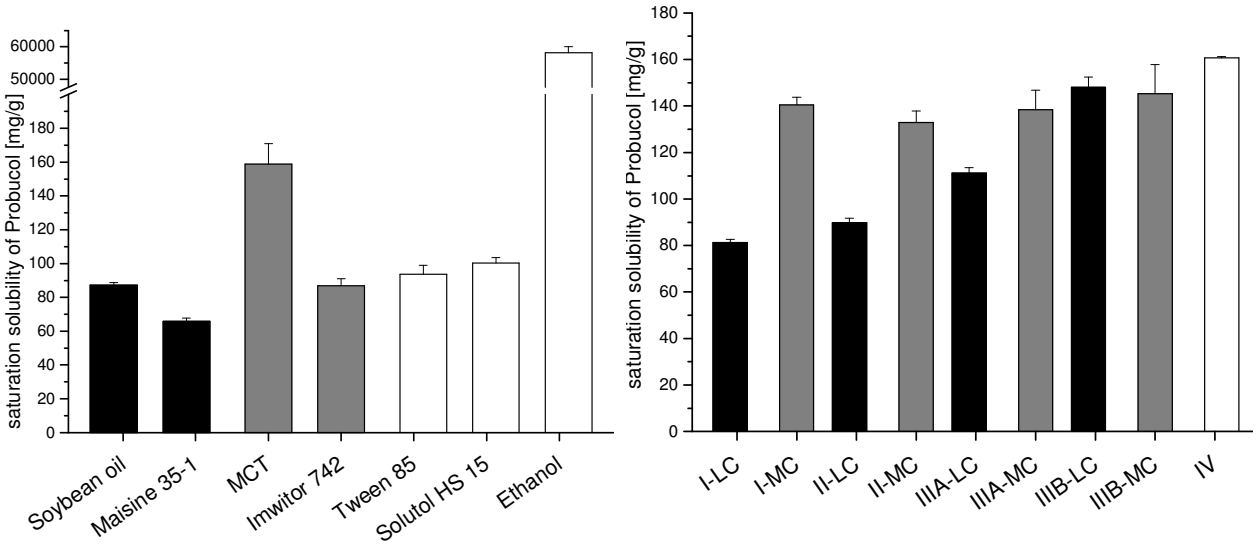


Figure 8 *Saturation solubility of Probucol in different excipients (left figure) and lipid formulations (right figure) (mean ± SD, n=3) determined at 37 °C, black bars represent long-chain based formulations, grey bars represent medium-chain based formulations, white bars represent surfactants based formulations and co-solvents*

Dispersion behaviour

Formulations I-LC and I-MC represent non-dispersing oils. The incorporation of mixed MG and DG was not sufficient to promote an effective self-emulsification. Upon dilution and shaking, crude emulsions were formed with oil droplets in the upper µm range. The inclusion of Tween 85 led to an effective self-emulsification. Milky dispersions were formed upon dilution of formulations II-MC and II-LC. In both cases, particle size distribution was rather broad but the dominating fraction was in the upper nm to lower µm range (Table 7). Upon dilution with phosphate buffer, formulations belonging to class III and IV of the LFCS formed opalescent to optical clear dispersions within 2 min. In all cases, monomodal particle size distributions were obtained by PCS measurements. There was no significant difference between the particle sizes obtained from LC- and MC-formulations. Furthermore, loading of the anhydrous formulations with Probucol had no influence on the dispersion behaviour and particle sizes.

Table 7 *Characterisation of the resulting dispersions obtained after dilution of lipid formulations*

Formulation	appearance of the dispersions	PSD of dispersions[a]	PSD of dispersions loaded with Probucol[a]
I-LC	crude emulsion	n.a.[b]	n.a.[b]
I-MC	crude emulsion	n.a.[b]	n.a.[b]
II-LC	milky dispersion	6.5 µm[c]	n.a.
II-MC	milky dispersion	5.1 µm[c]	n.a.
IIIA-LC	opalescent dispersion	42 nm[d] (PDI 0.067)	44 nm [d] (PDI 0.067)
IIIA-MC	opalescent dispersion	43 nm[d] (PDI 0.046)	n.a.
IIIB-LC	clear dispersion	18 nm [d] (PDI 0.033)	23 nm [d] (PDI 0.023)
IIIB-MC	clear dispersion	19 nm[d] (PDI 0.031)	n.a.
IV	clear dispersion	13 nm (PDI 0.063)	18 nm[d](PDI 0.039)

[a] ……formulations were dispersed in phosphate buffer (1% w/v) pH 6.8 at 37 °C under gentle rotation

[b] ……Particles distribution was too heterogeneous to be determined with accuracy

[c] ……volume weighted mean diameter was determined by laser diffraction measurements

[d] …...intensity weighed mean diameter was determined by photon correlation spectroscopy

n.a. ..not analysed

In order to investigate the influence of dilution on the solubilisation capacity of the lipid systems, each formulation loaded with Probucol at 70% of its saturation solubility was either diluted with phosphate buffer or simulated intestinal fluid without enzyme. At several time intervals, samples were withdrawn and analysed for Probucol content. The results of this experiment are summarised in Table 8.

As expected, no loss of solvent capacity was observed for the lipophilic formulation types I and II. However, the more hydrophilic formulation types III and IV were also able to maintain their solvent capacity in general (amount of precipitated drug below 10% with exception to IIIA-LC and IIIB-LC). Remarkably, there was no statistical difference whether the formulations were diluted in phosphate buffer or simulated intestinal fluid containing mixed micelles.

Table 8 *Probucol load in different lipid formulations and solubilised amount after dilution of the formulations*

Formulation	Drug load [mg/g][a]	Solubilised dose after 60 min dispersion [%][b]	
		Buffer pH 6.8	SIF
I-LC	56.9	99.74 ± 0.07	97.28 ± 1.08
I-MC	98.3	n.a	98.58 ± 0.37
II-LC	62.9	98.76 ± 0.47	99.24 ± 0.28
II-MC	93.1	n.a.	97.04 ± 1.88
IIIA-LC	77.8	88.14 ± 1.28	87.08 ± 2.07
IIIA-MC	96.9	96.47 ± 2.55	91.73 ± 0.39
IIIB-LC	103.6	89.16 ± 2.61	84.48 ± 1.35
IIIB-MC	101.6	97.38 ± 2.51	95.75 ± 0.60
IV	112.5	90.25 ± 2.16	92.19 ± 3.54

[a]…Probucol was incorporated at 70% of its saturation solubility in the formulations

[b]…Percentage of dose recovered in the aqueous phase after 60 min dispersion of 1% formulation in simulated intestinal medium (SIF) without enzyme (mean ± SD, n=3)

Regarding the qualitative dispersion properties no difference between the utilisation of LC or MC-glycerides could be observed. Investigations of the quantitative impact of dilution revealed some surprises. In the cases of the self-micro-emulsifying systems containing long-chain glycerides (IIIA-LC and IIIB-LC), drug precipitation of more than 10% was observed. On the other hand, self-micro-emulsifying systems containing medium-chain glycerides (IIIA-MC and IIIB-MC) effectively maintained Probucol in solubilised state.

One explanation for these results might be found in the combination of the single ingredients. The polarities of long chain glycerides and Solutol® HS 15 differ more than the polarities of medium chain glycerides and Solutol® HS 15. The hydrophilic surfactant Solutol® HS 15 seems to partially separate from the long chain glycerides during dissolution. This might also be triggered by the presence of bile salts in the simulated intestinal fluid since PSC measurements revealed no sign of instability at dispersion experiments in buffer. In consequence, the migration of the surfactant into the aqueous medium leads to a reduction of the solvent capacity and, thus, to the precipitation of the API. The difference in polarity of middle chain glycerides and the surfactant is less pronounced; hence, the solubilisation capacity was maintained under the experimental conditions.

Remarkably, the Solutol®-based formulation IV was able to remain its solvent capacity to more than 90%. Moreover, drug precipitation for the polar formulations of type IIIB was quite low. These findings were surprising. In accordance with Pouton`s assumption a strong loss of solvent capacity during dilution of formulations IV and IIIB was expected. Due to the high

solubility of Probucol in ethanol, a strong precipitation of the API caused by diffusion of ethanol into the aqueous phase was anticipated.

Solutol® HS 15 has a very low CMC of about 0.021%[144]. That means at the experimental conditions, micelles can be formed which in turn maintained the solvent capacity of formulation IV.

In comparable studies, Cuiné et al. investigated the influence of dilution and digestion of type III and IV lipid formulations of Danazol. Similar to our findings, solutions of non-ionic surfactants (Cremophor® RH 40 and EL, Brij® 97, Tween® 80) as well as SEDDS with high or low content of oil (long chain glycerides) effectively maintained drug in solubilised state after dispersion in simulated gastric medium[35,36].

In contrast to that, Mohsin et al. reported a strong precipitation of Fenofibrate after dilution of type IIIB (containing medium chain glycerides) and IV formulations[139].

In conclusion, the resistance to dilution seems to be not only affected by the interaction of single ingredients with water but also by the nature of the drug itself.

Susceptibility to fat digestion

Following the dispersion behaviour, the impact of enzymatic degradation on the solubilisation capacity of the formulations was evaluated. For that purpose, *in vitro* digestion was initiated by the addition of pancreatin powder to dispersed formulations in simulated intestinal medium.

In contrast to the dispersion experiments, *in vitro* digestion of the lipid formulations significantly changed the solubilising properties. Figure 9 illustrates the amount of solubilised drug during simulated digestion of the LC-based formulations.

Under the experimental conditions used in this study, drug precipitation was observed for all formulations. Lipophilic formulations I-LC and II-LC showed a similar precipitation profile. Remarkably, for formulation IIIA-LC containing 50% of oil and oil-free formulation IV no statistical difference was noted (level of significance 0.95). The highest and fastest drug precipitation was measured for formulation IIIB-LC.

Figure 10 shows the results of the digestion experiments for the MC-based formulations. In contrast to the LC-derivatives, solubilisation profiles were completely different. For formulation I-MC, a drastically drug precipitation of about 82% was observed after 4 h. Formulations II-MC and IIIA-MC, with similar oil content, lost about 45% of their solvent capacity while formulation IIIB, with the lowest content of MC glycerides, effectively maintained 77% of the dose in solubilised state. Surprisingly, the results obtained from IIIB-MC were in the same range of I-LC and II-LC. With exception to formulations IIIB, the kinetics of Probucol precipitation was faster from the MC-based systems than from LC-based systems within the first 60 min of digestion.

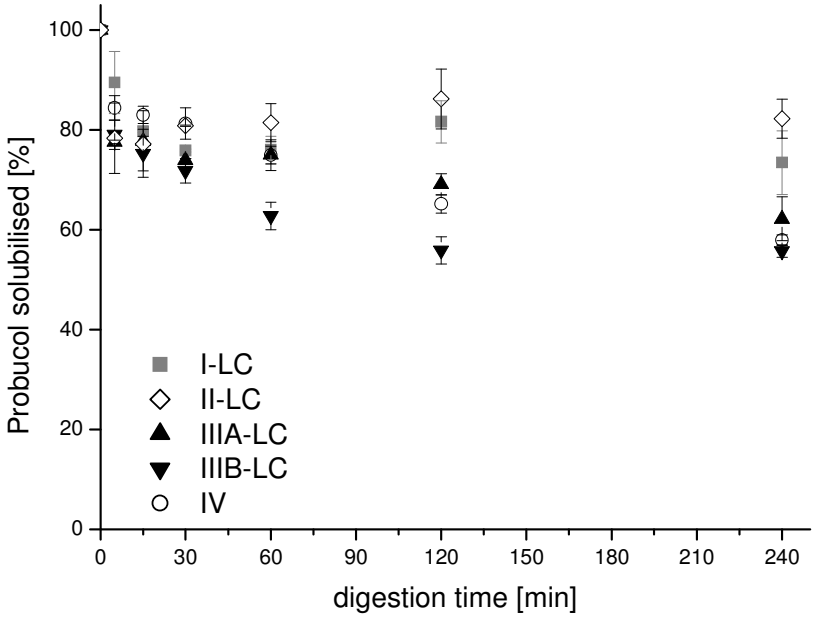


Figure 9 *Amount of Probucol solubilised during simulated digestion of different lipid formulations (compositions are given in Table 6). Data represent mean±SD, n=3*

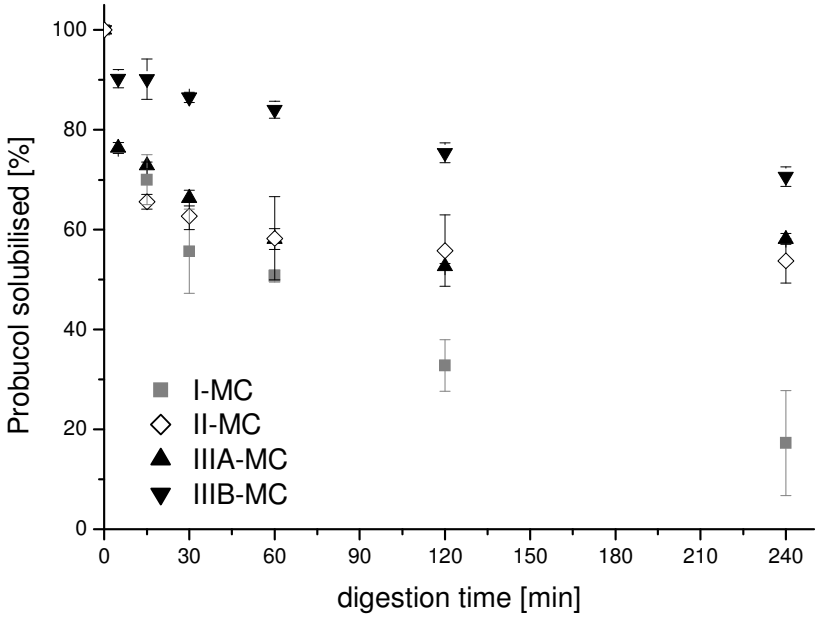


Figure 10 *Amount of Probucol solubilised during simulated digestion of different lipid formulations (compositions are given in Table 6). Data represent mean±SD, n=3*

The drug precipitation observed in this digestion study indicates that major changes of the formulations physicochemical properties must have occurred. For that reason, the kinetics and the extent of formulation degradation was further investigated. *In vitro* fat digestion was monitored conducting the widely-used pH-stat titration technique.

The FA titration profiles obtained during simulated digestion of the LC-based formulations are depicted in Figure 11a (top).

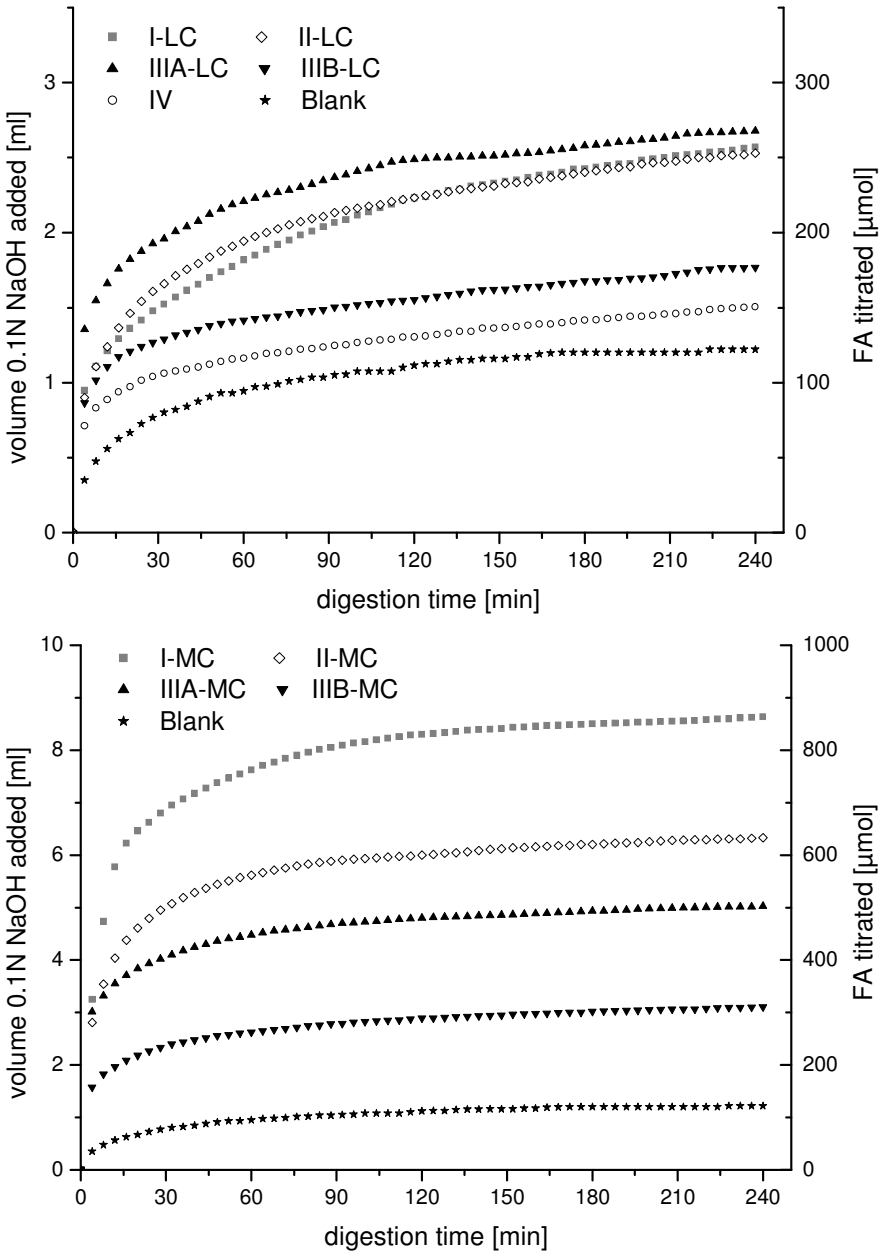
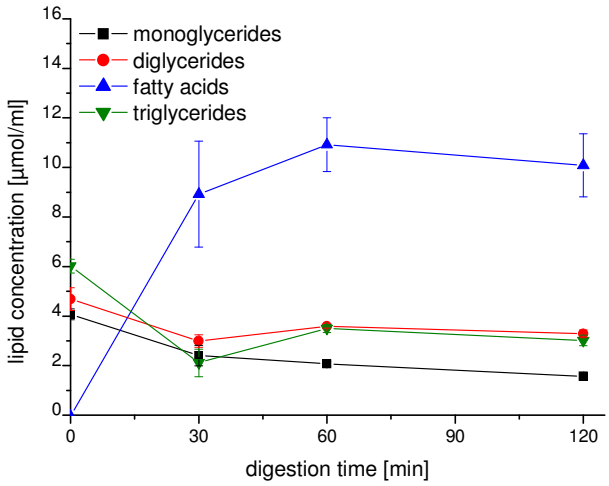


Figure 11 *Fatty acid (FA) concentrations determined by pH-stat titration during digestion of blank medium and different lipid formulations (mean of n=3)*
a) top: LC-based lipid formulations and surfactant-based formulation
b) bottom: MC-based lipid formulations

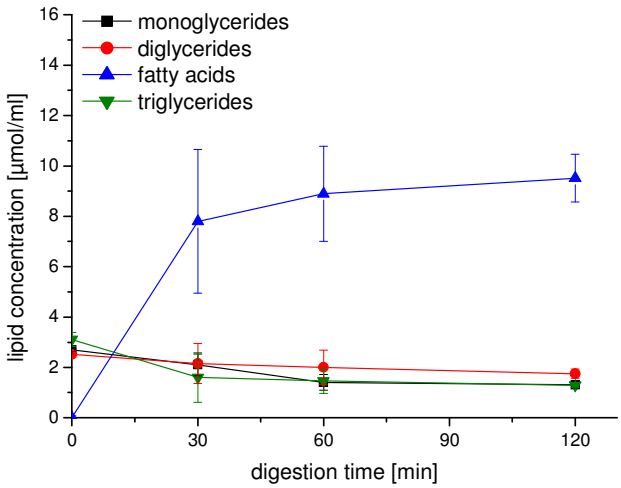
As expected, a drop of the pH during the simulated digestion of the blank medium containing mixed micelles could be detected. This result can be attributed to the degradation of phospholipids to free FAs and lysolecithin. In comparison to the blank experiments, higher amounts of sodium hydroxide were necessary to remain the constant pH value of 6.8 for oil-free formulation IV indicating that the surfactant itself is susceptible towards pancreatic

lipase. For formulation IIIB-LC with a low oil content of 15%, about 180 µmol FAs were detected after 4 h of simulated digestion.
The fastest progress of digestion was observed for formulation IIIA-LC. Within the first 30 minutes of simulated digestion about 200 µmol FAs were detected. However, after 4 h of simulated digestion a similar endpoint to the lipophilic formulations I-LC and II-LC is reached.
Surprisingly, the titration curves of I-LC- and II-LC were comparable although the formulations differ in the content of the glyceride mixture. For both formulations, the progress of digestion seems to be slower in comparison to formulation IIIA-LC. Furthermore, digestion seems to be still in progress after 4 h.

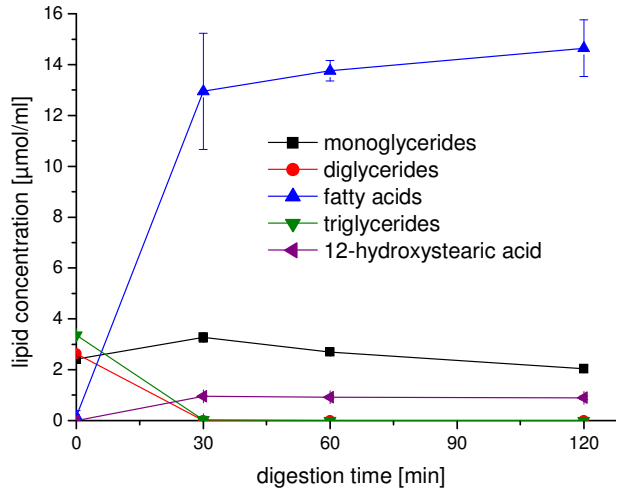
It is well known that the performance of the pH-stat titration technique in terms of quantifying the FAs produced during lipid digestion is highly dependent on their ionisation rate. The availability of a titratable fatty acid is in turn dependent on several factors including the chain length of the fatty acid, the pH of the media, the bile salt and electrolyte concentrations as well as the phase behaviour of the fatty acid. Sek et al. monitored the simulated digestion of LCT and MCT using pH-stat and a HPTLC method and compared the results. It was shown that the titration efficiency of the pH-stat was lower in assessing the digestion of LCT when compared with medium chain lipids[119]. In order to evaluate if the results obtained in this study were attributed to the measuring principle, additional analysis utilising HPTLC measurements were performed.
Figure 12 displays the lipid fractions determined as monoolein, diolein, triolein, oleic acid and 12-hydroxystearic acid by means of HPTLC analyses obtained during 2 h of simulated digestion of the LC-based lipid formulations. In all cases, transient increases in FA concentrations were observed, whereas TG and DG levels decreased. Unlike pure LCT digestion where an accumulation of monoglyceride can be noted during hydrolysis, monoglyceride levels decreased only slowly or were relatively constant for the soybean oil/ Maisine® 35-1 mixture which was used as oily component. This effect can be attributed to the differences in the MG isomer which is present in Maisine® 35-1. According to the product information, up to 90% of the monoglyceride is present as the 1-monoglyceride isomer. Pancreatic lipase has a relatively higher specificity for the outer chain ester bonds of triglyceride and is more active against the 1-monoglyceride than the 2-monoglyceride isomer[145]. Thus, 1-monoglyceride levels decrease whilst 2-monoglyceride derived from degradation of TG and DG increase.



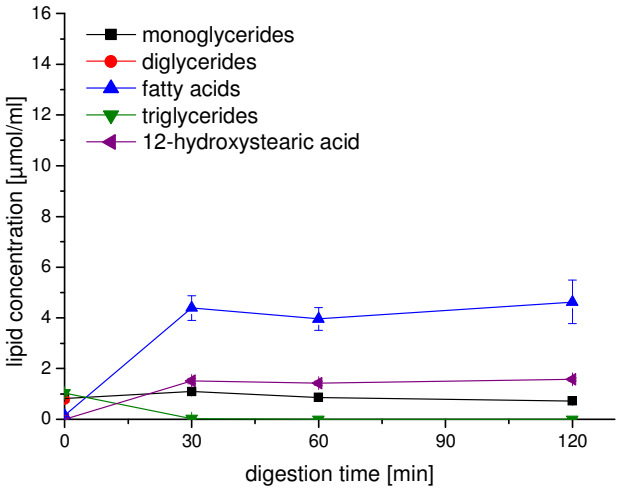
A: I-LC



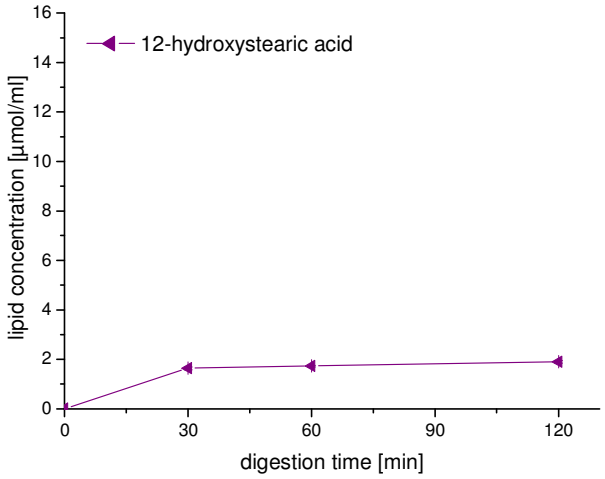
B: II-LC



C: IIIA-LC



D: IIIB-LC



E: IV

Figure 12 *Lipid fraction obtained after simulated digestion of different lipid formulations as analysed by HPTLC/in situ densitometric measurements (data represent mean±SD, n=3).*

In accordance with the results obtained by the pH-stat method, similar FA levels were detected for I-LC and II-LC. However, the digestion of TG and DG was not completed. In case of LC-I, 58% of the initial DG and TG was left after 120 min of simulated digestion. For formulation LC-II, 54% of the initial DG and TG were detected after 120 min. The results obtained in this study are in qualitative agreement with Sek et. al.[114,119].

In agreement with the results by the pH-stat method, the highest amounts of FA were detected for formulation IIIA-LC, the lowest for IIIB-LC. The degradation of DG and TG was complete for both formulations. In addition, for formulations IIIA, IIIB and IV, containing Solutol®, the formation of 12-hydroxystearic acid was detected revealing that the surfactant underwent enzymatic degradation itself. In the case of the surfactant-based formulation IV, about 1.8 µmol/ml 12-hydroxystearic acid as degradation product was detected after 120 min of digestion. Taking the sapofinication value of Solutol® into account, this would be equivalent to a degradation of 22%.

The incomplete digestion of the lipophilic formulations I and II may be attributed to the low concentration of bile salts and PL as used in this study. Stagnating TG and DG levels may result due to the saturation of the phospholipid/ bile salt micelles with lipolytic products. If lipolytic products can not be effectively removed from the oil-water interface, the rate of lipolysis decreases. These results are in agreement with Sek et al. who reported that efficient digestion and dispersion of long chain lipids was dependent on bile salt concentration[119].

Another effect influencing the rate of lipolysis at least during *in vitro* experiments is the particle size of the dispersions. Armand et al. reported that the pancreatic lipase activity was higher on a fine mixed MCT/LCT emulsion than on a coarse mixed MCT/LCT emulsion (mean droplet sizes 0.46 vs. 3.18 µm)[146]. The higher digestion efficacy of formulations IIIA-LC and IIIB-LC might reflect the more amphiphilic nature of these formulations, thereby facilitating an improved dispersion of the lipolytic products into the phospholipid/ bile salt micelles.

Nevertheless, overall fat assimilation in healthy humans was reported to be not affected by differences in initial droplet size due to efficient fat digestion by pancreatic lipase in the small intestine[147]. This thesis is further supported by Nielsen et al., who reported a non-significant difference between the oral bioavailability of Danazol which was administered as SNEDDS or SEDDS (mean particle sizes of the dispersion 45 nm vs. 4.8 µm) in fasted minipigs[46].

In addition, the fatty acid concentrations determined indirectly by the pH-stat and directly via HPTLC analysis as oleic acid and 12-hydroxystearic acid was compared. The percentage ratio of the analysed fatty acid concentration (pH-stat titration/HPTLC assay) determined during 60 min of simulated digestion is given in Table 9.

In accordance with the results by Sek et al., the efficacy of the titration method was lower in comparison to the determination by HPTLC measurements. This can be attributed to the low degree of ionisation of LC FA at the pH value as used in this assay.

Table 9 *Percentage ratio of the analysed fatty acid concentration (pH-stat titration/HPTLC assay) determined during 60 min simulated digestion of different lipid formulations. Data represent mean of n=3.*

formulation	I-LC	II-LC	IIIA-LC	IIIB-LC	IV
FA_{HPTLC} [mM]	10.9	8.9	13.7	3.9	1.7
$FA_{pH\text{-}stat}$ [mM]	5.6	6.3	7.7	3.3	1.8
FA titrated [%]	51	71	56	82	101

Figure 11b illustrates the FA titration profiles during digestion of MC-based formulations. In comparison to the LC-competitors, significant higher amounts of FA could be detected in all cases. As expected, the amount of fatty acids titrated could be correlated with the content of the oil in the formulation. In contrast to the LC formulations, the digestion of the middle chain acyl derivatives was complete.

These results are in good agreement with previous studies in which has been reported that the digestion of LCT compared to MCT progresses more slowly and undigested LCT can still be present after 60 min digestion[114,115,119,146,148]. This can be attributed to a number of factors such as aqueous solubility, acidity and phase behaviour of the resulting digestion products. The acidity of a compound is determined as acidity constant (pKa). The pKa value represents the ionic environment of the solution where 50% of the molecules are protonated. Table 10 summarises pKa values of fatty acids which have been reported in the literature.

Table 10 *Overview of pKa values of fatty acids in water as reported in the literature*

	pKa value			
Fatty acid	**Kanicky & Shah[149]**	**Kanicky et al.[150]**	**Heikkila et al.[151]**	**Cistola et al.[133]**
Octanoic acid (C_8)	n.a.	6.3-6.5	n.a.	n.a.
Decanoic acid (C_{10})	n.a.	7.1-7.3	n.a.	6.8
Lauric acid (C_{12})	n.a.	7.5	n.a.	8.0
Myristic acid (C_{14})	n.a.	8.1	7.9	n.a.
Palmitic acid (C_{16})	n.a.	8.6	9.7	n.a.
Stearic acid (C_{18})	10.15	n.a.	8.0	n.a.
Oleic acid ($C_{18\ \Delta 9}$)	9.85	n.a.	8.3	8.0-8.5
Linoleic acid ($C_{18\ \Delta 9,12}$)	9.24	n.a.	8.0	n.a.
Linolenic acid ($C_{18\ \Delta 9,12,15}$)	8.28	n.a.	n.a.	n.a.

n.a. …not analysed

Due to lower acidity constants, medium chain FA are ionised to a greater extent than long chain FA at intestinal pH values and at the experimental conditions of this study[133]. Hence, their solubility and amphiphilicity is increased, thus enhancing the potential for formation of sodium or calcium soaps, or incorporation into bile salt micellar systems.

Nevertheless, it should be noted that the apparent pKa value of middle and especially long chain fatty acids underlies high variations due to their interaction with water and the presence of other compounds like proteins or bile salts. The groups of Cistola, Hamilton and Small investigated the acidities of FA in different environments using ^{13}C-NMR spectroscopy[133,135]. They reported that above their monomer solubility limit, fatty acids form aggregates (e.g., micelles, bilayers, oil phases) which lead to higher pka values for decanoic acid. Furthermore, they observed that the apparent acidity constant for oleic acid (pKa 8-8.5) was dramatically lower when incorporated into vesicular structures (pKa 7.5) or bound to albumin (pKa 4.2).

Another reason for higher digestion efficacy of MC glycerides is that the aqueous solubility of MC digestion products is significantly higher in comparison to the LC analogues[152,153]. The solubilities of fatty acids have been determined by several groups. Table 11 summarises most of the reported results. Unfortunately, the data are inconsistent and hardly comparable. One explanation is the different methods which were used for the determination of the FA solubility. Many groups reported that especially higher FAs (above C_{16}) tend to form aggregates at very low concentrations which hampers an accurate determination of the monomeric solubility. Albeit the inconsistent values, it could be demonstrated that the solubility of middle chain FA is significantly higher in comparison to their long chain derivatives.

Table 11 *Overview of monomeric solubilities of fatty acids as reported in the literature*

	Solubility [µM] (temperature in °C)					
reference	**Octanoic acid**	**Decanoic acid**	**Lauric acid**	**Myristic acid**	**Palmitic acid**	**Stearic acid**
Raltson & Hoerr[154]	n.a.	1045 (30)	315 (30)	105 (30)	32 (30)	12 (30)
John & McBain[155]	n.a.	359 (25)	24 (25)	4.2 (25)	3.2 (25)	2.1 (25)
Eggenberger et al.[156]	5472 (30)	372 (30)	0.35 (40)	0.18 (50)	n.a.	n.a.
Nyren & Back[157]	n.a.	n.a.	34 (20)	< 8 (20)	n.a.	n.a.
Vorum et al.[158]	n.a.	n.a.	> 500 (37)	20-30 (37)	< 1 (37)	< 1 (37)
Bell[152]	2200 (25)	n.a.	11.5 (25)	0.8 (25)	0.12 (25)	n.a.

n.a. …not analysed

However, Hofmann investigated the solubility of monoglycerides in phosphate buffer and diluted micellar bile salt solutions. He reported that monoglycerides with a chain length of 12 carbon atoms had appreciable solubility in water (e.g. 3.8 mM for monooctanoin), whereas monoglycerides with higher chain length were practically insoluble in buffer but might form liquid crystalline states; these, however, disperse poorly. The solubility of monooctanoin in

sodium taurodeoxycholate (4 mM) was about 34 mM, whereas the solubility of monoolein was 10 times lower[153].

In conclusion, medium chain lipolytic products are more easily transferred from the oil-water interface forming simple solutions or colloidal species (especially simple or mixed micelles and vesicles[7,115]).

Sek at al. analysed the distribution of medium and long chain lipid digestion products across the different physical phases formed following 30 min digestion of LCTs and C_{18} monoglycerides/diglycerides as well as MCTs and C_8/C_{10} monoglycerides/diglycerides mixture[119]. The long chain lipid digests separated into an oily phase (containing undigested TG and DG), an aqueous phase (containing bile salt, FA and MG) and a pellet phase (containing FA, presumably as an insoluble soap) after ultracentrifugation. Higher proportions of long chain fatty acid and monoglyceride were dispersed into the aqueous phase with increasing bile salt concentrations.

In contrast, medium chain lipolytic products separated only into an aqueous phase and a pellet fraction in a bile-salt-independent manner[119].

For LCT digestion, the removal of the lipolytic products and, thus, the lipolysis rate was mainly dependent on the bile salt concentration and increases with higher concentrations[113]. The bile salt concentration used in this study was not high enough to completely remove LC digestion products from the oil-water interface. Thus, the progress of digestion was limited as proven by HPTLC measurements.

Drug solubility in the post-digestion medium is strongly dependent on the nature of the drug. Zangenberg et al. concluded that the solubilisation of highly lipophilic drugs like Probucol is dominated by the partition of the drug between the lipophilic delivery system and the aqueous phase. In contrast, the dissolution of the less lipophilic Danazol was affected by the solubilisation capacity of the resulting aqueous media[113]. Kossena et al. reported that drugs of lower and medium log P (e.g. Griseofulvin and Danazol) favoured solubilisation in reconstructed model digests containing medium chain lipids, whereas highly lipophilic drug Halofantrine reached significantly higher solubility in media with long chain lipids[7].

In this thesis, similar results were obtained for lipophilic formulations of Probucol (types I and II) but when comparing the more polar formulation types III and IV this effect becomes less distinctive.

Surprisingly, systems IIIA-LC and IV were comparable to I-LC and II-LC and able to retain more than 75% of Probucol in solubilised state after 60 minutes of simulated digestion. Formulation IIIB-LC was comparable to II-MC and IIIA-MC.

After 60 min, further drug precipitation took place in all cases. The further progress of drug precipitation after the decrease of the lipolysis rate has also been reported by the groups of Zangenberg and Christensen[113,117]. They attributed this phenomenon to changes in micellar size or precipitation of bile salts which affects drug solubilisation.

Summarising the digestion experiments, a trend emerges: When formulating lipid systems with long chain acyl glycerides, high amounts of oil are necessary to prevent Probucol precipitation. This trend is in accordance with results by Cuiné et al. They observed that increasing amounts of surfactant and decreasing amounts of LC-based oil resulted in higher precipitation of Danazol during digestion *in vitro* and a reduction of oral bioavailability *in vivo*[35].

In the case of middle chain acyl glycerides this phenomenon was vice versa. The higher the amount of MC-based oil in the formulation, the higher was the amount of precipitated drug. This effect can be attributed to the above mentioned higher tendency of medium chain lipolytic products to dissolve in aqueous media.
Surprisingly, the oil-free formulation IV was comparable to the formulations with high amount of LC glycerides and superior to the formulations with high amounts of MC glycerides. Although it could be shown that macrogol esters of fatty acids are a substrate for pancreatic lipase, degradation was not complete. At about 22% degradation, a plateau was reached where no further digestion took place. Nevertheless, the main fraction of the surfactant is still intact largely retaining Probucol in solubilised state.

These results are in accordance with previous studies. Cuiné et al. evaluated the applicability of different surfactants for the oral administration of Danazol. They concluded that the *in vitro* performance of type IV formulations was better if less digestible surfactants were used. Furthermore, *in vivo* studies demonstrated that single surfactant formulations suffered from poor dispersion and thereby poor bioavailability[36]. Pure surfactants often form viscous, highly-ordered liquid crystalline structures in aqueous media which constrain water penetration[36,40,159]. This phenomenon could be avoided by the addition of co-surfactants or co-solvents as shown in this study. Probucol precipitation from formulation IV can be attributed to changes in the colloidal structures formed upon dilution and partial digestion.

1.4 Conclusion

In this study, lipid formulations which meet the characteristics of the different lipid formulation classes were produced and loaded with the highly lipophilic drug Probucol. Long chain and medium chain glycerides were used for this purpose in order to evaluate their impact on drug solubilisation during dilution and *in vitro* fat digestion.
In terms of drug loading capacity, MC-based formulations were clearly superior towards LC-based formulations which can be attributed to the higher solubility of Probucol in the MC glycerides. Nevertheless, the more polar a formulation gets, the less distinctive is this effect.
Regarding the dispersion experiments, even the polar formulations were able to retain at least 85% of the API in solution. In terms of the digestion experiments, it was evident that all

formulations showed substantial changes in their physicochemical properties during digestion. The lipophilic formulations I-LC and II-LC showed good results during the digestions study but lipolysis was incomplete under the experimental conditions. Nevertheless, a complete degradation can be expected *in vivo*. A major drawback of these lipophilic formulations is that their *in vivo* performance is strongly dependent on individual physiological parameters e.g. native or postprandial bile flow, enzyme activity and others. This food dependency might lead to variations of the oral bioavailability which is especially problematic for drugs with a narrow relationship between toxicity and efficacy. For I-MC and II-MC, the results indicate that digestion will lead to a strong precipitation of the drug.

Promising results were obtained for formulation IIIB-MC and IV which combine high API loading capacity and the ability to maintain the drug in solubilised state during dispersion and simulated digestion. Thus, Solutol® HS 15 turned out to be a very interesting excipient for oral drug delivery. The formulation IIIA-LC represents a good alternative including LC glycerides which are known to trigger the absorption of lipophilic drugs via the lymphatic pathway.

The lipophilicity of the drug and the polarity of the formulations are crucial parameters influencing the performance of the lipid based drug delivery systems. The interactions that occur upon dilution in the intestinal environment and the digestion of lipid formulations are very complex and not easy to predict. The results of this study further underline the importance of dilution and digestion experiments in the early pharmaceutical development phase in order to design more biorobust formulations.

On the basis of these findings further investigative research work should be addressed on the susceptibility of surfactants towards digestion and its impact on drug solubilisation.

2 Susceptibility of surfactants towards pancreatic digestion

2.1 Introduction

In recent years, there has been a trend towards the design of self-emulsifying lipid-based formulations containing large amounts of hydrophilic surfactants (HLB>12) and co-surfactants or co-solvents in an attempt to increase the drug loading capacity and to decrease digestion-induced variability. Formulations comprising large quantities of surfactants belong to types IIIB and IV of the LFCS as proposed by Pouton. These formulation types typically produce fine dispersions when introduced in aqueous media. Particles of submicron size have been suggested to increase drug absorption[146,160].

Surfactants play a crucial role in the self-emulsification process: They reduce the interfacial tension and enable the dispersion process by providing a flexible film that can easily cover the lipid core of an emulsion droplet leading to the spontaneous formation of nano- or microemulsions[161].

Another aspect is that poorly water soluble drugs with intermediate partition coefficients (2<log P<4) are typically less soluble in most natural glyceride lipids than in more amphiphilic surfactants, co-surfactants and co-solvents. For these compounds, drug loading capacity clearly benefits of formulations with high amounts of surfactants. Hence, understanding the performance of formulations comprising larger quantities of surfactants is becoming increasingly important in early formulation development.

Most surfactants which are used in the design of lipid-based drug delivery systems can be classified as type IIIA according to Small's lipid classification system. Non-ionic surfactants are more frequently used than ionic ones due to safety issues[161].

Recently, Strickley has reviewed the non-ionic surfactants in commercially available oral formulations including polyoxyl 35 castor oil (Cremophor® EL), polyoxyl 40 hydrogenated castor oil (Cremophor® RH 40), polysorbate 20 (Tween® 20), polysorbate 80 (Tween® 80), *d*-α-tocopherol polyethylene glycol 1000 succinate (TPGS), macrogol 15 hydroxystearate (Solutol® HS-15), sorbitan monooleate (Span® 80), polyoxyl 40 stearate (Myrj® S40), and various polyglycolyzed glycerides such as Labrafil® M-1944CS, Labrafil® M-2125CS, Labrasol®, Gelucire® 44/14, and Softigen® 767 [51].

It has been demonstrated in many studies that the digestion of the lipid component and formation of the corresponding lipolytic product is a key factor for the *in vivo* performance of a lipid-based drug delivery system. Thereby, it has been shown that long-chain lipid digestion products tend to increase drug solubilisation in the GI tract more effectively than lipolytic products of medium chain TG[7,8,41,42,113,115,162].

Taking into account that most surfactants are esters of FA with polyethylene glycol (PEG) it becomes obvious that they are themselves potential substrates for pancreatic digestive

enzymes. Recent studies indicate that lipase-mediated digestion of surfactants affects the performance of SEDDS as well[35,36,122]. However, little is known about the formation of degradation products of surfactants and their interactions with endogenous lipids like bile salts, phospholipids and dietary lipids.

In this chapter, the susceptibility of a range of commonly used nonionic surfactants towards *in vitro* digestion and the effect of surfactant digestion on *in vitro* solubilisation of poorly soluble drugs was investigated.

2.2 Materials and Methods

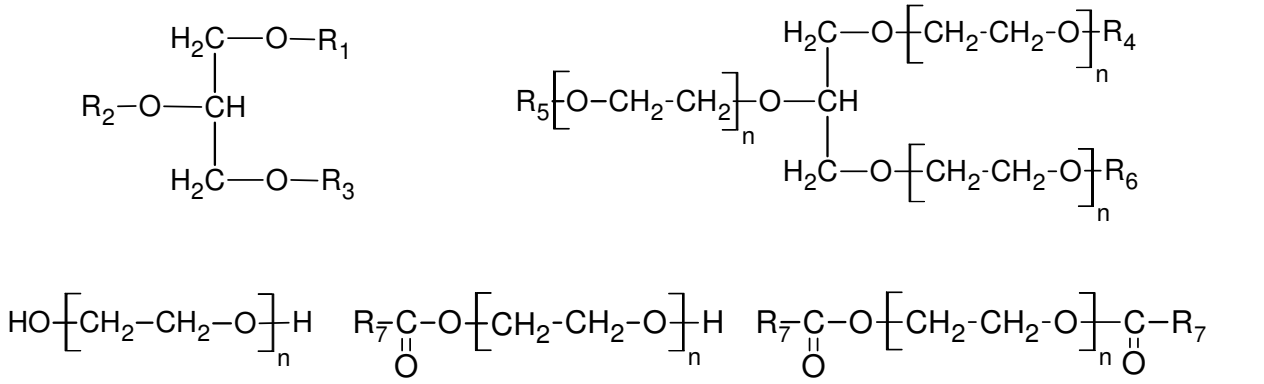
2.2.1 Nonionic surfactants

Cremophor® EL (Polyoxyethylene glyceroltriricinoleat 35, Ph. Eur.; Polyoxyl 35 Castor Oil, USP/NF, BASF)

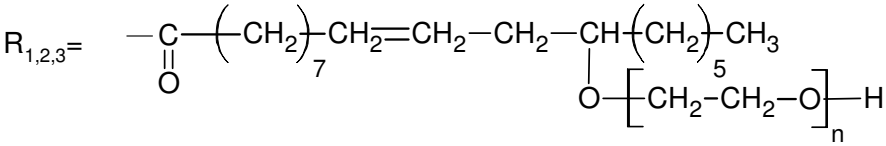
The main component of Cremophor® EL is glycerol-polyethylene glycol ricinoleate, which, together with fatty acid esters of PEG, represents the hydrophobic part of the product. The smaller, hydrophilic part consists of PEG and ethoxylated glycerol (Figure 13). Cremophor® EL is produced by ethoxylation of castor oil with 35 moles of ethylene oxide. The hydrophilic-lipophilic balance (HLB) lies between 12 and 14. The critical micelle concentration (CMC) is approximately 0.02%[163]. Cremophor® EL can be classified as type IIIA according to Small's lipid classification system.

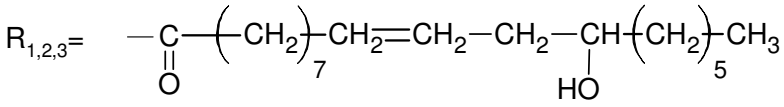
Cremophor® EL was developed by BASF for oral, topical and injectable formulations. Drawbacks of the product are its bitter taste and histamine-related toxicological side effects (anaphylactic reactions, drop of blood pressure, haemolytic reactions) after injection[163,164]. Cremophor® EL is one of the components of the Gengraf® (Cyclosporin A) microemulsion, the Norvir® (Ritonavir) oral solution and soft gelatine capsule, and the Kaletra® (Ritonavir and Lopinavir) soft gelatine capsules[51].

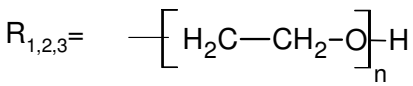
In 2012, BASF has expanded its dermatology and solubilisation range, and developed a new naming system for its products. The new Kolliphor® product family is now including the whole solubilisers range. The new trade name for Cremophor® EL is Kolliphor® EL. Nevertheless, the old trade names were used within this work.



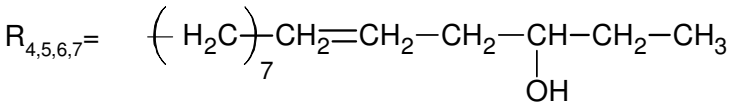
Cremophor EL



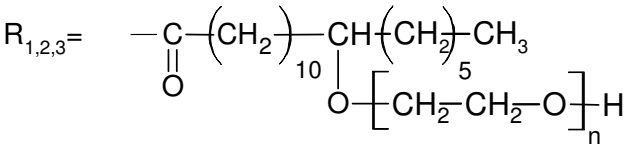


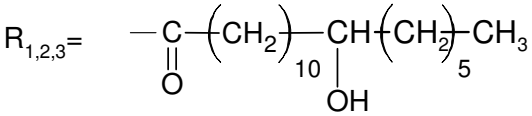


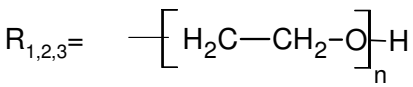
$R_{4,5,6}$= –H



Cremophor RH 40







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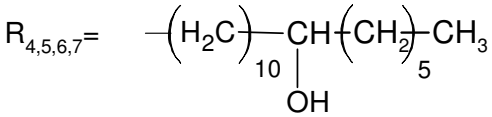


Figure 13 *Chemical structures of the main constituents of Cremophor® EL and Cremophor® RH 40. The products consist of a complex mixture of polyethylene glycols, polyethoxylated glycerols, polyethoxylated fatty acids, and mono-, di-, and tri-esters of glycerol that are polyethoxylated to different degrees.*

Cremophor® RH 40 (macrogol glycerol hydroxystearate, Ph. Eur.; polyoxyl 40 hydrogenated castor oil, USP/NF, BASF)

The main constituent of Cremophor® RH 40 is glycerol polyethylene glycol oxystearate, which, together with fatty acid glycerol polyglycol esters, forms the hydrophobic part of the product (75-83%). The hydrophilic part consists of polyethylene glycols and glycerol ethoxylate (17-25%, Figure 13). Cremophor® RH 40 is produced by ethoxylation of hydrogenated castor oil with 40 moles ethylene oxide. The HLB value lies between 14 and 16.

Cremophor® RH 40 was developed after Cremophor® EL, to produce a solubiliser for oral use that was not bitter[164,165]. Cremophor® RH 40 is one of the components of the Kaletra® (Lopinavir and Ritonavir) oral solution, and the Neoral® (Cyclosporin A) oral solution and soft gelatin capsule[51]. The new trade name for Cremophor® RH 40 is Kolliphor® RH40.

Solutol® HS 15 (macrogol 15 hydroxystearate, Ph. Eur., polyoxyl 15 hydroxystearate, USP; BASF)

Solutol® is a mixture of mono- and diesters of 12-hydroxy stearic acid and polyethylenglycol (Figure 14). Furthermore, about 30% free PEG is present in the mixture. Solutol® is produced by the reaction of 15 moles ethylene oxide per mole of 12-hydroxystearic acid. The hydrophilic-lipophilic balance lies between 14 and 16. According to the product information, the critical micelle concentration (CMC) lies between 0.005 and 0.02%. Gonzáles et al. reported an CMC of 0.021% at 37°C[144]. Solutol® was developed in 1995 by BASF as a low toxicity solubiliser for injection solutions but has also gained interest in the formulation of oral lipid dosage forms[34,130,144,164,166-168]. The new trade name for Solutol® HS 15 is Kolliphor® HS 15.

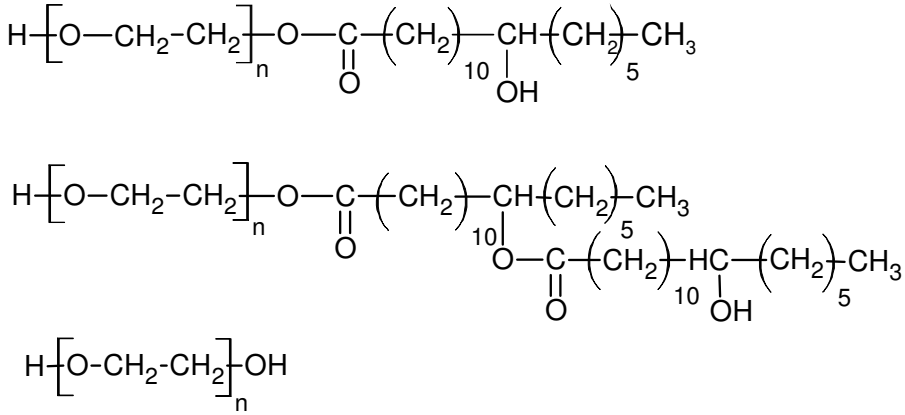


Figure 14 *Chemical structures of the constituents of Solutol® HS 15. The content of free macrogol is about 30%, about 65-70% are mono- and diesters of 12-hydroxy stearic acid and polyethylenglycol.*

Labrafil® M1944 (oleoyl macrogolglycerides, Ph. Eur., oleoyl polyoxylglycerides USP/NF, Gattefossé)

Labrafil® M1944CS is obtained by the partial pegylation of apricot kernel oil and consists of mono-, di-, and triglycerides as well as mono- and di-fatty acid esters of PEG-300 (Figure 15). The dominating fatty acid fractions are oleic acid and linoleic acid. Labrafil® M1944CS is able to self-emulsify on contact with aqueous media forming a coarse dispersion. The HLB value is 4. Labrafil® M1944CS is mainly used as vehicle for highly lipophilic APIs in order to increase their oral bioavailability[169,170].

Labrafil® is a typical example of a multi-component mixture which hampers adequate classification of the excipient into Small's LCS. However, Müllertz et al.[161] recently recommended to classify such mixtures according to the main component and intended use. According to this guidance, Labrafil® can be classified as a polar lipid (class II).

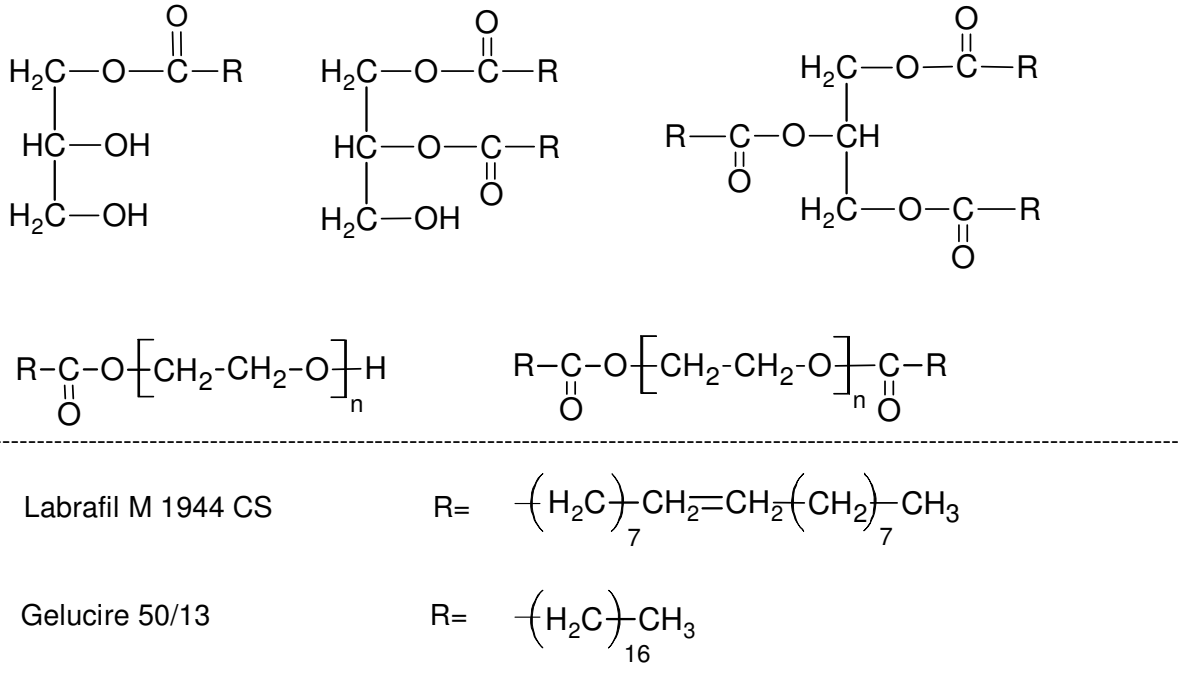


Figure 15 *Chemical structures of Labrafil® M 1944CS and Gelucire® 50/13. Both excipients are mixtures of mono- di and triglycerides as well as mono and diesters of fatty acids and PEG.*

Gelucire® 50/13 (stearoyl macrogolglycerides, Ph. Eur., stearoyl polyoxylglycerides USP/NF, Gattefossé)

Gelucire® 50/13 is a semi-solid mixture of mono- di and triglycerides (aprox. 20%) as well as mono and diesters of fatty acids and PEG 1500 (approx. 72%). In addition it contains about 8% free PEG (Figure 15). More than 90% of the fatty acid fraction are derivatives of stearic and palmitic acid.

Gelucire® 50/13 is obtained by partial alcoholysis of saturated oils using macrogol. It is able to self-emulsify on contact with aqueous media forming a fine dispersion. The HLB value is 13. This excipient is mainly used in formulations with focus on solubility and bioavailability enhancement[170,171]. Gelucire® 50/13 can be classified as type IIIA according to Small's lipid classification system.

2.2.2 Dispersion experiments

Surfactant dispersions were prepared by weighing appropriate amounts of the surfactant in a glass vial and adding Sørensen's phosphate buffer to obtain a 1% (w/v) solution. Agitation was achieved using a magnetic stirrer. Since most of the surfactants were solid or semi-solid at room temperature, the complete container was heated to 50-60 °C and molten samples were mixed properly prior to use. This step was further crucial to avoid inhomogeneity as all surfactants used in this study represent multi-component mixtures. All samples were allowed to disperse completely prior to analysis. The mean particle size of the dispersions was assessed using either photon correlation spectroscopy (PCS; Malvern HPPS, Malvern Instruments, UK) or laser diffraction measurements (LD, Malvern Mastersizer 2000, Malvern Instruments, UK).

2.2.3 Digestion experiments

An appropriate amount of surfactant was weighed into a reaction vessel. Sørensen's phosphate buffer and mixed micelle pre-concentrate was added. The solutions were allowed to equilibrate at 37°C. Constant agitation was provided either by a magnetic stirrer or by utilisation of the end-over-end shaker. In all cases, formulations were completely dispersed prior to initiation of simulated digestion.

Before start of the analysis, sodium chloride and calcium chloride corresponding to 150 mM and 5 mM respectively were added and the pH value of the mixture was adapted to 6.8 using a 1 N NaOH solution. The simulated digestion was started by the addition of pancreatin powder equivalent to 450 units of lipase activity per ml.

Two different monitoring methodologies were applied:

a) pH-stat titration which non-selectively detects the drop of the pH caused by the liberation of free fatty acid
b) analysis of fatty acids derived from the specific surfactant by means of HPTLC coupled with *in situ* densitometric analysis

A detailed description of the methods is given in chapter III.

Estimation of the extent of surfactant digestion

The proportional extent of the digestion of simple components can be achieved by comparison of the mol of digestion products relative to the mol of ester bonds initially present and, therefore, potentially digestible. This can be easily achieved for well defined compounds such as triglycerides but is more challenging for multi-component mixtures. The analytical method which was used for the monitoring of the *in vitro* digestion experiments was not capable to analyse the starting material and the whole spectrum of potential digestion products. In order to estimate the mol of cleavable FA present in 1 g of the surfactant, the ester value of each surfactant was used.

The *ester value* I_E is the number that expresses the quantity of potassium hydroxide in milligrams required to saponify the esters present in 1 g of the substance. It is calculated from the saponification value I_S and the acid value I_A according to the following equation:

$$I_E = I_S - I_A \qquad \textit{eq. 3}$$

The *saponification value* I_S is the number that expresses the quantity of potassium hydroxide (KOH) in milligrams to saponify the esters and neutralise the free acids in 1 g of a sample.

The *acid value* I_A is the number that expresses the quantity of potassium hydroxide in milligrams required to neutralise the free acids present in 1 g of the substance.

2.3 Results and discussion

2.3.1 Characterisation of surfactant dispersions

Surfactants were dispersed in Sørensen's phosphate buffer (1 g surfactant per 100 ml buffer). In the case of Gelucire® 50/13 the solution was heated up to 60 °C in order to facilitate dispersion.

The resulting 1% (w/v) dispersions of Solutol®, both Cremophors® and Gelucire® were clear solutions. When a laser beam was applied to the dispersions, the Tyndall effect could be observed. The mean particle sizes determined as z-average by PCS for Solutol®, Cremophor® RH 40 and EL solutions were 13.4±0.2 nm, 14.5±0.3 nm and 13.7±0.1 nm, respectively (refer to Figure 16A).

In the case of Labrafil® M 1944 CS, a milky dispersion was obtained. Particle size analyses using laser diffraction measurements or PCS indicated the presence of particles in the lower µm range. Since PCS is not applicable in this particle size range, laser diffraction measurements were applied. By means of LD, a heterogeneous particle size distribution was determined for the aqueous Labrafil® dispersion (Figure 16C).

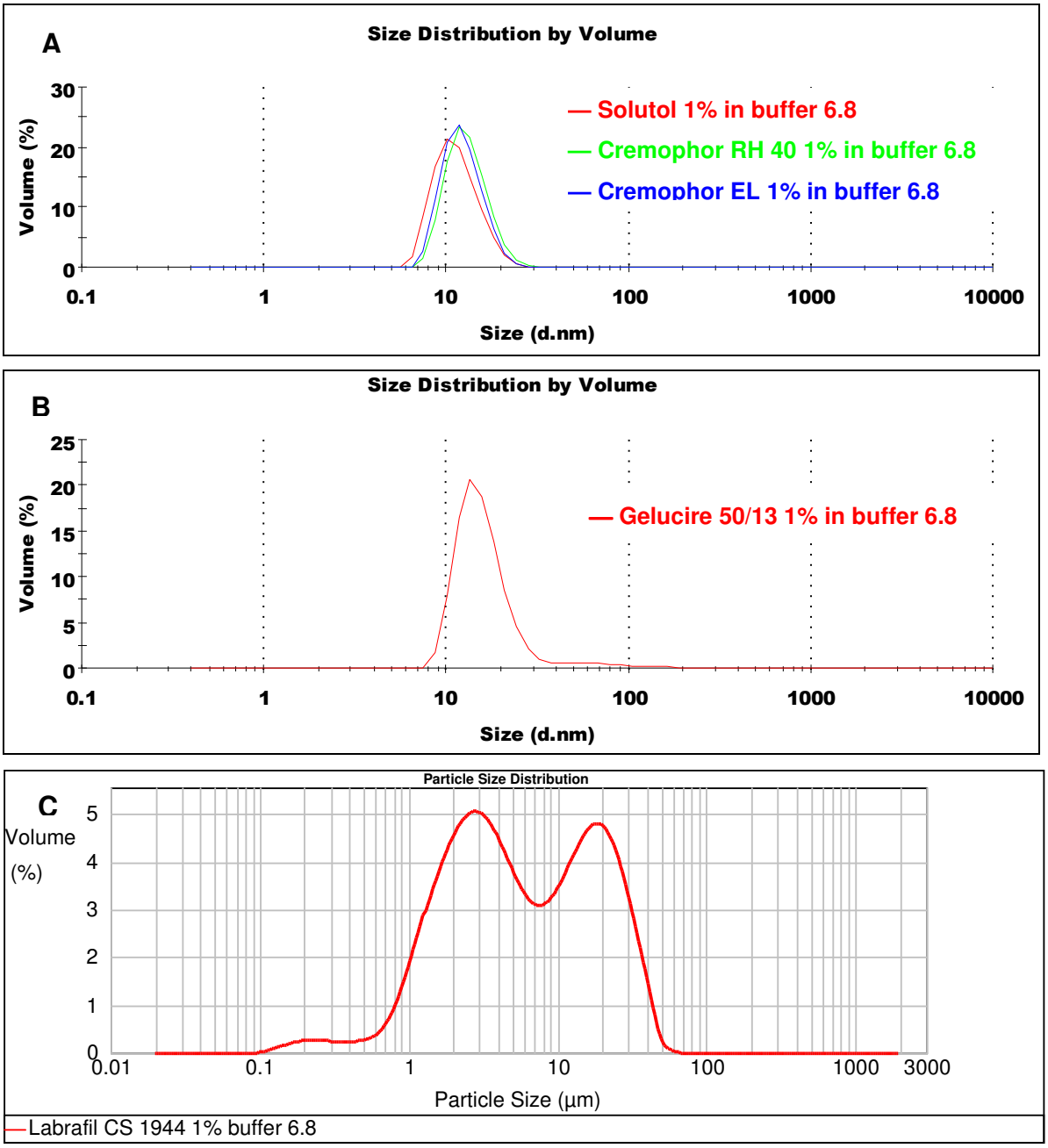


Figure 16 *Particle size distribution of surfactant dispersions as determined by PCS (A+B) and LD (C). surfactants were dispersed in phosphate buffer (1% w/v) pH 6.8 at 37 °C under gentle rotation prior to analyses*
A: *PCS data of Solutol® (red curve), Cremophor® EL (blue curve) and Cremophor® RH 40 solutions (green curve). PDI were below 0.1 in all cases.*
B: *PCS data of Gelucire® dispersion (red curve). PDI was 0.43.*
C: *LD data of Labrafil® dispersion*

The particle size determination of the Gelucire® dispersion failed due to the polydispersity of the sample. Two fractions of about 17 nm and 110 nm were detected but the polydisperisity index (PDI) was greater than 0.4. Hence, the results should be seen as a rough estimation (Figure 16B).

2.3.2 Susceptibility of non-ionic surfactants to simulated digestion

Susceptibility of Cremophor® RH 40, Cremophor® EL and Solutol® HS 15 towards lipolysis

The polar PEG fatty acid esters Cremophor® EL and RH 40 show a very similar chemical composition. Since Solutol® HS 15 can be regarded as a kind of further development of Cremophor® RH 40 and can act as an alternative ingredient in lipid formulations, the susceptibility of the three surfactants towards pancreatic digestion was compared within this chapter.

Two different monitoring methodologies were applied: pH-stat titration which detects the drop of the pH caused by the liberation of free fatty acid non-selectively and the quantification of the fatty acid derived from the specific surfactant following chromatographic separation.

Figure 17 shows the profiles of fatty acid titrated during pancreatin-mediated digestion obtained by the pH-stat technique. Within the very first minutes post initiation of digestion, the pH drops which in turn leads to the addition of sodium hydroxide solution. This process was evident for the blank digestion medium as well as for the surfactant samples. In terms of the blank digestion medium, the pH drop can be attributed to the hydrolysis of phospholipids leading to the formation of lysolecithin and free fatty acids. Furthermore, it could be observed that pancreatin powder, which was used as source of digestion enzymes, influenced the buffering capacity of the digestion medium leading to an additional consumption of NaOH.

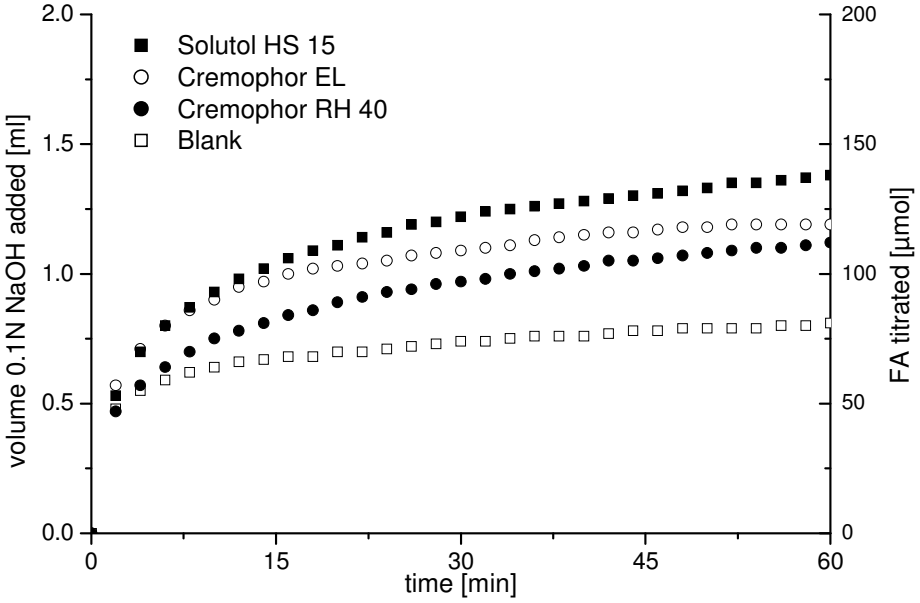


Figure 17 *Formation of fatty acids during lipase-mediated digestion of 3 surfactants and blank medium detected by pH-stat titration. Data represent mean of n=3.*

For all three surfactants, the addition of NaOH was considerably higher than for the blank medium (81±2 µmol) indicating that all surfactants are susceptible to duodenal digestion. The progress of digestion was fastest for Cremophor® EL and Solutol® as can be concluded by the slope of the curve within the first five minutes. Digestion progressed slower for Cremophor® RH 40. After 60 minutes, both Cremophors® reach a similar endpoint (119±1 µmol FA for Crem EL and 112±4 µmol FA for Crem RH 40) whilst for Solutol® higher FA (137±19 µmol) levels were detected.

Figure 18 shows the results of the specific fatty acid concentrations which were detected in the digestion medium utilising lipid analysis by HPTLC/ *in situ* densitometry. This method is more sensitive towards liberated FAs than the pH-stat technique. Interferences of FAs which are not derived by the surfactant but by digestion of the blank medium is nearly excluded because the chromatographic step separates polar hydroxy-FA from less polar aliphatic FAs. Furthermore, respective standards (12-hydroxystearic acid for Solutol® and Cremophor® RH 40 as well as ricinoleic acid for Cremophor® EL) were available for quantification. In agreement to the results obtained by the pH-stat methodology, the same trend was noted: The liberation of FAs was greatest for Solutol® (3.26±0.22 mM) > Cremophor® EL (2.77±0.09 mM) > Cremophor® RH 40 (2.06±0.12 mM).

The differences between the 12-hydroxystearic acid derivatives Cremophor® RH 40 and Solutol® was very surprising. The chemical structure of Cremophor® resembles the natural substrate of pancreatic lipase more than the compounds of Solutol®, but digestion was less effective for Cremophor® RH.

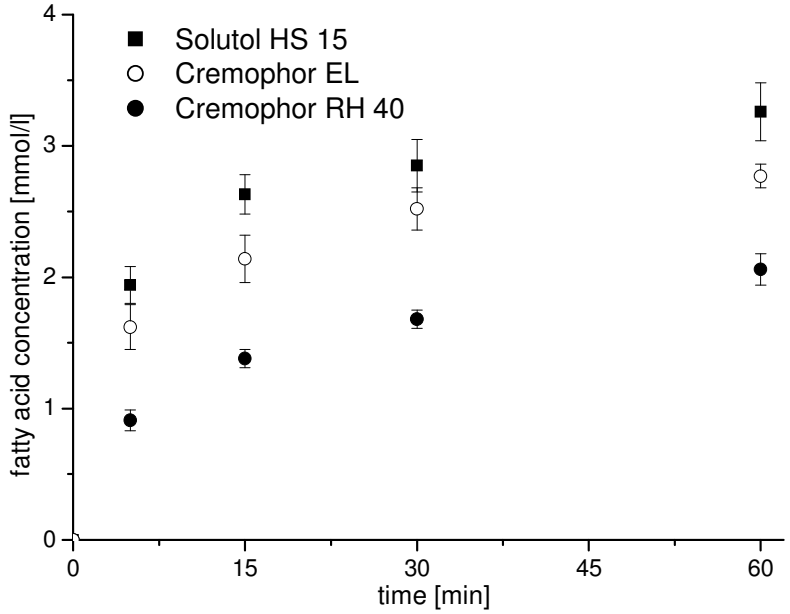


Figure 18 *Formation of fatty acid (12-hydroxystearic acid or ricinoleic acid) during lipase-mediated digestion of 3 surfactants detected by HPTLC-in situ densitometric analyses. Data represent mean±SD (n=3).*

An estimation of the extent of digestion based on the ester value of the respective surfactants is given in Table 12. According to these assumptions, Cremophor® EL contained the highest amount of potentially susceptible ester bonds per mass of the surfactant, followed by Solutol® and Cremophor® RH. Taking the amounts of FA which were detected during simulated digestion by HPTLC analysis into account, it can be concluded that the extent of digestion was similar for both Cremophors but higher for Solutol®. The results are further supported by the data obtained after 60 min of simulated digestion utilising the pH-stat technique. One reason for the incomplete digestion of the surfactants is their inhibitory effect towards pancreatic lipase[140,172]. This effect might be more pronounced for surfactants which show structural similarity towards triglycerides as natural substrate of pancreatic lipase.

These findings are in contrast to studies by Christiansen et al. and Cuiné et al. who reported a significant difference between the extent of digestion of Cremophor® RH and EL (14.4% vs. 6.1% and 30% vs. 7.5%, respectively)[36,140]. The reason for these conflicting results remains unclear. However, a comparison between the data is difficult due to substantial differences regarding the experimental set-up (e.g. substrate concentration, enzyme activity, buffer composition and pH) and the analytical method. Furthermore, both groups estimated the extent of digestion based on the average molecular weight and the average mol of FA per mol of the surfactant neglecting the percentage of hydrophilic reaction compounds as free polyethylene glycol and glycerol ethoxylates which are also present in the surfactant product.

Table 12 *Percentage of digestion calculated from the estimated amount of cleavable fatty acid esters present in one gram of surfactant*

Surfactant	***Ester value*** [a]	***mmol FA/g surfactant***	***mM FA/dig. medium***	***mM FA liberated over 60 min*** [b]	***Hydrolysis [%]***
Solutol® HS 15	61.0	1.09	10.87	3.26 ± 0.22	29.9
Cremophor® EL	66.8	1.19	11.91	2.77 ± 0.09	23.2
Cremophor® RH 40	53.9	0.96	9.61	2.06 ± 0.12	21.4

[a] …data obtained from certificate of analyses of the surfactant batches used in these experiments
[b] …data from HPTLC analyses after simulated digestion of a 1% surfactant solution

Comparison of fatty acid concentrations determined by pH-stat titration and HPTLC techniques

Figure 19 compares the fatty acid concentrations determined indirectly by the pH-stat and directly via the HPTLC analysis over the course of a 60 min experiment.

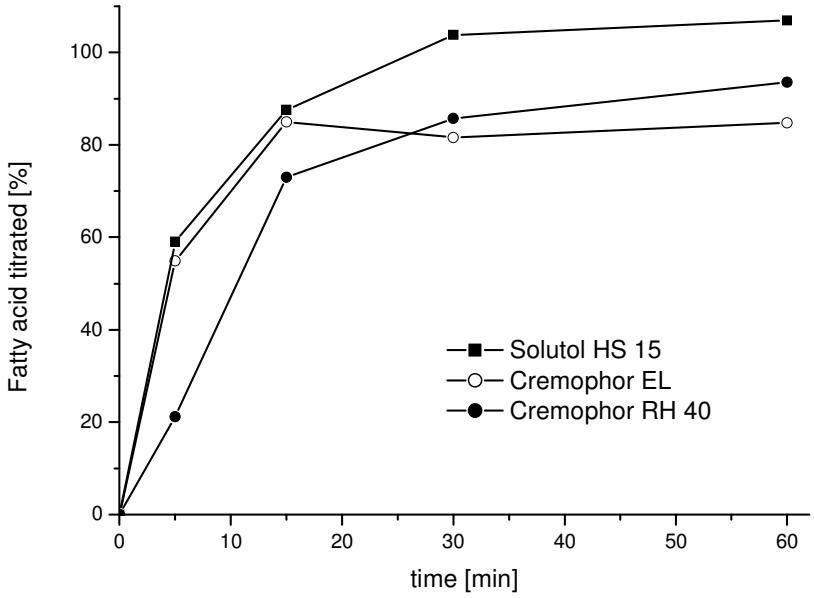


Figure 19 *Percentage ratio of analysed fatty acid concentration (pH-stat titration/HPTLC assay) determined during 60 min simulated digestion of 3 surfactants. Data represent mean, n=3.*

In all cases, the fatty acid concentration assessed by the pH-stat titration lagged behind the concentrations measured directly by HPTLC. By the end of the 60 min digestion period these differences were less evident. From 30 min onwards, a good correlation was observed between titration and HPTLC assay methodologies.

Regarding the digestion of Cremophor® EL, only 84% of the FA determined by HPTLC could be observed. For the 12-hydroxystearic acid derivatives Solutol® HS 15 and Cremophor® RH 40 106.9% and 93.5% of the FA could be detected. Similar results were reported by Sek et al. who observed a lag time between the FA concentrations determined during simulated digestion of LCT and MCT indirectly by the pH-stat and directly via HPTLC analysis[119].

The accuracy of the pH-stat titration technique in quantifying the amount of fatty acids produced during lipid digestion is highly dependent on the availability of titratable FA[119]. This is in turn dependent on the ionisation of fatty acid which is influenced by several factors including the chain lengths of the FA, the pH of the media, the bile salt and electrolyte concentrations as well as the phase behaviour of the FAs[133,135,173]. Enhanced titration efficiencies can be seen when conducting digestion experiments in the presence of bile salts reflecting an improved trafficking of FA to the aqueous phase of the digest as opposed to sequestration of the fatty acid in the oil phase[119,173,174]. In the present experimental setup, bile salt concentration was high enough to detect more than 80% of the FAs by titration.

Kinetics of simulated digestion of Solutol® HS 15

In order to achieve a better understanding of the kinetics of surfactant digestion, further experiments with different substrate concentrations were performed. Solutol® HS 15 was used as model surfactant due to its key role in the development of new lipid formulations. Moreover, very limited information about the performance of Solutol® formulations in the GI tract can be found in the literature until today.

Figure 20 shows the effect of the Solutol® concentrations ranging from 0.05% to 5% (w/v) on the efficacy of surfactant digestion. The higher the Solutol® concentration, the higher amounts of 12-hydroxystearic acid were detected utilising the HPTLC method. However, this relation was non-linear. As further underlined by the estimated extent of digestion, based on the ester value of Solutol®, it becomes clear that increasing amounts of surfactant led to a decrease in the efficacy of the surfactant digestion. Whereas about 37% of the surfactant were degraded at a surfactant concentration of 0.05% (w/v), only 15% degradation could be observed at the 100-fold concentration of 5% (w/v). The reason for this phenomenon might be an inactivation of the lipase by either denaturation of the enzyme at higher surfactant concentrations or by inhibition of its active site. Furthermore, the surfactant components (native components or lipolysis products) and bile salts can form colloidal structures which are not accessible for pancreatic enzymes.

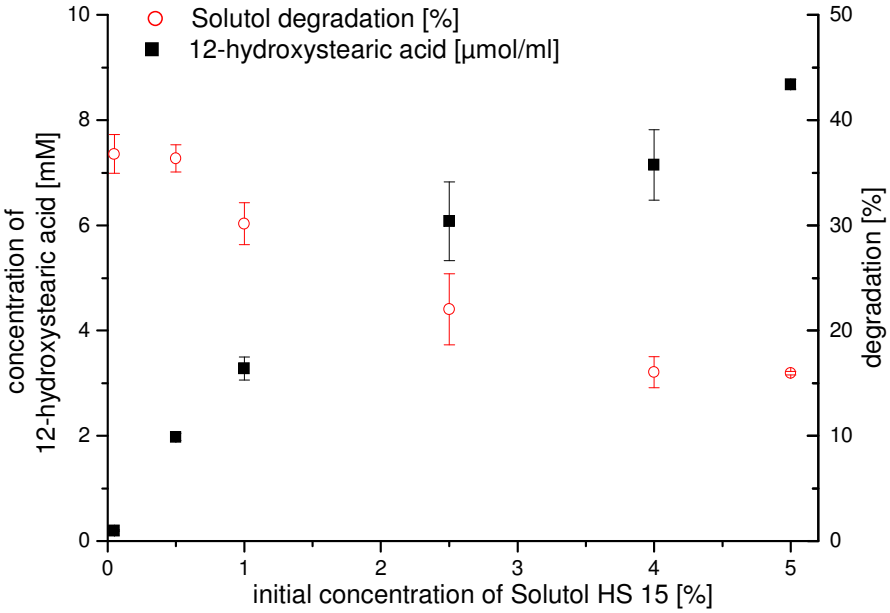


Figure 20 *Formation of 12-hydroxystearic acid after 120 minutes simulated digestion of Solutol® HS 15 in correlation to the substrate concentration ranging from 0.05% to 5% (w/v) (black squares). The red circles represent the percentage of surfactant digestion calculated from the estimated amount of cleavable fatty acid. Data represent mean±SD (n=3).*

In a previous study, Christiansen et al. demonstrated that the ability of various nonionic surfactants to inhibit the digestion of olive oil by pancreatic enzymes was concentration-dependent[140]. Gargouri et al. and Canioni et al. proposed that the inhibition of lipase results

from direct interaction with detergent molecules[175,176]. Furthermore, Hermoso et al. proved that the presence of pure micelles of the nonionic detergent tetra ethylene glycol monooctyl ether was able to activate the enzyme; a process that includes the movement of an N-terminal domain loop (opening of the lid), thereby inhibiting the action of lipase in a competitive way[177,178].

In order to achieve a better understanding on the mechanism behind the activity of pancreatic enzymes against the surfactant Solutol®, competitive inhibition experiments with Tetrahydrolipstatin (THL, Orlistat) were performed. Orlistat is a potent inhibitor of gastric lipase, pancreatic lipase and carboxyl ester lipase. However, it has no activity towards phospholipase A_2[179,180]. THL binds covalently to the serine residue of the active site of the lipase forming a long-lived enzyme-inhibitor complex[181,182]. Orlistat is an approved drug for the treatment of obesity (Xenical® or Alli®).
It could be demonstrated that the co-administration of THL led to a significant decrease of the formation of 12-hydroxy stearic acid (Figure 21). These results indicate that Solutol® is displaced by Orlistat on the active site of the enzyme.

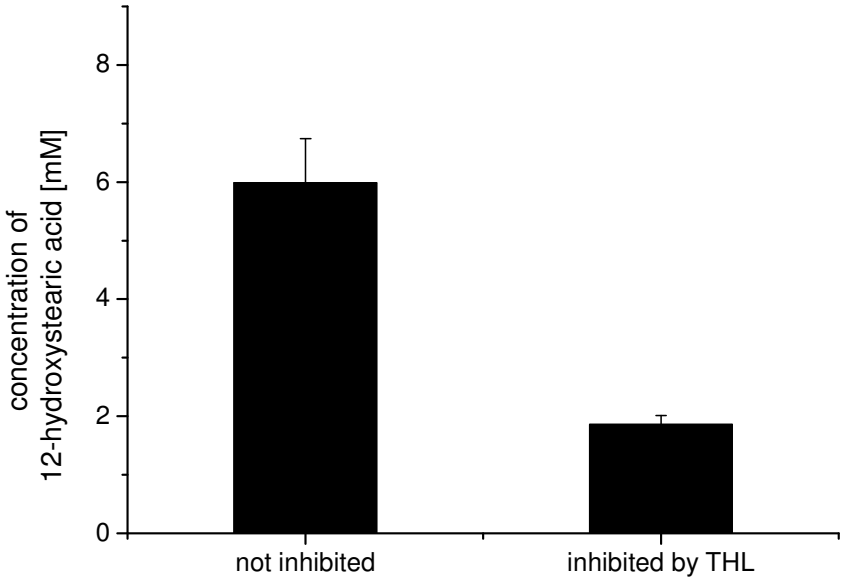


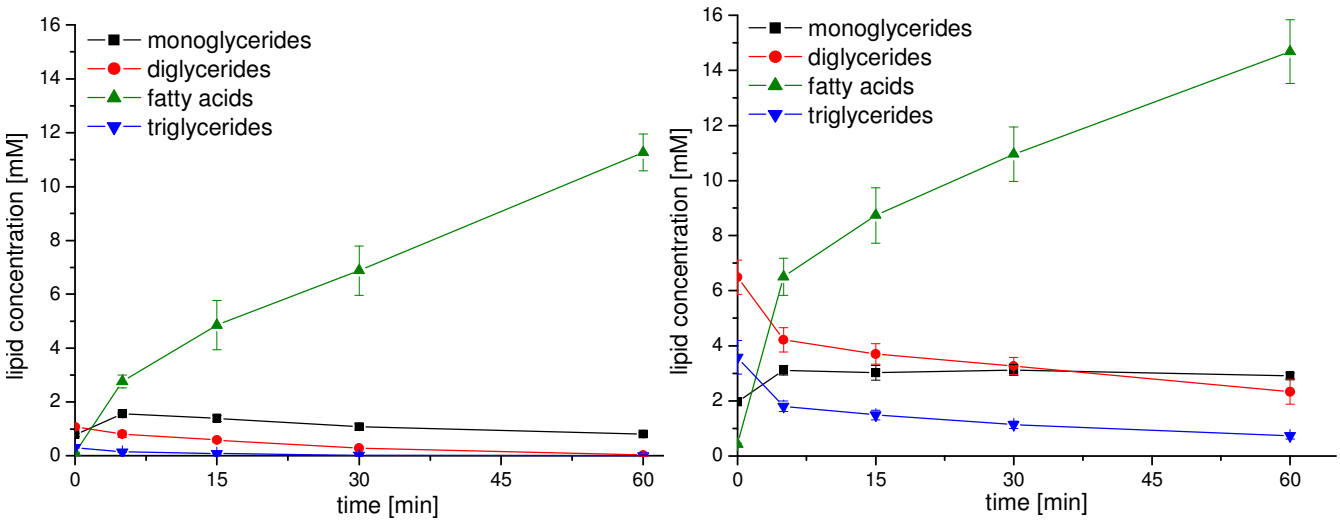
Figure 21 *Formation of 12-hydroxystearic acid after 120 min digestion of 2.5% Solutol® HS 15. The left bar reflects normal digestion conditions. The right bar reflects the results for surfactant solution which was loaded with 2 mg/ml tetrahydrolipstatin (THL) as competitive inhibitor of pancreatic lipase. The rate of inhibition by THL was 69%. Data represent mean±SD (n=3).*

Unfortunately, it is not clear which enzyme present in the porcine pancreatic extract was responsible for the degradation of Solutol® or Cremophor® compounds. Recently, Fernandez et al. investigated the activity of different digestive enzymes on the self-emulsifying excipients Labrasol® and Gelucire® 44/14 containing FA glycerides and PEG esters. They demonstrated that recombinant human pancreatic lipase (rHPL) and purified porcine lipase

(PPL) had a very low activity towards PEG esters, whereas carboxyl ester hydrolase (CEH) and pancreatic lipase-related protein 2 (PLRP2) showed high specific activities towards PEG esters and monoglycerides. Since PLRP2 has not been detected in porcine pancreatic extracts so far[120,121], it can be assumed that CEH rather than pancreatic lipase-colipase might play the key role in the degradation of PEG esters present in Solutol® and the Cremophors®.

Susceptibility of Gelucire® 50/13 and Labrafil® M 1944CS towards lipolysis

Gelucire® 50/13 and Labrafil® M 1944CS are mixtures of mono-, di- and triacylglycerides, mono- and diesters of PEG, and free PEG. The ratio between FA glycerides and pegylated FAs is different as further reflected by the HLB value of both excipients. Figure 22 displays the changes in the lipid concentrations of Gelucire® 50/13 and Labrafil® M 1944CS during *in vitro* lipolysis by pancreatic enzymes monitored by HPTLC analyses. The HPTLC method applied allows only the detection of MG, DG, TG and FA. Pegylated FAs do not migrate due to their hydrophilic nature. Hence, this approach gives no complete picture of the variations in the composition of both excipients during lipolysis. Nevertheless, for Gelucire® 50/13 it could be observed that TG and DG were completely digested within 60 min. The levels of MG increase within the first minutes followed by decreasing concentrations after this initial phase. As proven by HPTLC densitograms, this further degradation can be attributed to the presence of 1.3-diglycerides which can be completely cleaved to free glycerol and FAs. Furthermore, 1-MG may also be present in the initial Gelucire mixture. FA concentrations showed a strong increase even after 30 min of lipolysis. Mass balance calculations revealed that 303% of FA equivalents were obtained after 60 min of simulated digestion.



A: Gelucire® 50/13 B: Labrafil® M 1944CS

Figure 22 *Lipid fraction obtained after simulated digestion of Gelucire® 50/13 (left) and Labrafil® M1944CS (right) as analysed by HPTLC/in situ densitometric measurements (data represent mean±SD, n=3). FA concentrations were corrected by the FA levels as determined by digestion of the blank medium.*

Similar results were obtained during the digestion of Labrafil® M1944CS. The initial amounts of MG, DG and TG were significantly higher in comparison to Gelucire® reflecting the different composition of the two excipients. The DG and TG levels decreased very fast during the first minutes of digestion. However, the digestion of the oil components was not completed after 60 min. The MG levels increased initially, reaching then a kind of plateau with more or less constant levels of 3 mmol/l. In accordance with the results obtained by the Gelucire® digestion, FA levels further increased and mass balance calculations revealed that 123% of FA equivalents were obtained after 60 min of simulated digestion.

These results are in qualitative agreement with Fernandez el al. who investigated variations of the components of Labrasol® and Gelucire® 44/14 during lipolysis. Both excipients consist of MG, DG and TG and PEG-monoesters and PEG-diester. In addition to the MG, DG, TG and FA levels, Fernandez et al. were able to determine the concentrations of PEG-monoesters, PEG-diesters and free PEG during simulated gastric and duodenal digestion. They observed that PEG-mono- and diesters were cleaved to a great extent during lipolysis by porcine pancreatic extract leading to the formation of free PEG and FAs[122]. Since the degradation of PEG-esters cannot be monitored with the applied HPTLC method, it was assumed that the high amounts of FAs, which cannot be attributed to the degradation of FA-glycerides, were derived from the degradation of the PEG-esters. This was further underlined by the mass balance calculations which were significantly higher for Gelucire® 50/13 containing approximately 72% of PEG esters, in contrast to Labrafil® M 1944CS.
Figure 23 shows the FA profiles obtained during simulated pancreatin-mediated digestion as monitored by pH-stat titration. In both cases, there was a slow, transient increase of the FA concentrations, hence, the digestion did not seem to be completed after 60 min. In agreement to the results obtained by HPTLC analyses, higher FA concentrations were detected during the digestion of Labrafil® M 1944CS although the difference towards Gelucire® was less pronounced. The results obtained by the pH-stat titration provided very limited information about the susceptibility of the excipients towards digestion. Furthermore, a comparison of the FA levels detected by pH-stat titration and HPTLC analyses revealed that the titration efficacy was very low (40-41%) hampering substantial conclusions about the extent of excipient digestion. This can be attributed to the low degree of ionisation of long chain FAs at the pH value used in this assay.

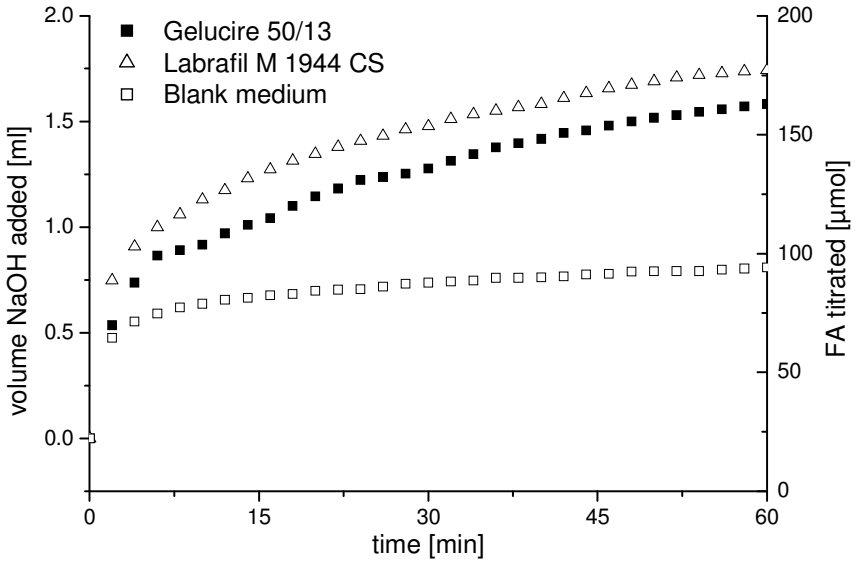


Figure 23 *Formation of fatty acid during pancreatin-mediated digestion of Gelucire® 50/13, Labrafil® M 1955CS and blank medium detected by pH stat titration at pH 6.8.*

Due to the complex lipid composition of both excipients, a clear conclusion about the extent of digestion could not be made. Hence, the extent of digestion was determined based on the ester value of both excipients as described in chapter 2.2.3 (Table 13).
Nevertheless, one should emphasize that a calculated hydrolysis of 100% would include the degradation of monoacylglycerides as well, however, this is generally believed to happen only for 1-monoacylglycerides.

Table 13 *Percentage of digestion calculated from the estimated amount of cleavable fatty acid esters present in one gram of surfactant*

Surfactant	*Ester value* [a]	*mmol FA/g surfactant*	*µmol FA /ml digestion medium*	*mM FA liberated during 60 min* [b]	*Hydrolysis [%]*
Gelucire® 50/13	74.67	1.33	13.30	11.27 ± 0.68	84.7
Labrafil® M 1944 CS	165	2.93	29.30	14.15 ± 0.53	48.3

[a]…data obtained from certificate of analyses of the surfactant batches used in these experiments
[b]…data from HPTLC analyses after simulated digestion of a 1% surfactant solution

Labrafil® with its high amounts of FA glycerides has the larger ester value and, therefore, the highest amount of cleavable FAs per gram. For Labrafil®, a hydrolysis level of 49% was obtained. This can be attributed to the incomplete digestion of TG and DG. 30% of the oily components (DG+TG) were still present after 60 min of simulated digestion. Moreover, the 2-monoacylglycerides accumulated as lipolytic products, which did not contribute to the hydrolysis level as explained earlier. Unfortunately, none of the methods applied was capable of analysing the PEG esters. Nevertheless it was assumed that PEG diesters and monoesters were at least partially degraded by pancreatic enzymes. Fernandez et al.

reported that CEH, which is supposed to be present in porcine pancreatic extract, has reasonable activity towards PEG mono- and diesters and may be the enzyme that contributes the most on the degradation of PEG esters[120,121]. The incomplete digestion of the oily components (TG and DG) may be attributed to the low concentration of bile salts and PL as used in this study. Stagnating TG and DG levels can be an effect of saturation of phospholipid/ bile salt micelles with lipolytic products. If lipolytic products cannot be effectively removed from the oil-water interface, the rate of lipolysis decreases. These results are in agreement with Sek et al. who reported that efficient digestion and dispersion of long chain lipids was dependent on the bile salt concentration[119].

For Gelucire®, containing large amounts of PEG-32-esters and only small amounts of FA glycerides, the ester value was significantly lower. Nevertheless, a hydrolysis level of 85% was determined for Gelucire® indicating that digestion was completed.
These results correlate well with the data obtained after simulated digestion of Gelucire® 44/14 by Cuiné et al. who reported a degradation level of 79% using a similar calculation approach based on the average molecular weight and average mol FA per mol surfactant[36]. Moreover, the results obtained by Fernandez et al. confirmed that more than 80% of the Gelucire® components were degraded during simulated digestion[122].

2.4 Conclusion

The data obtained in these studies further highlight the importance of considering the impact of pancreatic enzyme-mediated digestion on *in vivo* drug solubilisation and drug absorption when developing self-emulsifying drug delivery systems. More specifically, the results suggest that nonionic surfactants containing fatty acid esters are at least partially susceptible to pancreatin-mediated hydrolysis. Using the example of Solutol® it could be demonstrated that this effect was concentration dependent. Moreover, the data suggest that the hydrophilic PEG esters Cremophor® EL and RH as well as Solutol® might be able to retain their solvent capacity during digestion due to limited hydrolysis. This finding is further supported by the promising results of the hydrophilic formulations containing Solutol® as discussed in the previous chapter and by the results obtained by Cuiné et al.[36] for the Cremophors®. For Gelucire® and Labrafil®, a higher extent of digestion can be expected *in vivo* which might result in a higher variability of the formulation performance.
In conclusion, the knowledge of such effects contributes to a more rational choice of excipients for a successful development of lipid based delivery systems.

3 Susceptibility of sucrose ester formulations towards pancreatic enzyme-mediated digestion

3.1 Introduction

Sucrose esters (SEs) are non-ionic emulsifiers which are produced by esterification of sucrose and natural FAs derived from edible oils. As sucrose has eight hydroxyl functions, compounds ranging from mono- to octa- FA esters can be produced (Figure 24). The most common FAs used in SEs are lauric, myristic, palmitic, stearic, oleic, behenic and erucic acids.

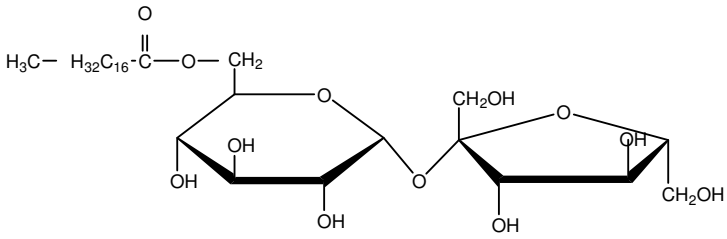


Figure 24 *Chemical structure of sucrose monostearate*

Sucrose ester cover a broad range of polarity spectrum (HLB from 1-16). The chain length of the FA (C_{12}–C_{22}) und the degree of esterification (one to eight) determines the properties of the product (Figure 25). In the pharmaceutical sector, SEs of lauric, palmitic and stearic acid are available as compendial material.

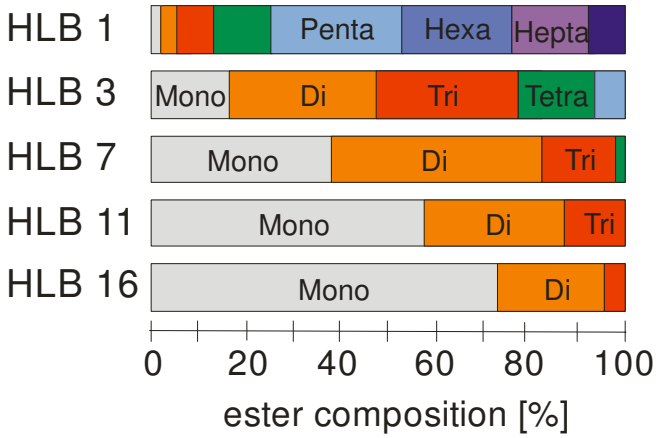


Figure 25 *Schematic representation of the correlation between ester composition and HLB value of sucrose ester products (adapted from Surfhope SE Pharma broschure [183]). The products are mixtures of molecules with different substitution degrees and, therefore, mixtures of substances with different properties (e.g. hydrophilicity, solubility, dispersibility, melting point).*

According to the lipid classification system by Small, sucrose monoesters are class IIIa lipids. They are soluble amphiphiles and undergo lyotropic mesomorphism. Higher esters are insoluble swelling amphiphiles belonging to class II of the LCS.
The application history of SEs can be found in the cosmetic and food industry where they have been used for years. Due to their surfactant and other versatile properties, SEs have gained strong interest by researchers and formulators in the pharmaceutical sector. In oral solid dosage forms, SEs have proven to be an alternative lubricant to magnesium stearate or stearic acid. Furthermore, formulations containing SEs as matrix agent for the sustained release of drugs can be found on the market and in various scientific research publications[184-187]. Moreover, SEs have been shown to improve drug dissolution and to enhance the oral, nasal and ocular drug absorption[186]. These properties make SEs exciting and promising excipients in the development of dosage forms for different applications.
As surfactants, they play a role in the solubilisation or stabilisation of drugs in different preparations. SEs of long-chain FAs are widely tasteless, odourless, non-toxic and non-irritant. Hydrophilic esters of middle chain FAs have stronger surface active properties, but also a bitter taste which limits their use. For that purpose, the focus of this work was set on SEs of stearic acid.

Despite their growing use in pharmaceutical formulations, little is known about the influence of digestive enzymes on the performance of SE formulations. Metabolism studies of pure SEs in rats indicate that higher fatty acid esters of sucrose such as the octa-, hepta- and hexa-esters are not absorbed but excreted unchanged, while the lower esters are partially hydrolysed and absorbed as sucrose and individual fatty acids[186,188-190]. Taking into account that digestion of lipidic excipients has been shown to influence the drug solubilisation capacity of various formulations, it is obvious that more investigative work has to be addressed towards the effect of digestive enzymes on SE preparations.
The aim of this work was to examine the susceptibility of different SE formulations towards pancreatic enzymes in order to contribute to a better understanding of the *in vivo* performance of SE preparations. Furthermore, the influence of biorelevant media including digestive enzymes on the drug dissolution kinetics from a commercially available sustained release tablet formulation based on sucrose palmitate was investigated.

3.2 Materials and methods

Sucrose ester

In this study, sucrose ester D1811 (Mitsubishi Kagaku Foods Corporation, Japan) was used. The code '18' stands for the dominating FA which is esterified: In this case it is stearic acid. The second number reflects the HLB value which is 11. The letter 'D' indicates the presence of a Drug Master File (DMF). Sucrose stearate D1811 contains at least 70% esters of stearic acid whereupon minimum 55% are monoesters, maximum 40% diesters and maximum 20% are higher esters.

In addition, esters of sucrose and stearic acid with a HLB of 3, 5, 7, and 16 (D1803, D1805, D1807, D1816, Mitsubishi Kagako Foods Corporation) were used for analytical investigations. The relationship between the HLB value of the SEs and their composition is given in Figure 25. All SEs were used as received.

3.2.1 Characterisation of the sucrose ester D1811

In order to allow a deeper characterisation of the SE composition during simulated digestion, an analytical method was developed that should be capable of determining sucrose mono- and diester levels.

HPTLC analyses for sucrose esters

For the separation of the SE, a modified HPTLC method to the one described by Jaspers et al.[191] was developed. For that purpose, the SEs were dissolved in chloroform/methanol (1:1) and separated on a silica gel 60 HPTLC plate (Merck, Germany) by Automated Multiple Development (AMD) using a 4-step gradient based on methanol, water and chloroform (Table 5, AMD 2, CAMAG, Switzerland).

Post-chromatographically, HPTLC plates were immersed into a copper sulfate solution (copper sulfate 10% (w/w), phosphoric acid 8% (w/w), methanol 5% (w/w) and water 77% (w/w)) and heated to 150 °C for 20 min in an oven. Following derivatisation, the lipids were quantified by *in situ* spectrodensitometric measurements using a CAMAG TLC Scanner 3 at 675 nm.

Identification of sucrose esters by Matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry (MALDI-TOF-MS)

The fractions obtained by chromatographic separation were analysed and identified using MALDI-TOF-MS. After separation of the SEs via HPTLC, the bands of interest were collected, extracted and re-dissolved in methanol or methanol/chloroform.

Appropriate volumes of the fractions were mixed with a matrix solution (2.5-dihydroxy benzoic acid 20 mg/ml in acetonitrile/water, 50:50). 1 µl of this solution was spotted on the

sample plate. Co-crystallisation was achieved by air drying. Blank experiments were performed in the same manner in order to identify signals derived from the silica matrix.
MALDI-TOF mass spectra were performed using the Voyager DePro (Applied Biosystems, Germany) in reflector mode. The samples were ionised by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with a time delayed extraction before entering the time of flight mass spectrometer. Detector operation was in the positive ion mode, ranging from 500 to 1500 amu. Sequazyme™ peptide standard kit (Applied Biosystems) was used for calibration in this range. Each spectrum represented the sum of at least 6x100 laser pulses.

3.2.2 Preparation of sucrose ester formulations

Different SE formulations were prepared in order to investigate the susceptibility towards pancreatic enzymes (Table 14). An aqueous dispersion of the SE was prepared by weighing appropriate amounts of D1811 in a glass vessel and adding water to obtain a 5% (w/v) dispersion. The mixture was heated to 60°C under agitation provided by a magnetic stirrer and subsequently cooled down to room temperature.
Furthermore, an emulsion consisting of 5% (w/w) liquid paraffin (Caelo, Germany) and 5% sucrose fatty acid ester D1811 as emulsifier was produced using a pneumatically driven high-pressure homogeniser (Stansted Fluid Power, UK), which worked in two-stage mode. Five cycles were run at 100 MPa (first stage) and 10 MPa (second stage, back-pressure module), followed by one cycle at 40 MPa. The samples were allowed to equilibrate for 24 h prior to further analysis.
In addition, the commercially available fat emulsion Lipofundin® 10% N containing 10% of soybean oil was diluted with an aqueous dispersion of D1811 to achieve soybean oil emulsions containing 0.5-2.5% of the sucrose ester. The Lipofundin®-sucrose ester test substrates were homogenised using an ultra-turrax (IKA, Germany).

Table 14 *Composition of different sucrose ester formulations*

Sample	$SE_{disp.}$	$SP_{emulsion}$	$SL1_{emulsion}$	$SL2_{emulsion}$	$SL3_{emulsion}$	$SL4_{emulsion}$
D1811 [%]	5	5	-	0.5	1.0	2.5
Paraffin [%]	-	5	-	-	-	-
Lipofundin®10% N [%]	-	-	100	99.5	99.0	97.5
Water [%]	95	90	-	-	-	-

3.3 *In vitro* digestion experiments

The *in vitro* digestion experiments were performed as described earlier in this thesis. The digestion was followed using a pH-stat titration unit (Mettler DL 21), which maintained the pH at 6.8. All emulsion substrates were incubated in the digestion medium reflecting an oil content of 3.33% (v/v).

3.4 Dissolution of Ibuprofen sustained release tablets in biorelevant media

Commercially available Ibuprofen sustained release tablets 'Ibuprofen AL 800 retard' (Lot: 82739) by Aliud Pharma GmbH & Co. KG (Laichingen, Germany) containing sucrose dipalmitate as release controlling agent were purchased in a local pharmacy. The qualitative tablet composition as labelled in the patient information leaflet is given in Table 15.

Table 15 *Key facts of sustained release 'Ibuprofen AL 800 retard'*

API, strength	Ibuprofen, 800 mg
List of inactive ingredients	glucose monohydrate
	magnesium stearate
	povidon K 30
	sucrose dipalmitate
	stearic acid
	talcum
Tablet mass	1,200 mg

For all dissolution tests, the USP Apparatus 2 (paddle method, Pharmatest, Germany) was used employing 900 ml of dissolution medium at a temperature of 37±1°C and a paddle speed of 75 rpm. The composition of the dissolution media is given in Table 16. The biorelevant media (FaSSIF and FeSSIF with pancreatin) were produced as described in a previous chapter. Samples of 2 ml were withdrawn at 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h. The aqueous samples were centrifuged at 12,100 g and an aliquot of the supernatant was further diluted with acetonitrile for subsequent analysis by HPLC. All experiments were run in triplicate.

Table 16 *Composition of the dissolution media*

Ingredient	buffer	Buffer with salts	FaSSIF	FeSSIF with pancreatin
Bile salts [mM]	-	-	5	15
Phospholipids [mM]	-	-	1.25	3.75
Sodium chloride [mM]	-	150	150	150
Calcium chloride [mM]	-	5	5	5
Na_2HPO_4/KH_2PO_4 [mM]	31.1/35.6	31.1/35.6	31.1/35.6	31.1/35.6
Pancreatin powder with lipase activity [U/ml]	-	-	-	450
pH value	6.8	6.8	6.8	6.8

HPLC analyses

The content of Ibuprofen was analysed by HPLC. The HPLC system consisted of a Merck Hitachi system equipped with an AS 4000A auto sampler, a L 6200A programmable pump, a L 4250 UV-Vis detector and a column oven. Ibuprofen was analysed using a LiChrospher® RP-select B (125x4.6 mm, 5 µm, Merck, Germany) column fitted with a C18 guard column (LiChroCART 4-4, Merck, Germany). The mobile phase was 55/45 (v/v) water with 0.2% phosphoric acid (85%)/acetonitrile. The flow rate was 1.5 ml/min leading to a retention time of 4.3 min. For detection the wavelength of 214 nm was used. The lowest concentration of Ibuprofen which could be analysed with acceptable accuracy and precision (±20%) was 2 µg/ml.

Statistical data analysis was performed using the Student's t-test with a minimal level of significance of 5% provided that data distribution was normal.

3.5 Results and Discussion

3.5.1 Analytical characterisation of the sucrose ester D1811

By means of HPTLC, SE D1811 could be separated into 10 fractions (Figure 26). According to Jasper et al., sucrose monoesters should have low migration distances, whereas diesters and higher esters migrate longer distances[191]. In order to verify the separation of the different esters, sucrose stearate products with an HLB range from 3 to 16 were spotted onto the HPTLC plate. The less polar SEs reflected by lower HLB values contain higher amounts of sucrose polyesters and less amounts of mono- or diesters. For polar SEs with high HLB values this effect was vice versa. For SEs with a HLB of 3, 5 or 7 the intensity of spots 8-9 was higher in comparison to the polar SEs with a HLB of 11 or 16. For the polar SEs with a HLB of 11 or 16, the intensity of spots 1-4 was much higher in comparison to the SEs with lower HLB values. The intensity of spots 5-7 was quite similar for all sucrose stearate types.

Figure 26 *Photograph of a HPTLC plate after separation via a 4-step development program based on methanol/water/chloroform and post-chromatographic derivatisation. Five sucrose ester (SE) preparations ranging from HLB 3 to 16 and stearic acid (SA) were spotted onto the silica plate representing 50 and 125 µg of the SE, respectively. The different SEs were separated into 10 fractions as illustrated in the drawing to the right. Less polar SEs reflected by lower HLB values contain higher amounts of sucrose polyesters and less amounts of mono- or diesters. For polar SEs with high HLB values this effect is vice versa. For SE with an HLB of 3 the intensity of spots 8-9 is higher in comparison to the polar SE with HLB 16. For the polar SE with HLB 16, the intensity of spots 1-4 is much higher in comparison to the SE with lower HLB values.*

Subsequent identification by MALDI-TOF mass spectra yielded into 4 peaks of monoesters (1-4) and 3 peaks of diesters (5-7) of sucrose and stearic as well as palmitic acid (Figure 26 and Table 17). Since there was no difference in the retention of stearic or palmitic derivatives, the presence of different peaks could be attributed to the different positions of esterification.

The lipophilic polyester suffered from poor ionisation. Peaks 8 and 9 could not be identified by MALDI-TOF-MS. According to Jaspers et al., these spots can be attributed to higher esters of stearic acid or palmitic acid with sucrose. Fraction 10 could be attributed to free fatty acids due to similar retention time of an external standard solution.

Table 17 *Identification of the different fractions obtained after chromatographic separation of sucrose ester D1811 by correlation of the masses determined by MALDI-TOF-MS*

Peak	Masses [Da]	identification
1	603.4	[sucrose monostearate + Na]$^+$
	631.4	[sucrose monopalmitate + Na]$^+$
2	603.4	[sucrose monostearate + Na]$^+$
	631.4	[sucrose monopalmitate + Na]$^+$
3	603.4	[sucrose monostearate + Na]$^+$
	631.4	[sucrose monopalmitate + Na]$^+$
4	603.4	[sucrose monostearate + Na]$^+$
	631.4	[sucrose monopalmitate + Na]$^+$
5	870.8	[sucrose palmitate-stearate + Na]$^+$
	898.8	[sucrose distearate + Na]$^+$
	842.6	[sucrose dipalmitate + Na]$^+$
6	870.8	[sucrose palmitate-stearate + Na]$^+$
	898.8	[sucrose distearate + Na]$^+$
	842.6	[sucrose dipalmitate + Na]$^+$
7	870.8	[sucrose palmitate-stearate + Na]$^+$
	898.8	[sucrose distearate + Na]$^+$
	842.6	[sucrose dipalmitate + Na]$^+$
8	n.a.	n.d.
9	n.a.	n.d.
10	n.a.	n.d. / fatty acid according to a reference solution

n.d. …not determined

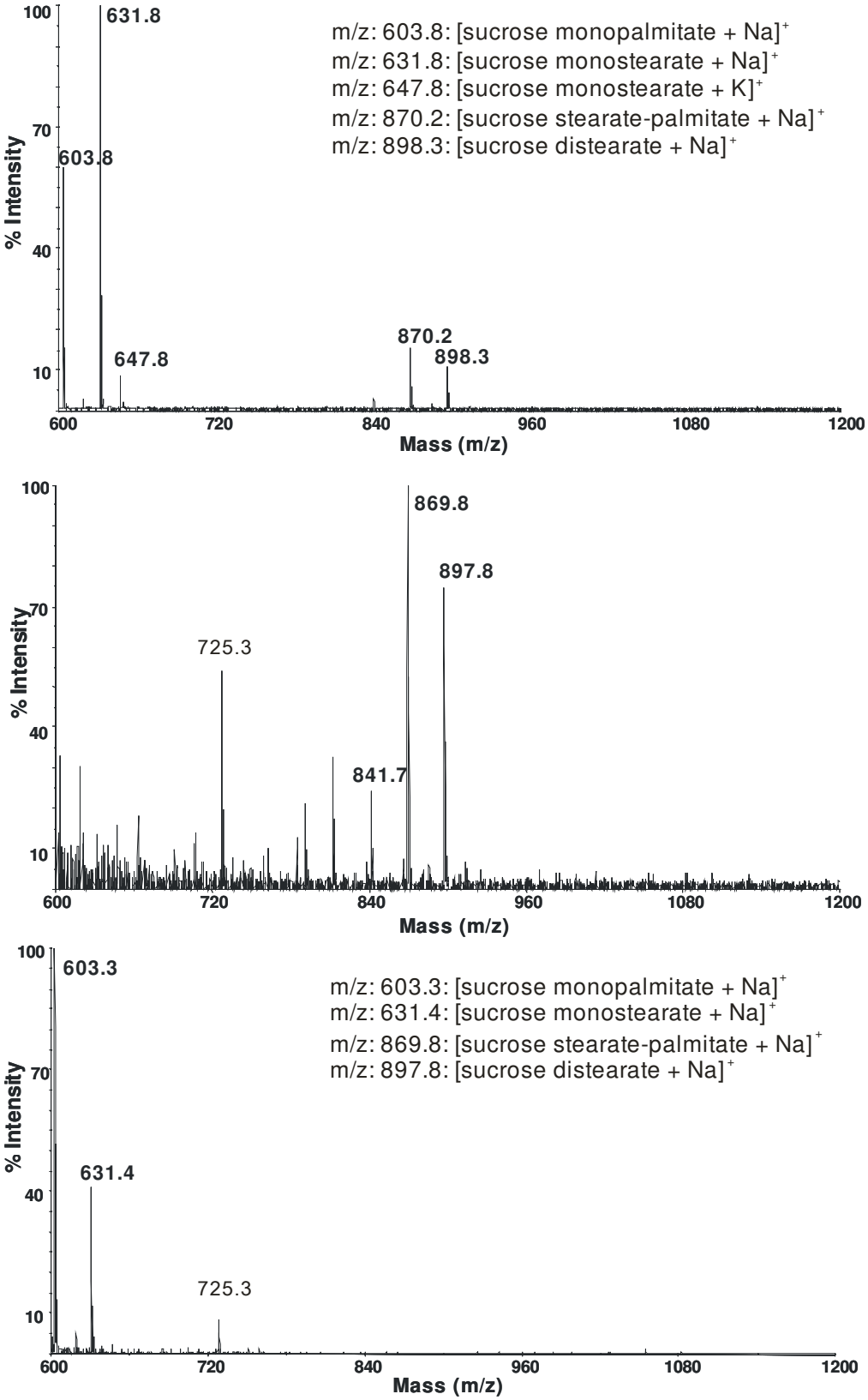


Figure 27 *MALDI-TOF mass spectra of sucrose ester D1811 (top) and exemplary spectra of isolated SE fractions 1 (bottom) and 5 (middle) after separation via HPTLC. Fraction 1 contains sucrose monoester and fraction 5 sucrose diester. Further peaks which were detected from the isolated SE fractions could be attributed to matrix components deriving from the silica plate (e.g. peak at 725 Da).*

3.5.2 Susceptibility of sucrose ester D1811 towards lipolysis

In a first trial, the influence of enzymatic lipolysis on the SE composition should be monitored using the HPTLC method described earlier. Unfortunately, strong interferences between the hydrophilic components of the blank medium (phospholipids and bile salts) and the sucrose monoester fraction was observed (Figure 28). Hence, monitoring of changes in the SE compositions was not possible using this method. Therefore, the pH-stat titration had to be applied in the following experiments.

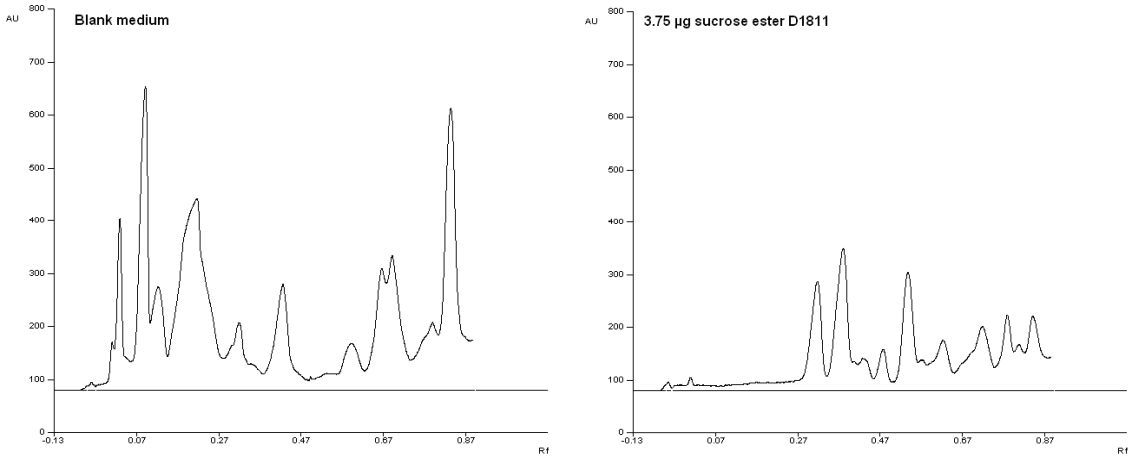


Figure 28 *HPTLC chromatogram of blank medium (left) and sucrose ester D1811 (right) after separation utilising a 4-step gradient based on methanol/water/chloroform. The components of the blank medium yield into many peaks interfering with the components of the sucrose esters. Hence, the monitoring of the in vitro digestion utilising this method was not possible.*

As illustrated in Figure 29, the FA titration during incubation of the SE dispersion in simulated digestion media was in the range of the results obtained during hydrolysis of the blank medium. These results indicate that SE was not digested by pancreatic enzymes.

In order to evaluate the impact of the colloidal state of the SE, an emulsion of paraffin and SE D1811 was prepared and introduced to the digestion assay. The aqueous SE dispersions contain SE micelles. In the emulsion, SE stabilises the surface of a paraffin droplet. Paraffin is an indigestible oil which was used to avoid interferences of other lipolytic products. In Figure 29, it appears that higher FA concentrations were detected during simulated digestion of the SE-paraffin emulsion in comparison to the results of the blank digestion. The experimental error was in the range of 10%.

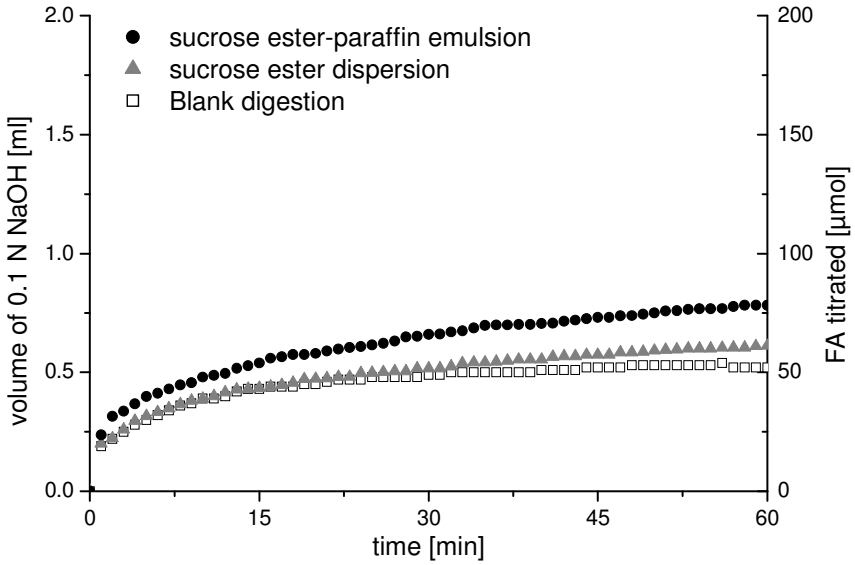


Figure 29 *Fatty acid concentrations as determined by pH-stat titration technique during a 60 min digestion period for mixed micelles (blank digestion), a sucrose ester D1811 dispersion (1% (w/v))) and a paraffin emulsion stabilised with 5% (w/v) of D1811 (data represent mean of 3 measurements). The release of FAs detected during simulated digestion of the SE substrate was in the range of the results obtained from blank experiments.*

In a further experiment, it should be evaluated if the presence of SEs would have any influence on the digestion of a natural TG substrate for pancreatic lipase. For that purpose, the commercial fat emulsion Lipofundin® was diluted with varying concentrations of a SE dispersion before the lipolysis assay was started.

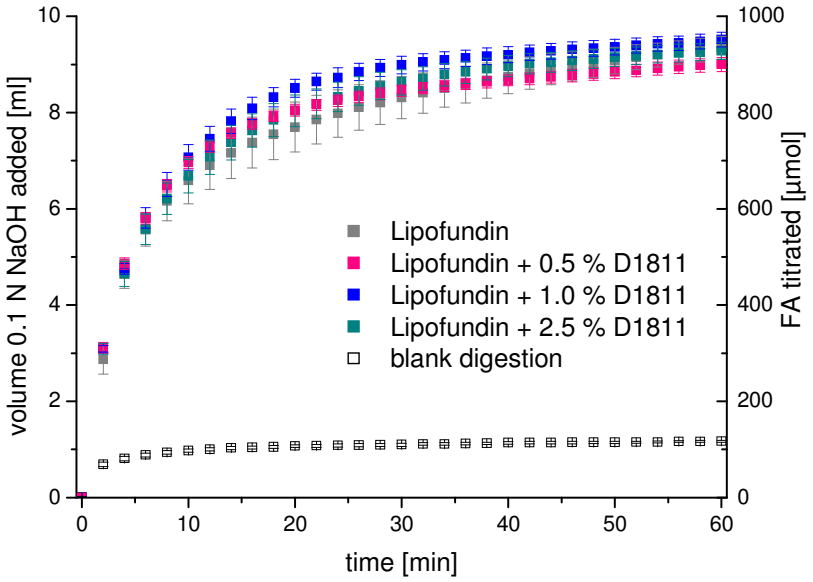


Figure 30 *Fatty acid concentrations as determined by pH-stat titration technique during a 60 min digestion period for mixed micelles (blank digestion) and the fat emulsion Lipofundin® which was mixed with different amounts of sucrose ester D1811 ranging from 0 to 2.5% (w/v (data represent mean±SD, n=3). All Lipofundin titration profiles were in the same range indicating that fat digestion was not influenced by the presence of the SEs.*

As illustrated in Figure 30, fat digestion was not influenced by the presence of the SEs under the experimental conditions. Neither a reduction of the FA concentration due to inhibitory effects nor increased FA levels due to a degradation of the SE itself were detected. This result further supports the thesis that SE D1811 was not susceptible to duodenal digestion.

In this study, sucrose ester D1811 did not undergo any detectable hydrolysis by pancreatic enzymes. These results were in contrast to Berry et al. who reported that the ester bonds of the sucrose ester mixture Sequol® 260 (containing 22% palmitic acid, 3.4% stearic acid, 22% oleic acid and 47% linoleic acid derivatives) were cleaved by pancreatic juice and isolated pancreatic lipase to an extent of about 25-30% after 60 min[188]. Moreover, Berry et al. concluded that the best hydrolytic activity was obtained with esters having a high content of unsaturated FAs. Nevertheless, they also observed that 15-20% of the esters of sucrose monostearate were cleaved by pancreatic lipase.
In contrast to these data but in agreement with the results obtained here, Christiansen et al. recently reported that sucrose laurate (HLB 16) did not undergo any detectable hydrolysis by pancreatic enzymes using a similar digestion assay and FA titration as used in this study[140].
The reason for these oppositional findings remains unclear. Differences in the enzyme composition between the porcine pancreas extract preparation as used in this study as well as by Christiansen et al. and the enzyme preparations used by Berry et al. might be one explanation. It should be taken into consideration that the different methods used for the analytical evaluation of digestion hamper an adequate comparison of the results. The FA titration method for monitoring *in vitro* lipolysis might not be sensitive and precise enough to obtain discriminative results at FA liberations below 100 µmol.

3.5.3 Dissolution of Ibuprofen sustained release tablets in biorelevant media

Following the investigations of aqueous SE formulations, a solid dosage form should be the focus of a further examination. It is well known that SEs can be used to modify the release of APIs. The mechanisms for this action are not yet completely understood. However, it has been discussed that the formation of H-bonds between the SE and the tablet filler causes the slow release of certain drugs[186]. Moreover, SEs form lyotropic mesophases upon contact with water, thus influencing the release of the drug[186,187]. In this section, dissolution testing as an established quality control method for oral solid dosage forms should be combined with the *in vitro* digestion assay. For that purpose, simulated digestion media were used as dissolution media.

During incubation in the different dissolution media, the Ibuprofen sustained release tablets underwent an erosion process: The size of the matrix tablets decreased in the course of the experiments (Figure 31). No initial swelling of the matrix system could be observed.

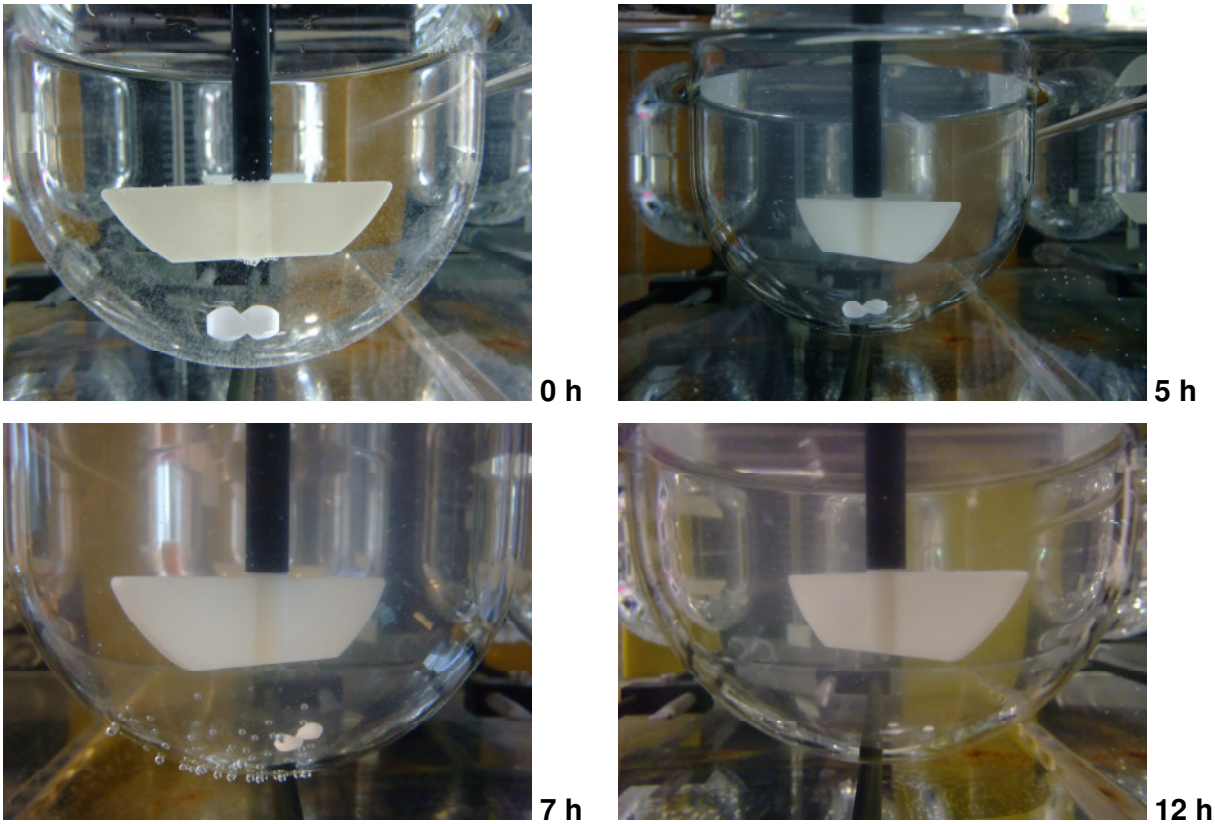


Figure 31 *Photographs taken during dissolution of ibuprofen sustained release tablets 'Ibuprofen 800 AL retard' in phosphate buffer (paddle apparatus, pH 6.8).*

Figure 32 shows the mean dissolution profiles of Ibuprofen sustained release tablets in various dissolution media. The dissolution profile in phosphate buffer showed a slow, non-linear drug release within 12 h. Half of the Ibuprofen content was released after about 3.8 h. The dissolution profile of Ibuprofen in buffer containing additional calcium and sodium chloride was slightly faster. 50% of the API was released after about 3.2 h and dissolution was already completed after 10 h. The dissolution of Ibuprofen in bioreleavant media containing bile salts and phospholipids was considerably faster compared to the dissolution in buffer. 50% of the API was released after about 2-2.2 h and dissolution was already completed after 8 h. Surprisingly, no difference in the dissolution profile was observed if low amounts of BS/PL reflecting fasted state intestinal conditions or high amounts of BS/PL reflecting fed state intestinal conditions were used. Furthermore, pancreatic enzymes which were present in the media reflecting fed state conditions had no influence on the dissolution of the API from the matrix tablets.

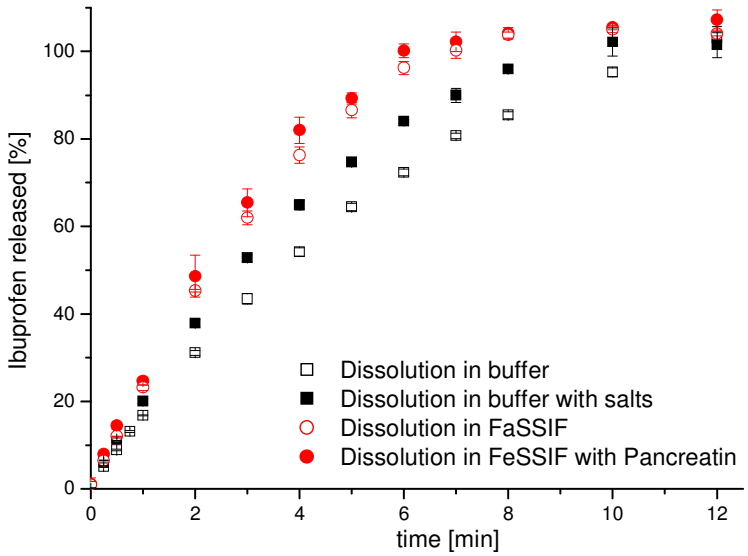


Figure 32 *Mean dissolution profile of Ibuprofen sustained release tablets 'Ibuprofen 800 AL retard' in phosphate buffer and biorelevant media (FaSSIF and FeSSIF with pancreatin). Experiments were run in triplicate using USP dissolution apparatus 2 (paddle) at a rotation speed of 75 rpm.*

The faster dissolution of the Ibuprofen tablets in buffer containing additional salts in comparison to the native buffer can be attributed to the higher ion strength of the dissolution medium. By inclusion of bile salts and phospholipids, the wettability of the solid dosage form was improved. Furthermore, it is well known that the saturation solubility of a drug can be substantially increased by the presence of mixed micelles[42]. These aspects contribute to a higher dissolution rate of Ibuprofen in biorelevant media. In accordance with the previous results obtained by *in vitro* digestion experiments with sucrose stearate, no difference in the dissolution profile of Ibuprofen in media with or without pancreatic enzymes was observed.

These results further underline the thesis that SEs of higher saturated FAs are poor substrates for pancreatic lipase.

3.6 Conclusion

Due to their versatile properties, SEs have gained large interest in the pharmaceutical technology. Metabolism studies in rats have shown that SEs are partially hydrolysed to sucrose and fatty acids in the GI lumen. However, in this study, dispersions of D1811 did not undergo any detectable hydrolysis by the enzymes present in porcine pancreas powder. Moreover, it could be shown that the presence of SEs did not affect the activity of pancreatic lipase towards its natural TG substrate.

A classical solid dosage form containing SEs as sustained release matrix agent was subjected to the *in vitro* lipolysis assay. Pancreatin-mediated digestion had no influence on the dissolution of Ibuprofen indicating the biorobustness of the formulation with respect to lipolysis.

On the basis of these findings, SE D1811 turned out to be an interesting surfactant for the development of new biorobust lipidic drug delivery systems.

4 Modelling gastric fat digestion: Development of a gastric lipolysis assay

4.1 Introduction

An increasing number of drug candidates which are poorly soluble in water represent a great challenge to the formulators of the pharmaceutical industry. One formulation strategy to improve the oral bioavailability of those compounds is the development of lipid based drug delivery systems. Lipid formulations circumvent the rate limiting step of absorption: the dissolution of the compound in the gastrointestinal tract. However, complex processes are involved in transferring the drug from a dissolved state in the formulation to colloidal phases formed during digestion of formulation components and then into mixed micelles[7,8,128,161].

One important aspect to consider when developing lipid based drug delivery systems is the performance of the formulation upon contact with gastrointestinal fluids including the digestion processes as well. Unless enteric-coated, a lipid formulation will disperse in the stomach. Classical dissolution testing for instance in acidic solutions as proposed by the pharmacopoeias has only limited applicability. Hence, during the last 15 years, much effort has been put into the development of more biorelevant media reflecting the composition and physical chemical characteristics of the gastric fluid as closely as possible[109,192-196]. Nevertheless, those systems are unable to provide detailed understanding of the fate of a lipid formulation and its co-administered compound upon exposure to gastric lipolysis.

Duodenal fat digestion has been the major field of research during the last 30 years. Nowadays, it is generally accepted that intestinal lipolysis plays a key role in the performance of lipid based drug delivery systems and *in vitro* lipolysis testing has been established in early formulation development. Although the pancreatic lipase-colipase complex is responsible for the main lipolytic activity of pancreatic juice on dietary triglycerides, several lipases are involved in the digestion of lipids in human[67,120]. One essential prerequisite for the subsequent action of pancreatic enzymes as well as the stimulation of bile salts is the initial degradation of lipids in the stomach. However, although the presence of a gastric lipase has been discussed as early as in 1900 by Volhard[197], gastric digestion is widely neglected during formulation development.

Fernandez et al. were the first who developed an *in vitro* method that took both the gastric and the duodenal steps of digestion into consideration[122]. They used recombinant dog gastric lipase (rDGL) which was provided from Meristem Therapeutics (Clermont-Ferrand, France). However, the main limitation is that rDGL or human gastric lipase (HGL) are not commercially available. Furthermore, the preparation of crude gastric extracts from animals is no option to most pharmaceutical laboratories. Hence, it was the objective of this study to establish an alternative gastric lipolysis model with lipases that are available and affordable

to many laboratories. Consequently, formulators should be able to evaluate the fate of a lipid formulation and its co-administered compound upon exposure to gastric lipolysis.

4.2 Scientific background

4.2.1 The physiology of the stomach

In order to design a predictive gastric model it is of crucial importance to pay attention to the physiological parameters of the stomach. This chapter will focus on the dominating parameters which will be the basis of any gastric model: pH value, gastric enzymes and motility of the stomach.

The pH value of the gastric fluid influences the solubility of APIs, the fate of acid-labile compounds and the activity of gastric lipase.

The variations of the gastric pH which occur during digestion have been studied in several test meal experiments[67,124,126,198-201]. Measurements of the pH value are performed either in aspirates achieved by sampling via nasogastric tubes or by using wireless pH monitoring systems (e.g. BRAVO™ capsule or SmartPill™). The cardia, the fundus, the corpus and the antrum of the stomach have different pH-values[199,202]. Hence, the position of the sampling device strongly influences the results of the measurements.

After the ingestion of a meal, the pH of the gastric contents range typically between 5.5 and 7 depending on the composition of the meal and on the position of the monitoring device. Then the chyme is diluted by gastric acid secretion leading to a reduction of the pH. Intragastric pH values in the range of 4-5 have been reported at half-gastric emptying time (~60 min) for healthy humans. When the gastric emptying process is completed (which can take 150 min and more), low pH values of 1-2 are reported[67,203].

Nevertheless, diseases like chronic pancreatitis, gastroparesis or medication with proton pump inhibitors show substantial alterations in the gastric pH profile[203-205]. Furthermore, the type of food ingested has a varying buffer capacity. In conclusion, the gastric pH underlies high variability, thus hampering the predictability of models simulating the stomach.

There are two major enzymes which are responsible for the gastric digestion processes: pepsin and human gastric lipase (HGL). HGL is produced in the fundic mucosa of the stomach where it is co-localised with pepsinogen (as pepsin precursor) in the chief cells of fundic glands[206]. Gastric lipase is stable at acidic pH levels, but its stability decreases at pH values below 1.5[207]. Its maximum activity is obtained at pH 5-5.4 on LC-TG[208]. Recently, N'Goma et al. have reviewed the mean concentrations of HGL observed during test meals. Usually, HGL levels increase after the ingestion of food due to the secretion of the enzyme as stimulated by the meal. At about 50% of gastric emptying time HGL levels of 15-20 µg/ml were reported followed by an increase after 70-80% of gastric emptying. Eventually, basal levels of 51 ± 50 µg/ml HGL were reached at the end of digestion. Gastric lipolysis is an

incomplete process. Pafumi et al. identified that the evolution of protonated FAs on the surface of the oil droplets entraps gastric lipase in a surface aggregate which restricts lipase access to the interface[209].

Due to the large variations in the gastric pH and HGL concentrations which occur during digestion, it is not easy to simulate the intragastric process of lipolysis *in vitro*[127]. The same is true for pepsin levels which have been reported to be in the range of 0.11-0.22 mg/ml in the fasted state and 0.26-0.58 mg/ml in the fed state[124]. Remarkably, HGL is not influenced by the proteolytic action of pepsin[65].

The third parameter which will be reviewed in this section is the motility of the stomach. The ingested food bolus entering the stomach experiences a number of mixing regimes. Mixing within the stomach is dominated by the progression of antral contract waves and retropulsive jet-like motions. Antral contract waves progress towards the pylorus and are strongest during the distal antral segment. The transpyloric and retropulsive flow within the antrum produces shear forces which are able to reduce the average particle size of emulsions down to 15-30 µm. In contrast to the antrum, mixing within the fundus and the body of the stomach is very weak[79,210-212].

The different components of the food are emptied from the stomach in a different way. After ingestion, liquids are rapidly distributed throughout the entire stomach. Emptying of liquids depends mainly on fundic pressure through the 'pressure pump' mechanism controlled by pyloric opening where the gastroduodenal pressure gradient is the driving force. Liquid meals often empty from the stomach according to first order kinetics; that is, the speed is directly proportional to the volume present in the stomach. In contrast, ingested solids are stored initially in the proximal stomach and move gradually into the distal stomach where solids are ground to particles of a size less than 1 to 2 mm before they are allowed to go through the pyloric opening. Indigestible material must wait for the interdigestive phase when the phase III contraction of the migrating motor complex empties the stomach[213-215].

Remarkably, the content of fat in a meal influences the gastric emptying time. If FAs enter the upper intestine, a hormonal feedback (release of cholecystokinine) leads to a retardation of the gastric emptying rate. Furthermore, the same effect was observed for liquid emulsions which were instable in the gastric environment leading to an aqueous phase which was quickly emptied from the stomach and an oily layer which was emptied from the stomach significantly slower[79,211]. Studies in medicine, pharmacy, and nutrition have demonstrated that food disintegration in the stomach is a complex process involving numerous variables, including particle size, meal volume, calories and composition of the meal, viscosity, and physical properties such as texture and structure.

4.2.2 Media simulating gastric fluid

A better understanding of the gastrointestinal environment combined with a growing interest for *in vitro* models in order to achieve a better characterisation of drug formulations has led to various publications of media simulating the stomach and upper intestine.

The basic media can be found in the European Pharmacopeia or the USP exclusively mimicking acidic pH and presence of pepsin[216]. The groups of J. B. Dressman and C. Reppas have focused their efforts on the development of biorelevant media which are suitable for dissolution studies during formulation development. Early attempts included the addition of surfactants like sodium lauryl sulphate (SLS) or Triton X to the pharmacopeial SGF in order to reduce the surface tension to more physiological data. However, these media have shown to overestimate gastric dissolution[111,195]. Recently, Vertzoni et al. developed a Fasted State Simulated Gastric Fluid (FaSSGF) which appeared to be more appropriate because the surface tension was reduced by physiological concentrations of pepsin and bile salts[194]. An overview about the composition and physicochemical properties of the dissolution media simulating fasted gastric conditions is given in Table 18.

Table 18 *Composition and physicochemical properties of the dissolution media simulating fasted gastric conditions and in vivo data as reported in the literature*

Physicochemical properties	**SGF[216]**	**SGF_{SLS}[111]**	**$SGF_{Triton\ X}$[195]**	**FaSSGF[194]**	***In vivo* data[124]**
Sodium lauryl sulphate (%,w/v)	-	0.25	-	-	-
Triton X100 (%, w/v)	-	-	0.1	-	-
Pepsin (mg/ml)	3.2	-	-	0.1	0.11-0.22
Sodium taurochloate (µM)	-	-	-	80	< 500
Lecithin	-	-	-	20	-
NaCl	34.2	34.2	34.2	34.2	-
Surface tension (mN/m)	-	33.7	32.0	42.6	41.9-45.7
Osmolarity (mosm/kg)	-	180.5±3.6	157.7±2.9	120.7±2.5	98-140
pH	1.2	1.2	1.2	1.6	1.7-2.4

Media simulating the postprandial stomach have been more difficult to develop due to the high variability *in vivo*. Although it has been suggested that the most suitable media representing the fed stomach would be the homogenised food which is used in clinical studies, this approach would hamper drug analysis. Alternative media included full fat milk (fat content of 3.5%) and the high caloric liquid meal 'Ensure® plus' but it could be shown that the disintegration of tablets in milk was negatively effected and drug binding to the milk fat led to an overestimated solubility of several compounds[109,196]. Recently, Jantratid et al. developed different 'snapshot' media reflecting the changing conditions in the postprandial stomach[109]. Based on these media, a general medium for the fed stomach was evolved.

An overview about the composition and physicochemical properties of the dissolution media simulating fasted gastric conditions is given in Table 19. Remarkably, none of these media contains digestive enzymes, since their target is dissolution testing of a drug from a classical solid dosage form. Hence, potential alterations that occur to excipients which are substrates to enzymatic degradation cannot be investigated.

Table 19 *Composition and physicochemical properties of the dissolution media simulating fed gastric conditions and in vivo data as reported in the literature*

Physicochemical properties	Ensure® Plus[196]	FeSSGF early[109]	FeSSGF middle[109]	FeSSGF late[109]	*In vivo* data[124]
Sodium chloride (mM)	-	148	237	122.6	-
Acetic acid (mM)/ Sodium acetate (mM)	-	-	17.12/29.75		-
Ortho-phosphoric acid (mM) Sodium dihydrogen phosphate (mM)	-	-		5.5/32	-
Milk/buffer	-	1:0	1:1	1:3	-
Ensure® Plus + 0.45% pectine (%)	100				
Pepsin (mg/ml)	-	-	-	-	0.28-0.58
Sodium taurocholate (µM)	-	-	-	-	< 500
Buffer capacity (mmol/lpH)	21±0.3	21.3	25	25	14-28
Surface tension (mN/m)	48.4	-	-	-	30-31
Osmolarity (mosm/kg)	730	559	400	300	217-559
pH	6.45	6.4	5	3	2.7-6.4

For the design of a gastric model intended to investigate the performance of lipid-based drug delivery systems, the addition of digestion enzymes is mandatory. These digestion models can be subdivided into static and dynamic models. Static models are single pot systems with constant experimental conditions. In such models, the products of digestion are not removed during the experiment, thus not mimicking physiological absorption processes. Moreover, physical processes that occur *in vivo* (e. g. different shear, mixing, hydration, changing conditions over time, etc) are rarely reflected.

In an attempt to achieve a better correlation between *in vitro* and *in vivo* behaviours, dynamic models have been developed. Dynamic models may or may not remove the products of digestion but have the advantage that they include the physical processing and temporal changes in the luminal conditions that mimic conditions *in vivo*. The most popular artificial model which is also used in some pharmaceutical labs is the computer controlled TNO intestinal model (TIM) developed by TNO Nutrition and Food Research (Zeist, The Netherlands). It consists of several compartments interconnected by valves and mimics the

body's temperature, peristaltic movements, acidity and electrolyte concentrations as well as the swallowing of saliva and the secretion of gastric and pancreatic juice[136,137,217]. According to TNO's protocols, the simulated gastric fluid consists of 53.04 mmol/l NaCl, 14.75 mmol/l KCl, 1.02 mmol/l $CaCl_2x2H_2O$, 7.14 mmol/l $NaHCO_3$, 0.28 g/l pepsin, 0.25 g/L lipase and 0.2 g/l trypsin. Furthermore, 1 M HCl is added at varying amounts in order to adjust the pH value[136,218].

The main drawback of this complex model is the high costs, thereby impeding routine application in pharmaceutical laboratories.

So far, only a few laboratories have taken the gastric step of fat digestion into account. Fernandez et al. developed an *in vitro* method that takes both, the gastric and the duodenal steps of digestion into consideration. The model included a 30 min gastric digestion step at pH 5.5 (NaCl 150 mM; NaTDC 4 mM; $CaCl_2$ 1.4 mM; Tris-HCl 1 mM) which is maintained via pH-stat titration and was used for evaluation of the changes in the composition of two excipients which are often used in lipid formulations (Labrasol® and Gelucire® 44/14) during their *in vitro* lipolysis[122].

The limiting factor for gastric lipolysis models is the availability of the gastric lipase. Fernandez et al. were able to receive recombinant dog gastric lipase (rDGL) from Meristem Therapeutics, France. Since this enzyme is not commercially available, other lipase sources have been evaluated in this work.

4.3 Materials and methods

4.3.1 Materials

Materials used for the preparation of the substrate emulsions

The commercially available parenteral fat emulsion Lipofundin® N 10 was kindly gifted by B. Braun Melsungen AG (Germany). For the preparation of test emulsions the following materials were used: olive oil was purchased from Caelo (Germany), polyvinyl alcohol was obtained from Sigma chemicals (Germany), Purity gum 2000 (OSA starch), an modified starch esterified with octenyl succinic acid was provided by National starch (Germany) and Poloxamer 188 (Lutrol® F 68) was donated by BASF (Germany).

Lipase

Lipase AP12, Amano Enzyme Inc. (UK), was chosen to be a promising enzyme preparation for the replacement of human gastric lipase in a gastric digestion model. Lipase AP is manufactured by fermentation of a strain belonging to *Aspergillus niger* followed by extraction and purification. Lipase AP12 is a fine brownish powder which is poorly wettable in water. According to the data provided by Amano Enzyme, lipase AP is stable at pH values from 2-10 showing an optimum activity at pH 5.0-7.0 (Figure 36). Hence, this enzyme may act satisfactorily in the stomach.

4.3.2 Preparation of the test emulsions

The commercial fat emulsion Lipofundin® N 10 was used as received.
Three further test emulsions were prepared as follows: The aqueous emulsifier solution was applied to a rotor-stator mixer (Ultra turrax®, IKA T18 basic, IKA-Works, NC). 10% of olive oil was added stepwise under mixing. The aqueous emulsifier solution and the oil phase were mixed for three minutes at 14,000 rpm before this coarse emulsion was introduced to a two-stage high-pressure valve homogeniser (nG7400.270 P, Standsted Fluid Power Ltd., UK-Stansted) to achieve droplet sizes in the nano range. Five cycles were run at 100 MPa (first stage) and 10 MPa (second stage, back-pressure module), followed by one cycle at 40 MPa.

The samples were allowed to equilibrate for 24 h prior to analysis. The particle size distribution of the emulsions was determined by means of laser diffraction using a Mastersizer 2000 (Malvern Instruments, UK). In accordance with the USP's assay for lipase activity (fat digestive power), the substrate was satisfactory if 90% of the particles did not exceed 2 µm and none exceeded 10 µm in diameter. Emulsions were diluted 1:1 with a buffer system comprising of varying amounts of citric acid (0.1 mol/l) and disodium hydrogen phosphate (0.2 mol/l) in order to investigate the stability of the emulsions at pH values relevant in the stomach (Table 20). After 1 h of incubation, the particle size distribution was determined by laser diffraction.

***Table 20**: Composition of the buffer systems ranging from pH 2.2-6.0*

pH value	citric acid (0.1 mol/l) [%] (v/v)	disodium hydrogen phosphate (0.2 mol/l) [%] (v/v)
2.2	98.8	1.2
3.0	80.3	19.7
4.0	62.0	38.0
5.0	49.0	51.0
6.0	37.4	62.6

4.3.3 Determination of the lipase activity

Lipase activity assay according to the Amano method
Amano Enzyme labels the lipolytic activity of lipase AP12 (Lot: LG 0751104) to be 12,300 u/g. In order to approve this value, lipase activity testing was reproduced utilising the procedure reported by Amano Enzymes. According to the protocol, lipase activity was determined by back-titration of the amount of FAs produced by hydrolysis of an olive oil emulsion at pH 6.0.
In brief, an emulsion of 25% olive oil stabilised with 2% of polyvinyl alcohol was prepared by running three cycles of high speed mixing using an Ultra turrax®. Since the emulsion tended to separate within 1 h, a subsequent high pressure homogenisation step involving four cycles at 100 MPa (first stage) and 10 MPa (second stage) was applied.

5 ml of the emulsion was incubated in 4 ml of phosphate buffer solution (0.1 M, pH 6) at 37 °C. Since the type of buffer was not described in the Amano Enzyme protocol, a buffer consisting of citric acid/ disodium hydrogen phosphate was used. One millilitre of enzyme solution (0.1 µg/ml; equivalent to a dilution factor of 10,000) was added to the substrate solution. After a 30 min incubation period, 10 ml of an ethanol-acetone mixture (1:1) was added to stop the reaction. Then 10 ml of 0.05 N NaOH followed by another 10 ml of the ethanol-acetone mixture (1:1) was added to the mixture. The excess of NaOH was titrated against 0.1 N HCl to pH 10. Blank experiments without the addition of lipase were performed in the same manner. The enzyme activity was calculated using equation 4.

$$\text{Lipolytic activity} = \frac{\text{Volume}_{\text{HCl blank}} - \text{Volume}_{\text{HCl sample}}}{0.3} \text{ x } 10 \text{ x dilution factor x factor}_{\text{HCl}} \qquad \textit{eq. 4}$$

According to the Amano Enzyme protocol, 10 units are defined as the amount of enzyme which is able to liberate 1 micro equivalent fatty acid per minute. Or in other words: 1 unit of enzyme is capable of cleaving 0.1 µmol FA per minute.

Lipase activity assay at different pH values

Since the Amano Enzyme method of determining the lipase activity was so different to other published protocols, a further method was applied for the determination of the lipase activity at different pH values. As the PVA-olive oil emulsion which was used in the Amano Enzyme protocol was not stable for more than a few hours, an OSA starch-olive oil emulsion was used as substrate in this study. The protocol was developed adapting parameters and conditions of lipase activity assay according to Fernandez et al[120].

In brief, 5 ml of the emulsion were mixed with 9 ml of buffer solution. The mixture was incubated at 37 °C for 10 minutes in a tempered glass vessel under constant agitation. Lipolysis was initiated by the addition of enzyme suspension (100 mg/ml). The digests were followed over five minutes using a pH-stat titration unit (Mettler DL 21, Germany), which maintained the pH at the desired value. The FAs produced by hydrolysis were titrated with 0.1 N NaOH. Due to the issue that FAs are not sufficiently ionised at lower pH values, back titration was necessary. Therefore, 100 µl of 5-bromophenylboronic acid (0.1g/ml in methanol) was added to inhibit the reaction and the pH was abruptly increased to 9.0 after the 5 min incubation period. The volume of NaOH was recorded. Blank experiments using surfactant solution instead of the emulsion were performed in the same procedure. In contrast to other protocols, blank experiments included the addition of enzyme since peptides and proteins have reasonable buffer capacity and may hamper the accurate determination of FAs produced by lipolysis.

It was confirmed that the release of free FAs was linear with time during at least 5 min. Experiments were performed in triplicate at various pH values. The lipase activities were

expressed as international units: 1 U = 1 µmol of FAs released per minute and were calculated using equation 5.

$$U/mg = \frac{(V_{sample} - V_{blank}) \times c_{NaOH} \times 1000}{c_{enzyme} \times V_{enzyme} \times t} \qquad \textit{eq. 5}$$

t…	incubation time [min]
c_{enzyme}…	enzyme concentration [mg/ml]
V_{enzyme}	volume of enzyme suspension [ml]
c_{NaOH}	concentration of sodiumhydroxide solution [mmol/ml]
$V_{sample/\ blank}$…	volume 0.1 N NaOH used for titration (5 min period + adjusting to pH 9)

4.3.4 Design of the gastric lipolysis model

After a broad review of the published media and assays for the simulation of the gastric digestion, the following parameters were chosen for the design of the gastric lipolysis model.

a) Electrolytes

The choice of electrolytes present in the gastric media was adapted from Salovaara et al. and consisted of 53.04 mmol/l NaCl, 14.75 mmol/l KCl, 1.02 mmol/l $CaCl_2x2H_2O$ and 7.14 mmol/l $NaHCO_3$[218]. In order to reduce the work for the preparation of the simulated gastric media, a stock solution of all electrolytes in 5-fold concentration was prepared.

b) Buffer

In analogy to the work presented by Jantratid et al., two pH values were chosen for modelling the postprandial stomach in middle and late stage: 5.0 and 3.0. A pH of 2.0 was chosen representing the fasted stomach[109].

A further challenge was to find a suitable buffer which can be used in the pH range from 2.0-5.0. Using only one buffer system has the disadvantage of strong variation of ion strength and buffer capacity. For that reason, four buffer systems were chosen using Van Slyke and Henderson-Hasselbalch equation in order to calculate the buffer concentrations needed to obtain the target buffer capacity of 25 mmol $l^{-1}\Delta pH^{-1}$. The ion strength of the buffers should not exceed 0.1 mol/l. The final composition is given in Table 21. In order to reduce the work for the preparation of the simulated gastric media, a stock solution of all electrolytes in 10 fold concentration was prepared.

For the gastric lipolysis assay, 5 ml of the olive oil emulsion was diluted with 9 ml of a pre-mixture of electrolyte solution, buffer solution and water. The mixture was heated to 37 °C. The desired amount of lipase AP12 was pre-dispersed in 1 ml of electrolyte-buffer solution and added to the substrate-buffer solution to start the digestion. The pH was adjusted using

1 N HCl or NaOH if necessary. This mixture was incubated at 37 °C in an end-over-end shaker at 10 rpm.

At 0, 5, 15, 30, 60 and 120 min, samples of the digest (50 or 100 µl) were taken and dissolved in a chloroform/methanol mixture (1:1). The organic samples were centrifuged and further diluted for analysis of the lipid concentrations by HPTLC and spectrodensitometric measurements as described earlier in this thesis. All lipids were calculated as oleic acid derivatives (monoolein, diolein, triolein, oleic acid).

Table 21 *Composition of the final gastric digestion media at 3 pH values simulating fasted, late, middle and early fed stomach*

pH value	**2**	**3**	**5**
Substrate	3.33% Olive oil stabilised with OSA starch or 3.33% Olive oil stabilised with poloxamer® 188		
Electrolytes [mmol/l]			
NaCl	53.04	53.04	53.04
KCl	14.75	14.75	14.75
$CaCl_2x2H_2O$	0.77	0.77	0.77
$NaHCO_3$	7.14	7.14	7.14
Buffer [mmol/l]			
Phoshoric acid	25.33	12.30	-
KH_2PO_4	19.00	92.27	-
Acetic acid	-	-	16.96
Na -acetate	-	-	30.17
Amount of enzyme [mg/ml]	6.67	3.33/6.67/ 13.33/26.66	6.67
Total volume [ml]	15	15	15

Since the total lipid mass is conserved throughout the experiment, mass balance calculations were performed using equation 6.

$$\text{Mass balance}\,[\%] = \frac{FA_{formed} + MG_{formed} + (2\,DG_{formed}) + (3\,TG_{remaining})}{(3\,TG_{initial})} \text{ x } 100 \qquad \textit{eq. 6}$$

The total FA equivalents present in the initial TG are equal to the FA equivalents derived from the remaining TG and FAs, MGs, and DGs formed during the lipolysis.

Monitoring gastric lipolysis with the pH-stat method was not applicable due to the poor ionisation of long chain FAs at acidic pH values.

4.4 Results and Discussion

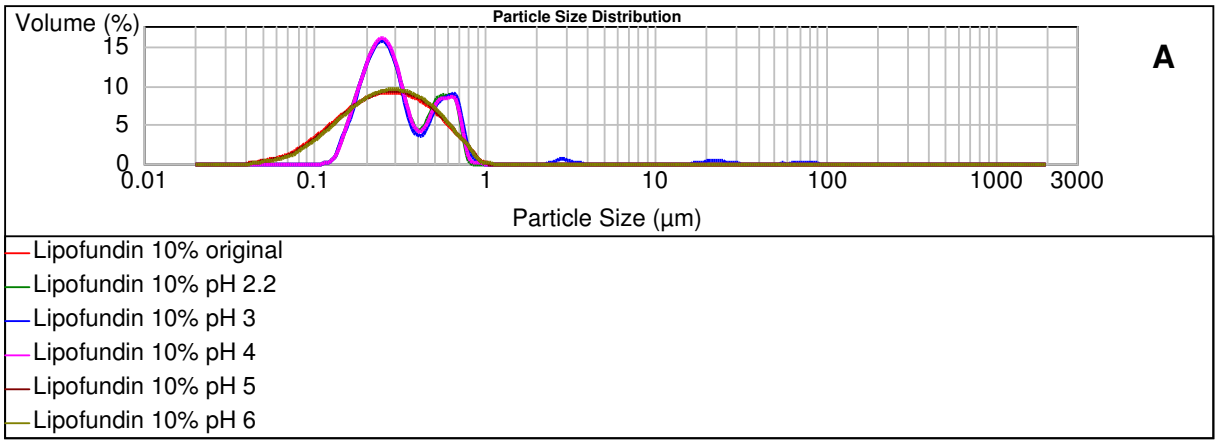
4.4.1 Characterisation and stability testing of the emulsions

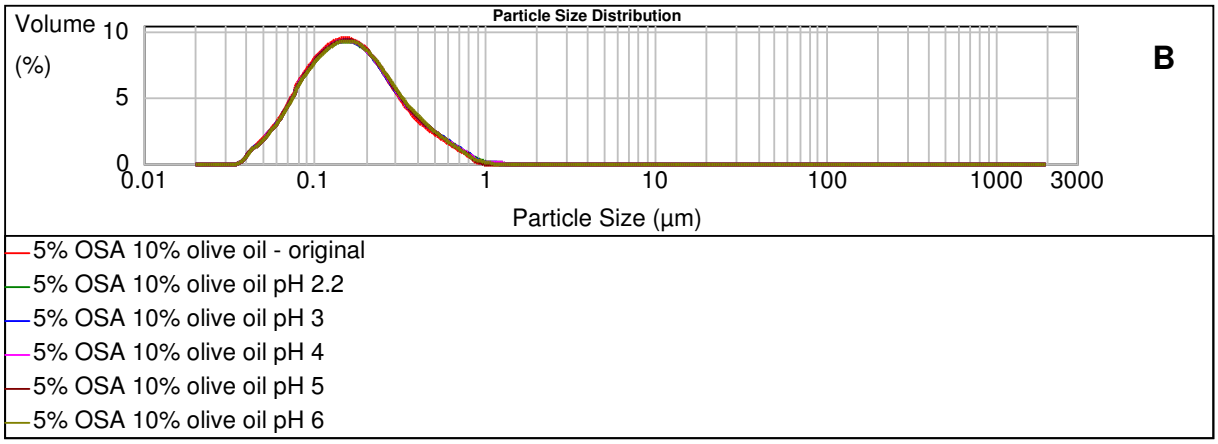
Different test emulsions were prepared by high pressure homogenisation. Since the OSA starch and the poloxamer differ in their emulsifying power, different concentrations of both excipients were chosen. Laser diffraction measurements revealed that similar PSD were obtained for both emulsions. Lipofundin® is a well characterised emulsion used in parenteral nutrition. The PSD of Lipofundin® was shifted to coarser particles (Table 22).

***Table 22**: Composition and particle size distribution of the test emulsions*

Ingredient	OSA emulsion	Poloxamer emulsion	Lipofundin® 10% N
Oil content [%] (source)	10 (olive oil)	10 (olive oil)	10 (soybean oil)
Emulsifier [%] (source)	5 (OSA starch)	2 (Poloxamer 188)	0.8 (lecithin)
water [%]	85	88	85.5
Particle size distribution (PSD)			
D (0.1) [µm]	0.069	0.066	0.114
D (0.5) [µm]	0.142	0.132	0.276
D (0.9) [µm]	0.320	0.271	0.570

The stability of the test emulsion at acidic pH is a prerequisite requirement for the development of a gastric digestion model. As shown in Figure 33A, Lipofundin® emulsion was not stable at pH values lower than 5. Coalescence of oil droplets could be observed. Therefore, Lipofundin® was discarded due to insufficient suitability as test emulsion. Emulsions stabilised with Poloxamer and OSA starch were stable in a pH range of 2.2-6.0 (Figure 33B-C).





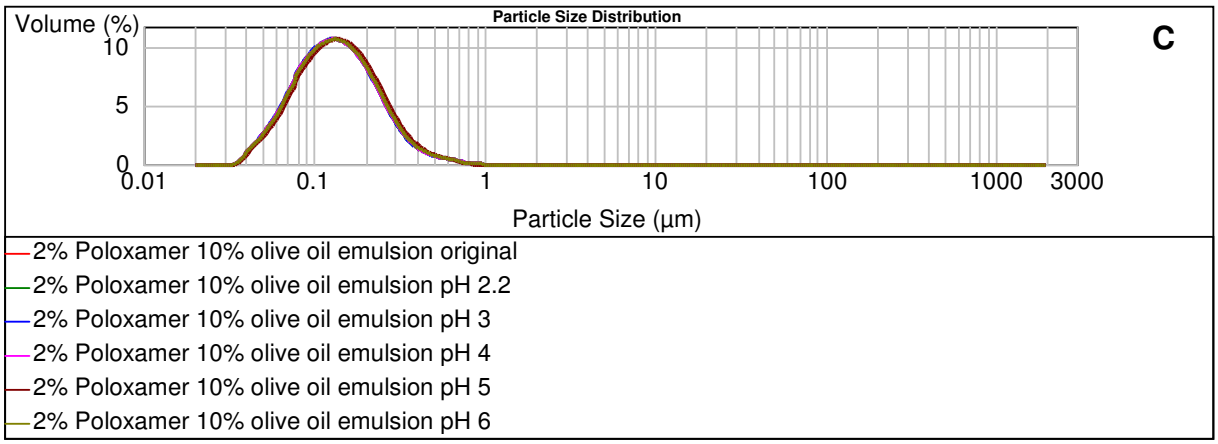


Figure 33 *Particle size distributions obtained by LD measurements of the native emulsion (red curve) and those obtained after dilution of the emulsion with buffer simulating pH 2.2 (green curve), pH 3 (blue curve), pH 4 (pink curve), pH 5 (brown curve) and pH 6 (dark yellow curve). Data represent mean of 5 measurements.*

4.4.2 Determination of the lipase activity

The activity of lipase AP12 determined by the protocol as suggested by Amano Enzymes Inc. was 19.4 U/mg. This value was higher compared to the manufacturer data of 13.4 U/mg. This discrepancy might be attributed to the substrate. The PVA-olive oil emulsion was a crude emulsion with strong tendency to phase separation. For that reason, a high pressure homogenisation step was performed which was not part of the Amano Enzyme protocol. This further manufacturing step leads to emulsions with fine particles in the sub-micron range that are characterised by a high surface area. Hence, the access of the enzyme towards its substrate is significantly increased leading to higher activity values. These effects were also reported by Armand et al. who determined a higher lipase activity on a fine mixed MCT/LCT emulsion than on a coarse mixed MCT/LCT emulsion (mean droplet sizes 0.46 vs. 3.18 µm)[146].

Due to the instability of the emulsion, an OSA starch-olive oil emulsion was used in the following investigations. Furthermore, the OSA starch-olive oil emulsion proved to be stable at the pH range of interest (2.2-5.0). Figure 34 shows an exemplary titration profile which was recorded during incubation of an OSA starch-olive oil emulsion with lipase AP12. Linearity was proven for at least 5 minutes indicating that the enzyme activities determined under these conditions were, therefore, independent from the incubation time.

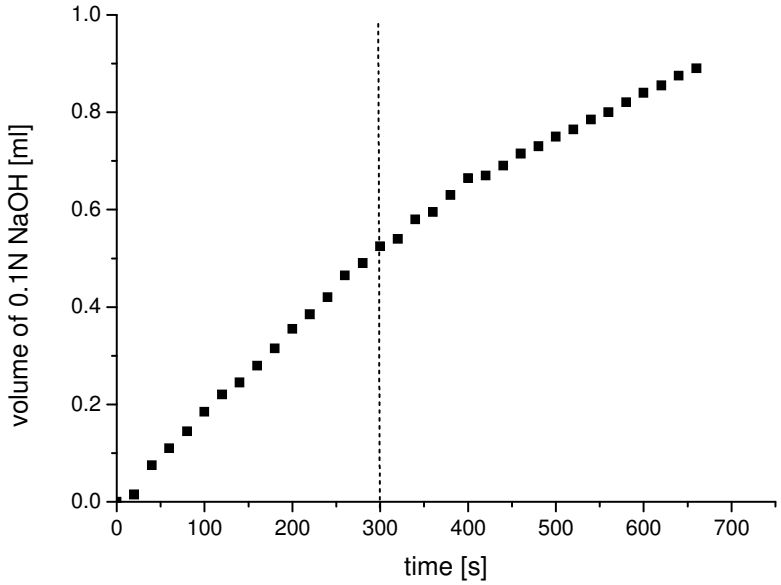


Figure 34 *Exemplary titration profile obtained during simulated digestion of OSA-starch-olive oil emulsion by Lipase AP12 at pH 6.8. Linearity of the curve is given at least within 5 minutes.*

The results of the lipase activity assay at different pH values are given in Figure 35. All measured values were an order of magnitude lower compared to the results of the Amano method. This can be attributed to the different measuring conditions used in the assays. Surprisingly, the highest lipase activity was observed at pH 5.0 and the lowest for pH 4.0 (68.5% compared with pH 5.0), whereas lipase was quite active at pH 2.2 and 3.0 (corresponding to 75.6% and 82% compared with the activity at pH 5.0, respectively). These results were in contrast to the data provided by Amano Enzymes (Figure 36) who reported highest activity of Lipase AP between pH 6 and 7 and decreasing activity at lower pH values[219]. Again, one should keep in mind that the methods which were used for the determination differed with regard to the buffer system involving buffer capacity, substrate concentration, particle size of the oily emulsion and the measurement of blank values. All these differences hamper an adequate comparison of the data.

Nevertheless, the results prove that lipase AP12 shows reasonable activity at pH values relevant to simulate gastric conditions. Since the lipase activity was in the range of 0.8-1.1 U/ml for the broad pH range of 2.2-6.0, no significant influence of the lipase activity on the digestion profiles during simulated gastric lipolysis at different pH values was expected.

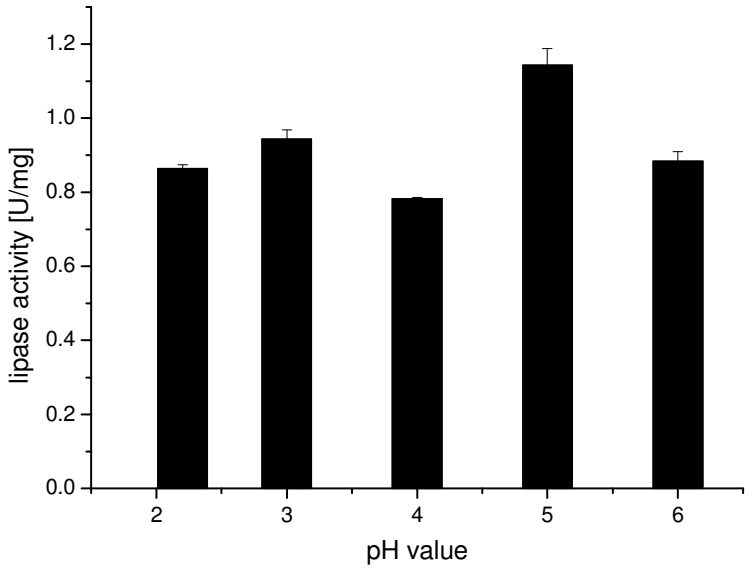


Figure 35 *Lipase activity of Lipase AP12 on OSA-starch-olive oil substrate at different pH values. Data represent mean±SD (n=3).*

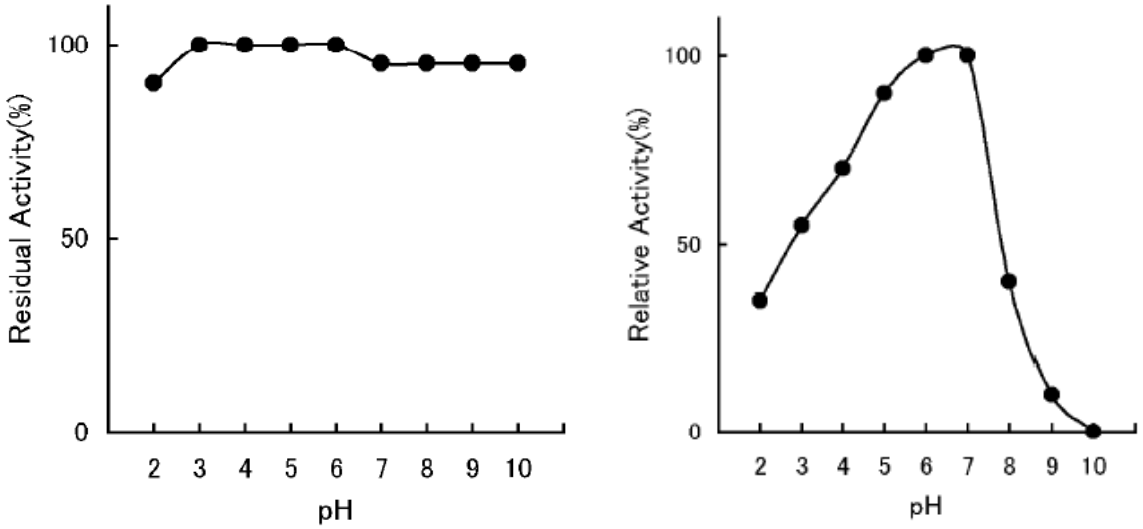


Figure 36 *Left: The residual activity of lipase AP as determined by Amano Enzyme Inc. after 30 min incubation at different pH values. After that period, the pH is increased to 6.0 and lipase activity is measured. The values are compared to the activity achieved at pH 6.*
Right: The influence of the pH value on the activity of Lipase AP as determined by Amano Enzyme Inc. According to the protocol, an olive oil substrate was prepared with different buffer solutions in the pH range between 2 and 10. Lipase acitivity was determined according to the procedure described in the text. The relative activity of 100% is shown at pH 7[219].

4.4.3 Results of the gastric lipolysis model

First experiments were performed using an emulsion of 10% olive oil stabilised with 5% of OSA starch as substrate solution. However, after 60 minutes of simulated digestion, an oily layer was formed on the top of the glass tubes indicating phase separation of the emulsion. Apparently, the ester linkage between the hydroxyl groups of the starch backbone and the octenyl succinic acid of the emulsifier was cleaved by the enzyme leading to a reduction of the emulsifying properties of Purity gum® 2000. This in turn led to coalescence of the oil droplets. This sign of instability was not observed during the 5 minutes lasting lipase activity testing. However, a potential degradation of the emulsifier was compensated by the blank experiments.

Since the variation of the particle sizes during the test may lead to misinterpretation of the results, another test emulsion had to be chosen which should be stable at low pH values and should be resistant to esterase. Hence, the test emulsion consisting of 10% of olive oil stabilised with 2% of Poloxamer® 188 was used in the following experiments.

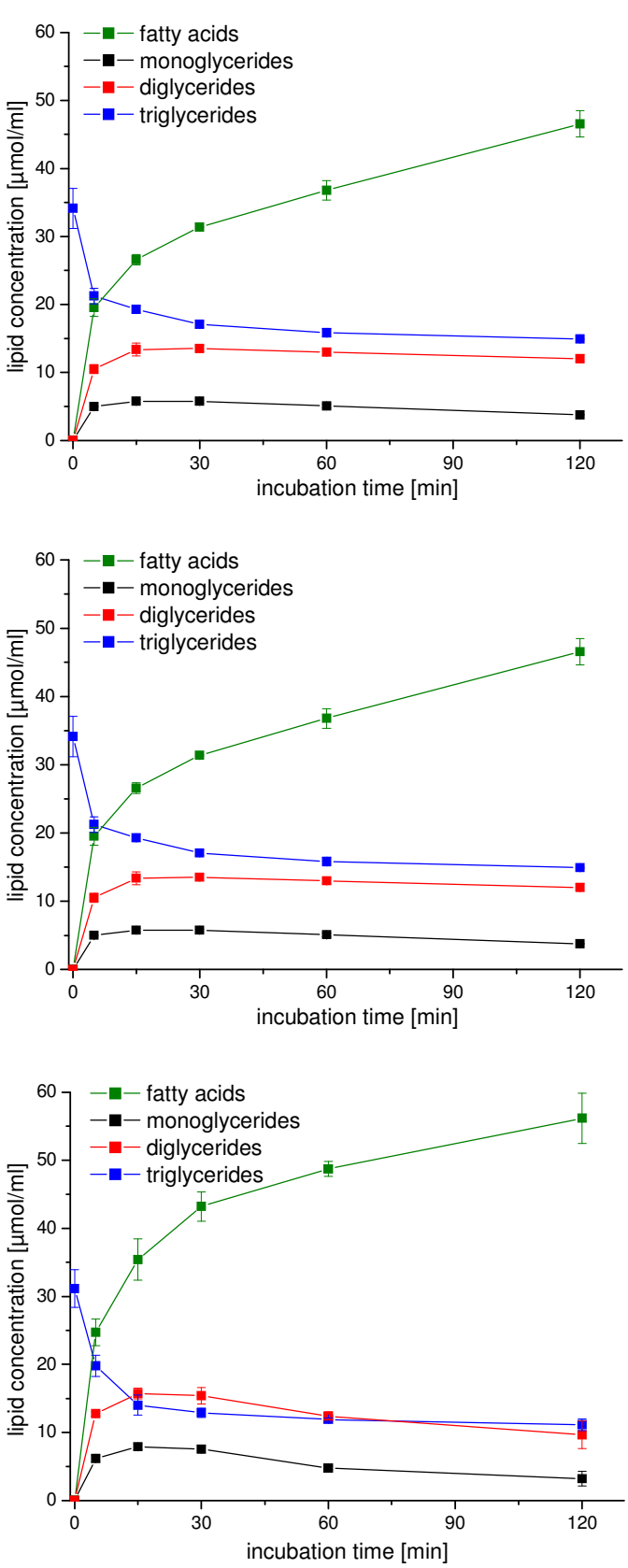


Figure 37 *Lipid concentrations obtained during simulated gastric digestion of an olive oil substrate by lipase AP12 (Amano Enzyme Inc.) at pH 2, 3 and 5. Lipid analysis was performed by HPTLC/in situ spectrodensitometric analysis. Data represent mean±SD (n=3).*

Figure 37 shows the lipid profiles obtained during incubation of an olive oil substrate in gastric digestion medium containing Lipase AP12 at pH 2, 3 and 5. In all cases, a transient increase of FA, MG and DG concentrations were observed during the first 30 min of digestion, whereas TG levels decreased. On further progression of the digestion, MG and DG levels decreased slightly indicating further degradation of both lipids. This effect was strongest at pH 5 which correlates well with the higher lipase activity at this pH value. Nevertheless, TG lipolysis was not completed after 2 h. In all cases, about 35-40% of the initial TG was still present. On comparison of the different pH values, the differences in the lipid profile during digestion at pH 2 and 3 were negligible. At pH 5, the rate of digestion was increased.

These results were quite promising. In humans, gastric lipolysis is inhibited by the evolution of FAs at the surface of an oil droplet. Hence, gastric fat digestion is incomplete.
Mass balance determinations for the samples from the digestion mixture at 120 min were between 106 and 110% indicating satisfactory accuracy of the method.

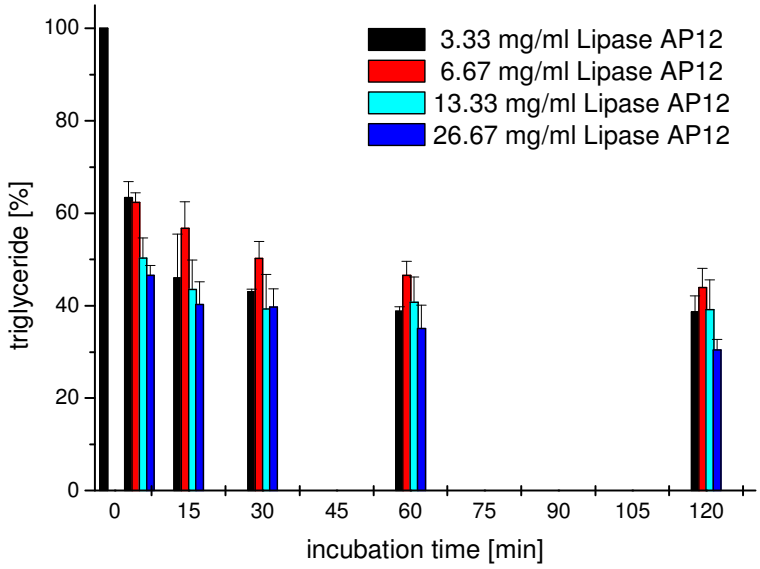


Figure 38 *Content of triglycerides (TGs) derived from an olive oil emulsion during simulated gastric digestion at pH 3.0 with varying amounts of Lipase AP12 (33.3-266.67 mg/ml). The initial amount of TG was set to 100%. For a better illustration, the initial value is only depicted for 3.3 mg/ml of lipase. Data represent mean±SD, n=3.*

Figure 38 shows the results of the TG digestion during incubation in digestion medium using different amounts of lipase AP12. TG digestion progressed faster within the first 5 minutes when higher amounts of lipase were present. When digestion proceeded further, similar TG concentrations were detected. Although not significant, the lowest TG levels remained when the highest amount of lipase was present. Surprisingly, even with high concentrations of 26.67 mg/ml of lipase AP12, TG digestion was not completed within 2 h. The lipid profiles

obtained during the course of digestion were in qualitative agreement to the profiles shown in Figure 37 (data not shown).

4.5 Conclusion

One important aspect to consider when developing lipid based drug delivery systems is the performance of the formulation upon contact with the gastrointestinal environment. In the recent years, many protocols have been published in order to simulate duodenal lipolysis, but the gastric step was mainly neglected with exception to a few research groups who were able to work with recombinant dog gastric lipase or extracts of gastric juice obtained from animals. Since these enzymes are not available to other research labs, an alternative lipase source was evaluated which should fulfil the following requirements:

a) stability at pH values relevant in the stomach; at least between pH 2-6
b) reasonable lipase activity (fat digestive power) at pH values relevant in the stomach
c) commercial availability

On the basis of peer reviewed literature, *in vivo* data and published assays, an *in vitro* gastric digestion model was developed which focused on the needs of lipid digestion rather than the digestion of proteins. Since the monitoring of gastric lipid digestion with the commonly used pH-stat method was not applicable due to the poor ionisation of long chain FA at lower pH values, lipid analysis by the established HPTLC method was applied. Back titration, i.e. acidimetric titration using sodium hydroxide solution up to pH 9.0, where all FAs are ionised and therefore detectable would be another option. However, this approach would be connected with a higher sampling effort as the preparation of a new sample for the monitoring of each time interval is necessary. Furthermore, lipid analysis by means of the established HPTLC method enables a deeper understanding of the lipid digestion progress as analysis includes TGs, DGs, MGs and FAs.

On the basis of the results presented in this chapter, lipase AP12 proved to be an interesting alternative for HGL or rDGL. The results indicate that lipase AP12 is stable at acidic pH values.

In vitro gastric digestion experiments indicated that the amount of enzyme added had an influence on the rate of TG digestion. Considerable differences were only observed when the lipase concentration differed in an order of magnitude. Taken the lipase activities determined at the OSA starch-olive oil substrate into account, the following activities have been investigated so far: 3.0, 6.0, 12.0 and 24.0 U/ml. These values would be in accordance with data by Armand et al. who reported lipase activities of 5.2±1.3 U/ml and 7.1±1.5 U/ml after low and high fat diet, respectively (activity measured on triolein after pentagastrin stimulation)[220]. Nevertheless, it should be noted that different assay conditions hamper an adequate comparison of the data.

In the literature, it is in general agreement that gastric lipase cleaves about 10-30% of ingested TGs. Hence, the lipase activities measured during simulated gastric digestion were too high. Similar results were obtained by Fernandez et al. who observed that about 90% of the TGs present in Gelucire® 44/14 and 80% of the TGs present in Labrasol® were degraded during the gastric lipolysis step[122]. Nevertheless, in continuative investigations, the enzyme concentration should be further decreased and digestion time should be limited to 30-60 min in order to simulate more physiological conditions.

In conclusion, it could be demonstrated, that a simulation of gastric digestion with commercially available lipase AP12 is possible. This will contribute to a deeper understanding of the performance of a lipid-based formulation upon exposure with the gastrointestinal environment.

V Summary and perspectives

Lipid based drug delivery systems represent a promising formulation strategy to improve the oral bioavailability of poorly soluble drugs. Nowadays, the term lipid formulation includes a wide spectrum of formulations ranging from simple oily solutions, self-emulsifying and self-microemulsifying systems to oil-free systems based on surfactants and co-solvents. Moreover, formulators have the choice out of numerous excipients with different physico-chemical properties. Following oral administration, the formulation should be able to maintain the drug in a solubilised state throughout its way through the GI tract. However, the interactions between the API, lipidic components of the formulation and endogenous lipids (e.g. bile salts and phospholipids) as well as the physicochemical alterations that occur during digestion are complex and not easy to predict. *In vitro* tests that mimic the fate of a lipid formulation upon exposure to the gastrointestinal environment are needed in order to rationalise formulation development of poorly soluble compounds and, hence, to reduce attrition rates.

The aim of this thesis was to gain detailed knowledge about the relation between the digestion of different lipid systems and their solubilisation capacity by utilising an *in vitro* lipolysis assay.

Different lipid formulations which met the characteristics of the four classes of the LFCS were manufactured and loaded with the highly lipophilic drug Probucol. Moreover, medium chain glycerides as well as long chain glycerides were utilised in order to investigate the impact of the glyceride source on the performance of the formulation.

In terms of drug loading capacity, MC-based formulations were clearly superior towards LC-based formulations which could be attributed to the higher solubility of Probucol in the MC glycerides. The polar formulation types III and IV were able to dissolve higher amounts of the API in comparison to the lipophilic formulations I and II.

Surprisingly, all formulations were able to maintain 85% and more of the drug in a solubilised state after a 100-fold dilution in buffer or simulated intestinal medium without enzymes. Even the polar formulation types III and IV showed good resistance towards dilution. Following the dispersion behaviour, the impact of the enzymatic degradation on the solubilisation capacity of the formulations was investigated. The utilisation of an *in vitro* lipolysis assay revealed more discriminative results. Drug precipitation was observed for all formulations, whereas the highest and fastest loss of solvent capacity was determined for the lipophilic MC-based formulations (I-, II- and IIIA-MC) as well as for the polar LC-based formulation IIIB- LC. The LC-based formulations I-LC, II-LC and IIIA-LC were able to maintain more than 75% of the API in a solubilised state during 60 min of lipolysis.

Remarkably, similar results were obtained for the oil-free formulation IV comprising of Solutol® HS 15 and ethanol.

In order to understand the mechanisms that cause the precipitation of Probucol, the susceptibility of the formulations towards enzymatic degradation was investigated. The lipolysis of the lipid formulations was monitored using the pH-stat titration technique. The results of the lipolysis were in good correlation to the data obtained by the solubilisation study. The MC-based systems which were highly susceptible to pancreatin-mediated digestion suffered from a strong loss of solubilisation capacity. This was attributed to the higher tendency of medium chain lipolytic products (FAs and MGs) to disperse in the aqueous medium instead of being incorporated in mixed micelles or vesicular structures. In addition, the digestion of MCT progressed much faster than the degradation of LCT which was correlated to the fast precipitation of Probucol. In the case of the LC based formulations, the results indicated that formulations with a higher susceptibility towards lipolysis were correlated with a better ability to maintain Probucol in a solubilised state. In order to achieve a more detailed picture of the chemical alterations that occur during simulated digestion, lipid analysis by HPTLC coupled with densitometric measurements was applied. The data revealed that the digestion of TGs and DGs was not completed. The incomplete degradation could be attributed to the experimental conditions. However, a complete digestion of TGs and DGs can be expected *in vivo*.

The results of the lipid analyses revealed that Solutol® HS15 was susceptible towards pancreatin-induced digestion. Nevertheless, its solubilisation capacity for Probucol was widely maintained.

Ongoing research should focus on the interactions between the API and the lipid formulation. For that purpose, a range of poorly soluble compounds with different physicochemical characteristics especially melting point, aqueous solubility and log P value should be incorporated in the different lipid formulation types and investigated for API precipitation after dilution and simulated digestion. Moreover, the investigations should be expanded to basic as well as acidic APIs. Thereby, a framework can be designed from which formulators can choose the most promising formulation approach for an API with similar characteristics.

Furthermore, *in vivo* studies should be performed in order to evaluate the correlation between the *in vitro* results and *in vivo* oral bioavailability.

Due to the promising results of the Solutol® based formulations in the first part of this thesis and as a tribute to the high interest in the development of formulations containing high amounts of surfactant, the susceptibility of a range of pharmaceutical relevant excipients was further investigated. The focus was set on the pegylated non-ionic surfactants Solutol® HS 15, Cremophor® RH 40, Cremophor® EL, Gelucire® 50/13 and Labrafil® M1944CS.

At the beginning of the experimental work, there was a lack of knowledge whether surfactants are influenced by the action of pancreatic enzymes and if this affects the performance of lipid based delivery systems. Utilising the *in vitro* lipolysis assay, it was demonstrated that esters of FAs and PEG were at least partially hydrolysed by pancreatic enzymes. The extent of degradation of the excipient was estimated using the ester value of

each surfactant. Based on this approach, a degradation of 22-30% was determined for the pegylated excipients Cremophor® RH 40, Cremophor® EL and Solutol® HS 15. Using the example of Solutol®, it could be demonstrated that the extent of degradation was concentration dependent.

For Gelucire® 50/13 and Labrafil® M 1944CS composed of mixtures of MAGs, DAGs and TAGs as well as mono- and diesters of FAs with PEG, higher degradation levels of about 80% and 50% were detected, respectively.

These results indicate that the hydrophilic PEG esters Cremophor® EL and RH as well as Solutol® might be able to retain their solvent capacity during digestion due to limited hydrolysis. For Gelucire® and Labrafil®, a higher extent of digestion can be expected *in vivo* which might result in a higher variability of the formulation performance. This knowledge is of crucial importance aiming a more rational choice of excipients for the successful development of lipid based drug delivery systems.

In order to achieve more detailed results, an analytical method for the quatification of the PEG esters should be developed. HPLC coupled with an evaporative light scattering detector could be one approach for this purpose. Moreover, ongoing research should focus on the interactions between an API and the surfactant-based formulation.

The investigations were expanded to sucrose esters. Following a long application history of in the cosmetic and food industry, SEs have gained large interest by researchers and formulators in the pharmaceutical sector. Despite their growing use in pharmaceutical formulations, little is known about the influence of digestive enzymes on the performance of SE formulations. Metabolism studies of SEs in rats indicate that higher fatty acid esters of sucrose such as the octa-, hepta- and hexaesters are not absorbed but excreted unchanged, while the lower esters are supposed to be partially hydrolysed and absorbed as sucrose and the individual FAs[186,188-190]. However, the results of this study indicate that sucrose ester D1811 was not degraded by pancreatin-mediated lipolysis. Moreover, it was shown that the presence of sucrose stearates did not influence the extent of digestion of a triglyceride emulsion. The investigations were expanded to a classical solid dosage form containing Ibuprofen in a sustained release matrix tablet formed by sucrose dipalmitate. By coupling dissolution testing using a compendial dissolution apparatus with the *in vitro* lipolysis assay it could be shown that the Ibuprofen release was independent from the presence of pancreatic enzymes. Thus, the results further support the thesis that SEs of long chain FAs and sucrose are poor substrates for pancreatic enzymes.

In further research work, more sensitive analytical methods should be applied in order to be capable of detecting small changes in the chemical composition of SEs and to verify the data. The biggest challenge will be to separate signals derived from the amphiphilic components of the digestion medium which have similar polarity as the analytes themselves.

In the recent years, many protocols have been published in order to simulate duodenal lipolysis, but the gastric step was mainly neglected with exception to a few research groups who were able to work with recombinant dog gastric lipase or extracts of gastric juice obtained from animals. Since these enzymes are not available to other research labs, an alternative lipase source was evaluated which should fulfil the following requirements:

a) stability at pH values relevant in the stomach; at least between pH 2-6
b) reasonable lipase activity (fat digestive power) at pH values relevant in the stomach
c) commercial availability

On the basis of peer reviewed literature, *in vivo* data and published assays, an *in vitro* gastric digestion model focusing on lipolysis rather than the digestion of proteins was developed. Hence, the test conditions include electrolytes, pH value, buffer capacity and lipase as core parameters of gastric lipolysis. Pepsin might be included in further trials if necessary, although the stability of the lipase against pepsin has to be proven in advance. The issue of gastric motility has also not been addressed in this assay, since it is assumed that lipid formulations disperse quickly upon contact with gastric fluids and are, therefore, more or less independent from the grinding forces of the stomach.

Since the monitoring of gastric lipid digestion with the commonly used pH-stat method was not applicable due to the poor ionisation of long chain FAs at lower pH values, lipid analysis using the established HPTLC method is recommended.

On the basis of the results, lipase AP12 by Amano Enzyme Inc. proved to be an interesting alternative for HGL or rDGL. The investigations showed that lipase AP12 is stable at acidic pH values. Moreover, an incomplete digestion of a triglyceride emulsion was achieved using 3-24 U/ml of lipase activity. Nevertheless, in continuative investigations, the enzyme concentration should be further decreased and digestion time should be limited to 30-60 min in order to simulate more physiological conditions. The lipase AP12 is commercially available and affordable to many laboratories. Hence, all above mentioned requirements are fulfilled.

In conclusion, it could be demonstrated that a simulation of gastric digestion with lipase AP12 is possible. This will contribute to a deeper understanding of the performance of lipid-based formulations upon exposure with the gastrointestinal environment.

Furthermore, next steps should include the combination of the gastric model proposed here with the established duodenal lipolysis model. For that purpose, the pH value needs to be increased after a certain time interval in order to mimic the duodenal pH. The addition of pancreatin in a buffer consisting of disodium hydrogen phosphate and mixed micelles would be an ideal option. Moreover, the addition of calcium is another prerequisite for duodenal lipid digestion.

Following this approach would not only allow to analyse the influence of the two digestion steps on the formulation, but also to address the fate of the drug during the change of the pH and upon digestion of its vehicle.

Zusammenfassung und Ausblick

Eine vielversprechende Strategie um die orale Bioverfügbarkeit von schlecht wasserlöslichen Wirkstoffen zu verbessern ist die Entwicklung von Lipidformulierungen. Der Begriff Lipidformulierung umfasst eine Bandbreite von Systemen, beginnend bei einfachen öligen Lösungen, über selbstemulgierenden und selbstmikroemulgierenden Formulierungen, bis hin zu ölfreien Systemen, welche auf Tensiden und Co-Tensiden beruhen. Darüber hinaus haben die Galeniker die Auswahl aus einer Vielzahl von Hilfsstoffen mit unterschiedlichen physikochemischen Eigenschaften.

Nach oraler Applikation sollte eine Lipidformulierung in der Lage sein den Wirkstoff während der Magen-Darm-Passage in solubilisierter Form zu halten. Die Interaktionen, die dabei zwischen dem schwerlöslichen Wirkstoff, den Formulierungsbestandteilen beziehungsweise deren Verdauungsprodukten sowie endogenen Lipiden (z.B. Gallensäuren und Phospholipiden) stattfinden, sind jedoch sehr komplex und nur schwer vorhersehbar. Zudem beeinflussen Verdauungsprozesse die physikochemischen Eigenschaften der Formulierung. Daher wurde in den letzten Jahren verstärkt daran gearbeitet *in vitro* Tests zu entwickeln, die es erlauben, den Einfluss der Lipolyse auf das Solubilisierungsvermögen von Lipidformulierungen zu untersuchen. Dadurch soll die Effizienz einer Formulierung in Mensch und Tier besser verstanden und vorhergesagt werden können, um die Versagerquoten in kostenintensiven klinischen Studien zu minimieren.

Zielsetzung der vorliegenden Arbeit war es daher, detaillierte Erkenntnisse über den Zusammenhang zwischen Verdauung unterschiedlicher Lipidformulierungen und den resultierenden Auswirkungen auf deren Solubilisierungskapazität mit Hilfe eines *in vitro* Lipolyse Assays zu erlangen.

Im ersten Teil der Arbeit wurden Lipidsysteme hergestellt, die den unterschiedlichen Klassen des Lipidformulierungsklassifizierungssystems von Pouton entsprechen. Dafür wurden sowohl mittelkettige als auch langkettige Glyzeride verwendet, um den Einfluss der Ölquelle auf die Solubilisierungskapazität näher zu beleuchten. Der Cholesterolsenker Probucol diente dabei als stark lipophile Modelsubstanz.

In Bezug auf die Wirkstoffbeladung waren jene Formulierungen, welche auf mittelkettigen Glyzeriden beruhten, den langkettigen Vertretern deutlich überlegen. Dies konnte auf die höherere Löslichkeit von Probucol in mittelkettigen Glyceriden zurückgeführt werden. Durch den höheren Anteil an Emulgator und Cosolvens konnte die Wirkstoffbeladung in den hydrophileren Formulierungen des Typs III und IV deutlich gesteigert werden.

Erstaunlicherweise waren alle Formulierungen auch nach 100-facher Verdünnung in Puffer oder künstlicher Darmflüssigkeit ohne Enzymzusatz in der Lage mehr als 85 % des Wirkstoffes in Solubilisierung zu halten. Selbst die polaren Formulierungen des Typs III und IV, bei denen die Gefahr einer Wirkstoffpräzipitation infolge der Diffusion der hydrophilen Formulierungsbestandteile in das wässrige Medium besonders hoch ist, zeigten gute Ergebnisse. Im Anschluss an das Dispergierverhalten wurde der Einfluss der Verdauung auf

die Solubilisierungskapazität der Systeme untersucht. Eine Präzipitation von Probucol wurde in allen Fällen nachgewiesen, jedoch war diese in unterschiedlichem Maße ausgeprägt. Die Formulierungen, welche auf mittelkettigen Glyzeriden basierten (I-, II- und IIIA-MC), sowie die polare Formulierung IIIB-LC zeigten den deutlichsten und schnellsten Verlust an Solubilisierungskapazität während der künstlichen Verdauung. Die Formulierungen, welche auf langkettigen Glyzeriden basierten (I-LC, II-LC und IIIA-LC), waren dagegen auch nach 60-minütiger Verdauung in der Lage, mehr als 75% des Wirkstoffes zu solubilisieren.
Erstaunlicherweise wurden für die ölfreie Formulierung IV, basierend auf Solutol® HS 15 und Ethanol, ähnliche Ergebnisse erhalten.
Um die Zusammenhänge zwischen Verdauungsvorgängen und den Veränderungen in der Solubilisierungskapazität der Formulierungen näher zu beleuchten, wurde deren Anfälligkeit gegenüber Verdauungsenzymen untersucht. Zu diesem Zweck wurde die Lipolyse mittels pH-Stat Verfahren verfolgt. Die Resultate der Lipolyseexperimente zeigten eine gute Übereinstimmung mit den Ergebnissen der Wirkstoffsolubilisierung während der simulierten Verdauung. Die Formulierungen, welche auf mittelkettigen Glyzeriden basierten, wurden durch die Pankreasenzyme schnell und effektiv abgebaut. Lipolyseprodukte von mittelkettigen Di- und Triglyzeriden (mittelkettige Fettsäuren und Monoglyzeride) besitzen eine moderate Wasserlöslichkeit. Anstelle eines Einbaus in Mischmizellen oder Vesikel, dispergieren diese Endprodukte eher in Wasser. Dies führt zu einem starken Verlust der Solubilisierungskapazität der Formulierungen. Im Falle der Formulierungen, welche vorwiegend aus langkettigen Glyzeriden bestehen, war die Geschwindigkeit des enzymatischen Abbaus deutlich geringer als bei den mittelkettigen Vertretern. Formulierungen, die einem enzymatischen Abbau unterlagen, konnten dennoch ihre Solubilisierungskapazität weitgehend bewahren.
Um ein genaueres Bild von den chemischen Veränderungen während der Verdauung der Lipidsysteme zu erhalten, wurden HPTLC/Densitometrie Untersuchungen durchgeführt. Es konnte gezeigt werden, dass der enzymatische Abbau der langkettigen Ölkomponenten nicht vollständig verlief. Dieses Phänomen ist jedoch den Versuchsbedingungen geschuldet und ist *in vivo* nicht zu erwarten.
Zudem konnte gezeigt werden, dass das Tensid Solutol® HS 15 einem enzymatischen Abbau unterliegt. Nichtsdestotrotz blieb die Solubiliserungskapazität für Probucol weitgehend erhalten.
Weiterführende Untersuchungen sollten sich eingehender mit den Wechselwirkungen zwischen Wirkstoff und Lipidformulierung beschäftigen. Zu diesem Zweck sollten die verschiedenen Formulierungstypen mit Wirkstoffen unterschiedlicher physikochemischer Eigenschaften (insbesondere Schmelzpunkt, Wasserlöslichkeit und Lipophilie) beladen werden und auf Wirkstoffpräzipitation nach Verdünnung und Verdauung untersucht werden. Weiterhin sollten die Untersuchungen auch basische und saure Wirkstoffe beinhalten. Dadurch soll ein Arbeitsrahmen geschaffen werden, aus dem sich Formulierer die

vielversprechenste Formulierung für einen Wirkstoff mit ähnlichen Eigenschaften wählen können.
Außerdem sollten *in vivo* Studien durchgeführt werden, um die Korrelation zwischen den *in vitro* Ergebnissen und der oralen Bioverfügbarkeit zu bewerten.

Aufgrund der vielversprechenden Ergebnisse der Solutol®-basierten Formulierungen aus dem ersten Teil der Arbeit und um dem steigenden Interesse an hydrophilen Lipidformulierungen mit hohem Tensidanteilen Rechnung zu tragen, wurde die Stabilität einer Reihe pharmazeutisch relevanter Tenside im Magen-Darm-Trakt untersucht. Dabei wurde der Fokus auf die pegylierten Tenside Solutol® HS 15, Cremophor® RH 40, Cremophor® EL, Gelucire® 50/13 und Labrafil® M 1944CS gerichtet.
Es konnte gezeigt werden, dass Ester aus Fettsäuren und PEG zumindest zu einem gewissen Anteil enzymatisch gespalten werden. Das Ausmaß des Abbaus wurde anhand der Esterzahl bestimmt. Hierbei ergab sich, dass 22-30 % der Fettsäureester in Cremophor® RH 40, Cremophor® EL und Solutol® HS 15 abgebaut wurden. Am Beispiel von Solutol® HS 15 konnte gezeigt werden, dass der Abbau konzentrationsabhängig ist.
Für Gelucire® 50/13 und Labrafil® M 1944CS, welche Mischungen aus Mono, Di und Triglyzeriden sowie Mono- und Diestern aus PEG und Fettsären darstellen, wurden 80 % beziehungsweise 50 % Abbau nachgewiesen.
Diese Ergebnisse deuten darauf hin, dass Cremophor® EL und RH sowie Solutol® in der Lage sein könnten die Solubilisierungskapazität während der Magen-Darm-Passage zu erhalten. Für Gelucire® and Labrafil® ist dagegen eine stärkere Beeinflussung durch Verdauungsvorgänge zu erwarten. Dies führt zudem zu einer größeren Variabilität der Bioverfügbarkeit. Diese Erkenntnisse sind für eine rationalere Auswahl der Hilfsstoffe für eine effektive Lipidformulierung von wichtiger Bedeutung.
Um detailliertere Ergebnisse zu erzielen, ist es ratsam eine analytische Methode für die Quantifizierung der pegylierten Fettsäureester zu entwickleIn. Eine HPLC-Methode kombiniert mit einem Lichtstreudetektor (HPLC-ELSD) wäre hierfür ein möglicher Ansatzpunkt. Zudem sollten die Wechselwirkungen zwischen Wirkstoff und Tensidformulierung näher beleuchtet werden.

Die Untersuchungen der Stabilität pharmazeutisch relevanter Tenside im Magen-Darm Trakt wurden auf Sucroseester ausgedehnt. Nach einer langen Einsatzhistorie in der Kosmetik- und Lebensmittelindustrie ist das Interesse an Sucroseestern auch im pharmazeutischen Sektor gestiegen. Trotz des zunehmenden Einsatzes in Arzneimittelformulierungen ist bislang wenig über den Einfluss von Verdauungsenzymen auf die Effektivität der Formulierung bekannt. Metabolismusstudien in Ratten deuten an, dass höhere Fettsäureester nicht resorbiert sondern unverändert ausgeschieden werden. Stattdessen geht man davon aus, dass niedere Ester zumindest partiell hydrolysiert und als Sucrose und Fettsäure resorbiert werden[186,188-190]. Die Ergebnisse der vorliegenden Arbeit zeigten, dass

kein Abbau von Sucrosestearat D1811 durch Pankreasenzyme erfolgte. Weiterhin konnte gezeigt werden, dass Sucroseester keinen Einfluss auf die Effektivität der Verdauung einer Triglyzeridemulsion haben.
Die Untersuchungen wurden auf eine klassische feste Darreichungsform ausgeweitet. Ibuprofentabletten, die Sucrosedipalmitat als Matrixbildner für eine retardierte Freisetzung enthalten, wurden einem Freisetzungstest in simulierter Darmflüssigkeit mit und ohne Pankreasenzymen unterzogen. Es konnte gezeigt werden, dass die Freisetzung von Ibuprofen enzymunabhängig erfolgte. Dies stützt weiterhin die Annahme, dass Sucroseester langkettiger Fettsäuren schwache Substrate für Pankreasenzyme darstellen.
In weiterführenden Arbeiten sollte eine empfindliche analytische Methode entwickelt werden, um auch minimale Veränderungen der chemischen Zusammensetzung der Sucroseester bestimmen zu können. Die effektive chromatographische Trennung der amphiphilen Matrixbestandteile stellt hierbei die größte Herausforderung dar.

In den vergangenen Jahren wurden zahlreiche Verfahren publiziert, die den duodenalen Lipidabbau simulieren. Die gastrische Lipolyse hingegen wurde jedoch aufgrund einer erschwerten Verfügbarkeit gastrischer Lipase weitgehend vernachlässigt. Daher sollte im Rahmen dieser Arbeit eine Enzymquelle evaluiert werden, die die humane gastrische Lipase ersetzen soll. Folgende Voraussetzungen sollten dabei erfüllt werden:

a) Stabilität bei sauren pH-Werten, mindestens pH 2-6
b) Aktivität der Lipase bei sauren pH-Werten
c) Kommerzielle Verfügbarkeit

Auf Basis von Fachpublikationen, *in vivo* Daten und bereits publizierter Methoden wurde ein *in vitro* Assay zur Simulierung der gastrischen Lipolyse entwickelt. Die Testbedingungen reflektieren Elektrolyte, pH-Wert, Pufferkapazität und Lipasekonzentration als Kernparameter der gastrischen Lipoylse. Pepsin kann in weiterführenden Untersuchungen berücksichtigt werden, sofern die Stabilität der Lipase gegenüber Pepsin belegt wurde. Da davon ausgegangen werden kann, dass Lipidformulierungen schnell und somit weitestgehend unabhängig von den Kontraktionen des Magens dispergieren, wurde die Motilität des Magens in der vorliegenden Arbeit nicht berücksichtigt.
Aufgrund der schwachen Ionisation der Fettsäuren bei saurem pH, war das pH-stat Verfahren zur Verfolgung der gastrischen Lipolyse nicht anwendbar. Stattdessen wurde die HPTLC-basierte Lipidanalytik angewendet.
Die Ergebnisse zeigen, dass Lipase AP12 (Amano Enzyme Inc.) eine interessante Alternative für die Simulierung der gastrischen Lipolyse darstellt. Dieses Enzym ist bei sauren pH Werten sowohl stabil als auch aktiv. Eine initiale Verdauung einer Triglyzeridemulsion wurde mit Enzymaktivitäten von 3-24 U/ml erreicht. Dennoch sollte sowohl die Enzymkonzentration als auch die Inkubationszeit in weiterführenden Arbeiten reduziert werden, um die physiologischen Bedingungen noch besser zu simulieren. Die

Lipase AP12 ist für viele Entwicklungsabteilungen kommerziell erhältlich und finanziell erschwinglich. Damit erfüllt das Enzym alle gesetzten Voraussetzungen.
Zusammenfassend konnte gezeigt werden, dass die Simulation der gastrischen Lipolyse mit dem Enzym Lipase AP12 möglich ist. Mit Hilfe solcher Simulationen kann das Verhalten einer Lipidformulierung im Magen-Darm-Trakt besser vorhergesagt werden.
Weiterführende Arbeiten sollten die Kombination des gastrischen Assays mit dem Dünndarmmodel thematisieren. Um dies zu erreichen, sollte nach einer definierten Inkubationszeit der pH-Wert des Mediums erfolgen. Dafür bietet sich die Zugabe eines Pankreatin und Mischmizellen enthaltenden Dinatriumhydrogenphosphatpuffers an. Darüber hinaus sollte Calcium zugegeben werden, um die Effektivität des Fettverdaus zu gewährleisten.
Dieser Ansatz ermöglicht es, den Einfluss beider Verdauungsphasen auf die Effektivität einer Formulierung im Magen-Darm Trakt zu simulieren. Mit Hilfe der vorgestellten Methoden können dabei sowohl das Schicksal des Wirkstoffes als auch die Veränderungen der Formulierung analysiert werden.

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Erklärung

Hiermit erkläre ich gemäß § 5 der Promotionsordnung der Naturwissenschaftlichen Fakultät I der Martin-Luther-Universität Halle-Wittenberg, dass ich die Ergebnisse der vorliegenden Dissertationsarbeit „*In vitro* lipolysis assay as a prognostic tool for the development of lipid based drug delivery systems“ am Institut für Pharmazie der Martin-Luther-Universität Halle-Wittenberg unter Anleitung von Herrn Prof. Dr. Karsten Mäder selbständig erarbeitet und die Dissertation ohne fremde Hilfe verfasst habe.

Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

Weiterhin habe ich diese Arbeit bisher an keiner in- oder ausländischen Fakultät als Dissertationsschrift vorgelegt.

Halle (Saale), den

Sandra Klein

Curriculum vitae

Personal details

Name	Sandra Klein
Date and place of birth	1982 in Halle/Saale
Nationality	German

Work experience

Since 04/2010	Formulation Manager in the R&D department of Losan Pharma GmbH, Neuenburg, Germany
01/2006-03/2010	Ph.D. student in the group of Prof. Dr. K. Mäder, Pharmaceutical Technology, Martin-Luther-University Halle-Wittenberg, Germany

Education

01/2006	Diploma degree in Pharmacy, Martin-Luther-University Halle-Wittenberg, Germany
12/2005	Approbation as a pharmacist, Martin-Luther-University Halle-Wittenberg, Germany
05/2005-10/2005	Practical training at the Apotheke Altmarkt-Galerie, Dresden, Germany
10/2004-04/2005	Diploma thesis in the group of Prof. Dr. K. Mäder, Pharmaceutical Technology, Martin-Luther-University Halle-Wittenberg, Germany
08-09/2001	Practical training at the Pharmazeutisches Kontroll- und Herstellungslabor Halle, Germany
10/2000-10/2004	Pharmacy studies at Martin-Luther-University Halle-Wittenberg
05/2000	Albert-Schweitzer-Gymnasium in Halle/Saale (Abitur)

List of publications

Research articles

Rübe, A.; Klein, S.; Mäder, K.: Monitoring of in Vitro Fat Digestion by Electron Paramagnetic Resonance Spectroscopy; Pharmaceutical Research Vol. 23, No. 9, 2006

Abdalla, A.; Klein, S.; Mäder, K.: A new self-emulsifying drug delivery system (SEDDS) for poorly soluble drugs: Characterization, dissolution, in vitro digestion and incorporation into solid pellets; European Journal of Pharmaceutical Sciences 35, 457-464, 2008

Book chapter

Kegel, C., Klein, S., Mäder, K.: Neue Hilfsstoffe - Mehrwert oder nur Mehrkosten?, chapter in: Mäder, K., Weidenauer, U.: Innovative Arzneiformen, WVG Stuttgart, 2009

Presentations

Klein, S., Rübe, A., Mäder, K.: EPR investigations on pancreatin induced degradation of nanocapsules and the impact on drug localisation; 33rd Annual Meeting of the Controlled Release Society, Wien, 2006 (Poster)

Klein, S., Rübe, A., Mäder, K.: EPR investigations on polymeric nanocapsules and the impact on drug localisation; International Symposium on Polymer Therapeutics 2007 in Berlin (Poster)

Klein, S., Mäder, K.: Fate of pharmaceutical surfactants during digestion; Controlled Release Society, German Chapter Annual Meeting 2007 in Freiburg (Poster)

Klein, S., Mäder, K.: Impact of digestion on pharmaceutical surfactants; 34rd Annual Meeting of the Controlled Release Society, Long Beach, California, 2007 (Poster)

Klein, S., Rübe, A., Mäder, K.: EPR investigations on dynamics of distribution processes and in vivo fate of polymeric nanocapsules; 34rd Annual Meeting of the Controlled Release Society, Long Beach, California, 2007 (oral presentation)

Klein, S., Mäder, K.: Pancreatin-induced digestion of lipid formulations: Kinetics and impact on solubilization of lipophilic drugs; Controlled Release Society, German Chapter Annual Meeting in Braunschweig, 2008 (oral presentation)

Klein, S., Mäder, K.: Pancreatin-induced digestion of Solutol® HS 15:Kinetics and impact on solubilization of lipophilic drugs; 6th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Barcelona, Spain, 2008 (Poster)

Klein, S., Wolf, R., Mäder, K.: The fate of sucrose ester during in vitro digestion; 36rd Annual Meeting of the Controlled Release Society, Copenhagen, Denmark, 2009 (Poster)

Klein, S., Mäder, K.: Design of lipid formulations for oral drug delivery: Impact of dilution and digestion on the solubilisation capacity, Mitteldeutsches Doktorandentreffen in Leipzig, 2009 (oral presentation)

Klein, S., Mäder, K.: Preparation and characterization of lipid formulations of Probucol: Influence of digestion on the solubilization behaviour, 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Valetta, Malta, 2010 (Poster)