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Research paper

Simultaneous lipolysis/permeation *in vitro* model, for the estimation of bioavailability of lipid based drug delivery systems

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ABSTRACT

The simultaneous processes of lipid digestion and absorption together determine the oral bioavailability of drugs incorporated into lipid based drug delivery systems (LBDDS). A number of slightly different protocols for *in vitro* lipolysis are widely accepted; however, the permeation process has so far not been included into the models due to the harsh conditions of lipid digestion compromising permeation barriers. The present study for the first time combines biomimetic permeation and lipolysis of LBDDS.

The focus of the current work was on the functional stability of the barrier - Permeapad® during lipid digestion. Using calcein as a marker molecule the investigations demonstrated that the barrier was able to maintain its permeation properties in the presence of the SNEDDS (self-emulsifying drug delivery system) formulation, the lipolysis medium, and the lipolysis medium while digesting the SNEDDS. Furthermore, the permeation of cinnarizine (CINN) from SNEDDS was demonstrated to be lower, if the formulation as such was applied as compared to the digested formulation. This support the general perception that meaningful *in vitro* evaluation of lipid based formulations requires consideration of both, the digestion and absorption, i.e. lipolysis and permeation.

1. Introduction

Many of the newly developed chemical entities in the pharmaceutical industry have a high permeability, but a poor aqueous solubility, hence being classified as class II drugs in the biopharmaceutical classification system (BCS) [1]. Dependent upon the physical-chemical characteristics that make the compounds poorly aqueous soluble, they can be denoted as either "grease balls" or "brick dust" [2]. Brick dust are in general terms, used to describe compounds with both a high log P and a high melting point, whereas grease balls are highly lipophilic compounds (Log P > 4) with a low melting point (<100 °C). While the solubility of grease balls type materials in aqueous media is low, it is frequently observed that these compounds are highly soluble in lipids, though this rule of thumb does not apply in all cases [3]. Generally, this makes grease ball type molecules very suited for lipid based drug delivery systems (LBDDS) such as e.g. self-nano-emulsifying drug delivery systems (SNEDDS). For poorly aqueous soluble drugs, LBDDS offers the advantage over conventional dosage forms such as tablets and capsules to present the molecule in a pre-solubilized form into the intestine, thereby omitting the potential rate determining step for absorption – namely the dissolution of the compound. Once taken orally the lipids will be digested in

Abbreviations: ACN, acetonitrile; NaTC, sodium taurocholate; LBDDS, lipid based drug delivery system; SNEDDS, self-nano-emulsifying drug delivery systems; CINN, cinnarizine; PAMPA, parallel artificial membrane permeability assay; PVPA, phospholipid vesicle-based permeation assay; MCDK, Madin-Darby Canine Kidney; PBS, phosphate buffer saline; TG, triglycerides; FFA, free fatty acids.

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the gastro intestinal tract (GI-tract). The digestion of lipids will result in formation of surface active mono-glycerides and di-glycerides, and the drug will be liberated from its pre-solubilized form. Drug molecules solubilized inside these micelles and in other colloidal structures present in the gastro intestinal fluids first need to be released from such structures and become freely (truly) molecularly dissolved, before their uptake [4]. This fact is widely discussed to be the reason for poor predictability of bioavailability from apparent solubilities [5,6]. Therefore the digestion step of the LBDDS is very important for drug release and subsequently the absorption.

Different models have been suggested for simulating the intestinal lipid digestion *in vitro*. The lipid digestion process is facilitated by different pancreatic enzymes, such as co-lipase, dependent pancreatic lipase [7], which can be mimicked *in vitro* using an *in vitro* lipolysis model [8,9]. The pancreatic lipase hydrolyzes the triglycerides (TG) in a LBDDS into two free fatty acids (FFA) and one mono-glyceride, thereby allowing transfer of the dissolved drug into mixed intestinal micelles. The composition of *in vitro* lipolysis medium consists primarily of bile salts and phospholipids, often in the ratio of 4:1 [10]. Porcine pancreatin is often used as enzyme blend due to its similarity in enzymatic composition in human pancreatic fluid [11]. It is widely accepted, that release of a compound, solubilized in a LBDDS can be investigated in an *in vitro* lipid digestion model, and discussions about standardization is also prevalent [12]. It has been reported that the drug release of cinnarizine (CINN) from a SNEDDS formulation during digestion was high [13]. The precipitation of CINN observed *in vitro* was of limited importance since the *in vivo* data did not show any difference between the formulations, despite that some of the formulations lead to precipitation *in vitro* and others did not [14,15]. It has further been suggested that the precipitate, being amorphous, would obstruct absorption less than a crystalline precipitate, assuming that the compound has a significant absorption during its passage through the gastro intestine. Hofmann and coworker [16] suggested therefore a model combining *in vitro* lipolysis with *ex-vivo* intestinal permeation; however no correlation was obtained when compared to *in vivo* bioavailability data from rats [16]. More recently Porter and coworkers [17] have described an *in vitro* lipid digestion model coupled with a single pass *in situ* intestinal perfusion setup in order to better investigate and understand the interplay between drug solubilisation, precipitation and absorption [17]. However, this model is time-consuming and complicated, not to mention, the high variability, when using animals and the associated ethical considerations.

Common alternative permeation studies are cell-based studies, such as Caco-2 and Madin-Darby Canine Kidney (MDCK) [18,19], or the non-cell based studies, such as parallel artificial membrane permeability assay (PAMPA) and phospholipid vesicle-based permeation assay (PVPA) [20,21]. However, none of these methods has been demonstrated to be able to withstand the harsh conditions of the lipolysis medium and pancreatic enzymes, not to mention the different surfactants and solvents used in LBDDS and other enabling formulations. Permeapad® has in earlier studies shown good resistance to a number of surfactants and solvents [22]. An *in vitro* model combining lipolysis and permeation using the biomimetic barrier may be an approach to better understand the drug uptake from LBDDS. The model may provide formulation scientists in an early phase of development with better prediction tools of drug uptake, given the fact that the uptake/permeation of drug molecules will affect the equilibrium of solubilized and freely dissolved drug.

The aim of this study was, therefore to combine *in vitro* lipid digestion and permeability in a simplified experimental setup and to investigate the usability of Permeapad® for lipolysis/permeation studies, using a LBDDS containing CINN as the model drug.

2. Materials and methods

2.1. Materials

Brij® 97, calcein, calciumcloride dihydrate ($CaCl_2{\cdot}2\ H_20$), cinnarizine, Cremophor® RH 40, oleic acid, pancreatin from porcine, sesame oil, sodium taurocholate, trismaleate, and 4-bromobenzene boronic acid (4-BBA) were all obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Soy phosphatidylcholine (PC) S-100 was a generous gift from Lipoid GmbH (Ludwigshafen, Germany). Sodium dihydrogen phosphate dihydrate ($[NaH_2PO_4]{\cdot}2\ H_2O$), di-sodium hydrogen phosphate dodecahydrate ($[Na_2HPO_4]{\cdot}12H_2O$), maleic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl) and sodium chloride (NaCl) used for the different buffers were all obtained from Sigma-Aldrich. All solvents used were of analytical grade and bought from Sigma-Aldrich. Water used for all the experiments was obtained from a MilliQ purification system.

2.2. Methods

2.2.1. Preparation of biomimetic barrier

The Permeapad® (Certificate No. 014557268) barrier was prepared as previously described by Di Cagno and Bauer-Brandl [23] using soy phosphatidylcholine S-100 as the lipid layer. In brief, a thin layer of lipid was applied to a hydrophilic support sheet (Pütz GmbH, Taunusstein, Germany). The lipid was dissolved in an organic solvent, which was allowed to evaporate after applying the organic solution to the support sheet, thereby forming the barrier. The final barrier therefore consisted of support layer and lipid layer. Alternatively the empty support sheet was used as a barrier. It is a hydrophilic polymer membrane (cellulose hydrate) which acts as a diffusion barrier. All barriers employed in this work were stored at room temperature protected against sunlight.

2.2.2. Preparation of the SNEDDS and lipolysis medium

The SNEDDS were prepared by weighing and mixing the components, see Table 1. Ethanol was added as the last excipient to minimize evaporation. The mixture was stirred until homogeneous. 50 mg/g cinnarizine was added to the mixture, and stirred until it was completely dissolved.

The lipolysis medium was prepared according to the protocol of [24], with some slight modifications, by dissolving the components listed in Table 2 in MilliQ water. The medium was prepared one day prior to the lipolysis experiments and was let to equilibrate at 37 °C until the next day, when pH and volume was finally adjusted.

Pancreatic extract solutions for the lipolysis experiments were prepared 10 min prior use to limit denaturation. The extract was prepared by gently vortexing pancreatic extract into Milli Q water until homogenously mixed. Enough pancreatic extract was added to obtain a lipase activity of 600 USP units (USP/mL). When

Table 1
Composition of the formulation and drug concentration used in the lipid based drug delivery system (SNEDDS).

SNEDDS composition	
Components	Ratio (w/w)%
Cremophor RH 40	45
Oleic acid	15.4
Brij 97	9
Sesame oil	20.6
Ethanol	10
Cinnarizine	50 mg/g

Table 2
Composition of the intestinal *in vitro* lipolysis medium.

Compound	Concentration (mmol/l)
NaCl	50
Tris base	25
NaTC	5
S-100 PC	1.25
$CaCl_2 \cdot 2\ H_2O$	5

homogenous, the mixture was centrifuged (5804 R centrifuge equipped with a F-34-6-38 rotor, Eppendorf, Hamburg, Germany) at 4000 rpm for 7 min, at 37 °C. The supernatant was collected, and pH adjusted to pH 6.5 with 0.5 M NaOH.

2.2.3. Solubility studies

The solubilities of CINN in buffer and in SNEDDS were determined using the shake-flask method. Excess of CINN was added to 10 mL buffer or 2 g SNEDDS, respectively, and mixed thoroughly. The suspensions in buffer and SNEDDS were allowed to equilibrate at 37 °C and 25 °C respectively for a minimum of 3 days. Both the suspension in aqueous buffer and in SNEDDS were then centrifuged at 11.000 rpm, at 25 °C or 37 °C for 30 min. Samples of the aqueous buffer suspensions were withdrawn and filtered through a 0.1 μm pore-size Anotop® 25 syringe filter (GE Healthcare, Little Chalfont, UK). Accurately weighed filtrates were transferred to HPLC vials and diluted with ACN (1:1). Quantification was carried out according to Section 2.2.4.

For the suspensions in SNEDDS, accurately weighed samples of the supernatant were transferred to a volumetric flask and diluted with chloroform:ACN (1:1) to 5 mL. Aliquots were diluted further with ACN to suitable concentrations and analyzed. Analysis was carried out according to Section 2.2.4, with the following alterations: the mobile phase was 100% ACN at a flowrate of 0.2 mL/min.

2.2.4. Permeation studies with cinnarizine suspension and cinnarizine in SNEDDS

The permeability of CINN when dispersed in an aqueous buffer and when solubilized in the SNEDDS was determined by permeability studies conducted in side-by-side diffusion cells (Ussing chambers, SES GmbH-Analysesysteme, Bechenheim, Germany).

The CINN suspensions were prepared by weighing 30 mg of CINN and adding 30 mL of buffer followed by placement in a shake-bath at 37 °C for 3 days. 1 g of the SNEDDS solution (containing 50 mg/g CINN) was dispersed in 40 mL of buffer by magnetic stirring until a homogeneous dispersion was obtained. In both cases, the donor side for the permeation experiment consisted of 7 mL of the respective formulation, namely either a CINN suspension or a SNEDDS solution containing CINN, while the acceptor chamber always contained 5 mL FaSSIF-V2-Blank buffer (fasted state simulated intestinal fluids) [25]. The permeability studies were carried out at 37 °C for 5 h, and samples of 500 μL were withdrawn at 30, 60, 90, 120, 150, 180, 210, and 240 and 300 min and immediately replaced with fresh buffer. 700 μL ACN was added to all withdrawn samples prior to analysis. The pH values in both the acceptor and donor compartments were measured prior to and after the permeability experiment.

Samples were analyzed using a UFLC-FL (Shimadzu, USA) with the following components: DGU-20A RR degassing unit, LC20AD pump, SIL-30AC autosampler, RF-20A xs detector, and a CTO-10 AS column oven. The separation was obtained using a Phemomenex Kinetex 1.7 μm EVO C18 column (150 mm × 2.1 mm) and a mobile phase consisting of ACN:water (40:60) at a flowrate of 0.15 mL/min, and a column temperature of 30 °C. The CINN content in the samples was quantified by fluorescence (ex: 249 nm, emm: 311 nm). The identified peak areas were converted to concentrations by standard curves in the range of 5–250 ng/mL.

2.2.5. Validation of lipolysis/permeation studies employing biomimetic barrier

Not all the components of the chosen SNEDDS formulation had previously been tested for their compatibility with Permeapad® and the respective combination of surfactants and solvents had not been studied either. The lipolysis media contain various pancreatic enzymes and lipids, resulting in a harsh environment in terms of lipid degradation and thus possibly unwanted alteration of permeation properties.

The barrier's ability to maintain its barrier function in the presence of the excipients included in the SNEDDS, the SNEDDS and the lipolysis media prior to the development of combined lipolysis/permeation studies. All validation studies were carried out using calcein as a hydrophilic marker in various dispersions with SNEDDS and/or lipolysis media, with PBS at 37 °C as the reference control. The control experiment was conducted as previously described [22], using side-by-side diffusion chambers instead of Franz cells, in order to maintain a temperature of 37 °C in both chambers.

The barrier was firstly validated with respect to the SNEDDS and its components. 1 g of SNEDDS was dispersed in 35 mL of PBS by magnetic stirring until a homogenous dispersion was obtained. 5 mL of a 40 mM calcein solution in PBS was added, resulting in a concentration of 5 mM calcein and a total volume of 40 mL. 5 mL of the dispersion was transferred to the donor compartment of the side-by-side chambers and the permeation study initiated. The acceptor compartment consisted of 74 mM PBS pH 7.4 ± 0.05, 285 ± 5 mOsm/kg. The permeation studies were carried out as described earlier [22].

Thereafter the barrier was validated towards i) the lipolysis media alone and, ii) with the presence of both SNEDDS and lipolysis media. i) 30 mL of lipolysis media (Table 1) and 3.3 mL of a 60 mM calcein solution were mixed by magnetic stirring in a thermostated vessel at 37 °C. ii) 1 g of SNEDDS was dispersed in 30 mL of lipolysis media by magnetic stirring and 3.3 mL of a 60 mM calcein solution was added. In both cases the pH of the solution was adjusted to 6.5 by addition of 0.5 M NaOH within 3 min and 6.7 mL of pancreatin extract was thereafter added to initiate the lipolysis in the thermostated vessel. The pH of the lipolysis experiment was maintained at 6.5 for 10 min by continuous manual titration adding 0.5 M NaOH. After 10 min, 5 mL of the lipolysis media, containing calcein, were transferred to the donor compartment of the side-by-side chambers and a permeation study initiated while the lipolysis was still progressing. The acceptor chamber consisted of 5 mL FaSSIF-V2-Blank buffer. The permeation study itself was carried out over 4 h as described in Section 2.2.4.

In order to determine if the integrity of the barrier was still maintained after the lipolysis/permeation study an explorative barrier functionality test was carried out after the experiment with SNEDDS alone, lipolysis media alone, and a combination of both. In all these cases when the lipolysis/permeation study was finished the acceptor and donor compartments of the side-by-side diffusion cells were emptied. Both compartments were washed with 5 mL PBS 3 times, until all residues of calcein were removed, and thereafter a standard calcein permeation experiment in aqueous solution was carried out on the very same barriers as previously described [22]. Calcein samples were analyzed by fluorescence spectroscopy using a BMG Fluostar Omega 96 plate reader with excitation at 485–512 nm and emission at 520 nm (BMG Labtech GmbH, Ortenberg, Germany).

2.2.6. Lipolysis/permeation studies using SNEDDS and model drug cinnarizine, employing biomimetic barrier

The lipolysis/permeation studies, using a SNEDDS formulation, containing CINN (50 mg/g) were conducted employing the side-by-side diffusion chambers. The lipolysis/permeation setup was chosen to be semi-dynamic, meaning that the lipolysis was initiated in a thermostated beaker, prior to transfer into the Ussing chamber. The donor chamber consisted of 7 mL lipolysis media, with SNEDDS, and the acceptor chamber consisted of 5 mL FaSSIF-V2-blank buffer.

The biomimetic barrier was employed in all the studies with a surface area of 1.77 cm^2. The lipolysis/permeation studies were carried out over 4 h at 37 °C. Samples of approx. 4 mL were withdrawn from the acceptor side with a glass syringe every 20 min for 4 h and 4 mL fresh buffer was added to the acceptor chamber after each sample to maintain sink conditions. The samples were weighed and 4 mL ACN was added to each sample prior to analysis. The pH values of the both the acceptor and donor solution were measured prior to and after the lipolysis/permeation experiment. Samples were analyzed according to Section 2.2.4.

The same lipolysis/permeation study was conducted using the empty support sheet as barrier instead of Permeapad® barrier, to establish the effect of the lipid layer.

2.2.7. Data analysis/permeability calculations

The cumulative amount of permeated drug (dn) was plotted as a function of time (t) and the surface (A), according to Eq. (1):

$$J = \frac{dn}{A \cdot dt} \quad (1)$$

The linear part of the slope corresponded to the steady-state flux (J). In some of the permeation studies a lag-time was observed, in which case the lag time was excluded when the flux was determined, hence all calculations were made using the steady-state flux. The obtained flux values were used to calculate the apparent permeability coefficient (P_{app}) using Eq. (2), where the flux, was divided by the initial concentration of the drug in the donor chamber:

$$Papp = \frac{J}{C_0} \quad (2)$$

2.2.8. Statistical analysis

Significant differences of permeabilities were evaluated by a two-sided student's t-test and ANOVA Tukey Post-hoc test. $P \leq 0.05$ was considered as significantly different. Potential outliers were evaluated using a Thomson Tau test.

3. Results and discussion

3.1. Solubility studies

CINN was chosen as a model drug in the present study due to its low solubility at pH values present in the intestine and high solubility in LBDDS. The LBDDS can help to maintain CINN in a solubilized state in the intestine and may avoid recrystallization or phase separation and thereby potentially promote the bioavailability. The solubilities of CINN in the lipid formulation (SNEDDS) and in FaSSIF-V2-Blank buffer were found to be 72.9 ± 17.6 mg/mL and 0.119 ± 4.4 mg/mL respectively. These values were in good accordance with literature values [2,15,26].

3.2. In vitro lipolysis model

Prior to the lipolysis/permeation setup an *in vitro* lipolysis protocol needed to be defined. First a background lipolysis was carried out with the lipolysis medium alone, i.e. without the addition of the lipid formulation. The degree of hydrolysis of the digestible components in the SNEDDS was calculated by determining the background NaOH consumption, via back titration to pH 9 [11,27]. As can be seen in Fig. 1 a clear difference in the NaOH consumption was observed in the presence and absence of the lipid formulation (SNEDDS), hence the formation of FA was more extensive when the SNEDDS was added to the medium, due to hydrolysis of the TGs from sesame oil contained in the SNEDDS. The amount of hydrolyzed sesame oil was calculated to be 60.2% and 84.3%, of the added amount, after 30 and 90 min, respectively. The hydrolysis process for the triglycerides in the formulation must therefore be expected to be ceased within the 5 h study.

Recent studies from Heider and co-workers [28] have questioned if the pH- stat method with back titration as a non-specific method can accurately quantify the degree of enzymatic digestion of lipids in LBDDS. Results from their study showed that the back titration method might lead to overestimation of lipid digestion due pKa values of the produced fatty acids interfering with the expected pH changes, as compared to a high performance thin layer chromatography (HPTLC) method, which is highly specific for the fatty acids. The disadvantage of HPTLC, is, however that it is limited to long chain fatty acids (LCFA) [28]. The pKa values of LCFA, can vary from 4 to 10 depending on the microenvironment – and a determination of a FA's pKa value in the presence of digestion medium (bile salts, proteins, micelles) is very difficult due to the complexity of the system [28]. Since the aim of this study was to determine the use of the Permeapad® barrier for evaluation of drug absorption for LBDDS, the specificity of the TG digestion was not the main focus here. It was therefore decided that the use of back titration method to determine TG digestion to be accurate enough for studies in early development phases due to its simple and fast setup.

The composition of the lipid formulation, SNEDDS, chosen for this study is shown in Table 1. The main TG present was sesame oil, which mainly consists of linoleic acid (41%), oleic acid (39%), palmitic acid (8%) and stearic acid (5%) after Codex Alimentarius [29]. This formulation was chosen, since it has previously been studied in great detail regarding lipid digestion, and was known to increase the apparent solubility of CINN [14,30,31]. Also these studies have shown that the precipitate of CINN from this formulation was in a non-crystalline form, indicating that the precipitate might be in an amorphous form [13].

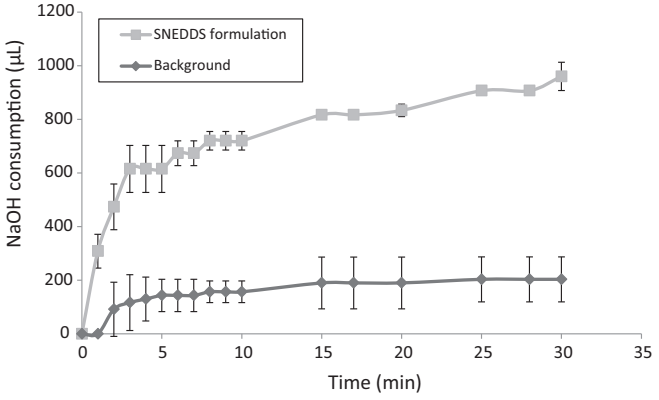

Fig. 1. The NaOH consumption in the presence and absence of the lipid based formulation, SNEDDS, in the *in vitro* lipolysis setup. Data presented as mean ± SD (n = 3).

3.3. Validation of lipolysis/permeation studies employing biomimetic barrier

Prior to using Permeapad® in combined lipolysis/permeation studies, the functional resistance of barrier needed to be validated under the harsh conditions of lipolysis, i.e. the presence of the SNEDDS formulation, the lipolysis medium and pancreatic enzymes, and after the finished experiment. The necessary experiments were carried out using calcein, a marker frequently used, to determine the integrity of barriers.

The results and P_{app} values for the validation studies are shown in Table 3. First, control permeation studies with aqueous calcein solution at 37 °C were conducted. Thereafter the permeability of calcein in the presence of the SNEDDS formulation was determined and results were compared to the control (pure calcein solution). No significant difference ($P > 0.05$) was observed when the SNEDDS was added to the donor chamber, however, a much larger standard deviation was observed as compared to the control experiment. Due to the higher standard deviation obtained, it was decided to carry out explorative barrier functionality studies after the end of the experiment: the side-by-side cells were emptied after the permeability study, the barrier washed, and another permeation study with calcein solution was carried out on the very same barrier that had previously been exposed to the SNEDDS. Results of these explorative barrier functionality studies showed no significant difference ($P > 0.05$) and the integrity of the barrier was therefore considered unchanged. The high variability of calcein permeation in the presence of SNEDDS was probably due to different colloidal structures formed when the SNEDDS was suspended in PBS, which may have different formation kinetics depending on the shear forces in the stirred chamber. Furthermore, such colloidal structures may also interact with the surface of the barrier.

As a next step, the functionality of the barrier was validated towards the lipolysis medium and the pancreatic enzymes. The permeability of calcein in the permeation study was not significantly different from the control, but a trend towards a lower P_{app} was observed, see Table 3. A lower P_{app} value could also in this situation, be a reflection of the formation of different colloidal structures present in the lipolysis medium. Again the explorative barrier functionality studies were carried out. The results were in line with the control ($P = 0.84$) and again the barrier was therefore considered functionally unchanged and hence found suited for the *in vitro* lipolysis experiments.

Furthermore, the effect of the combination of SNEDDS and lipolysis media in the donor chamber on the barrier functionality was also studied. Calcein permeation studies were conducted to establish if Permeapad® could be used for combined lipolysis/permeation studies. Two permeability studies were conducted over 4 and in addition over 24 h, respectively, to establish the barrier integrity also after very long exposure times. In both cases the barrier functionality studies with calcein were carried out on the very same barrier that was exposed to the SNEDDS and lipolysis media as described above. After the 4 h permeation study, the barrier showed a significant decrease in calcein permeation compared to the control ($P = 0.007$), as shown in Table 3. In contrast, the 24 h permeation study showed results in line with the control. Fig. 2A shows the steady state increase in flux over 24 h for calcein in the presence of SNEDDS and ongoing lipolysis. The explorative barrier functionality studies of calcein resulted in a permeability in line with the permeability of the calcein control as shown in Table 3. The barrier was therefore found to keep its functionality in all the investigated cases. As the permeability of calcein was lower than the control and not higher, the values after 4 h were attributed to supramolecular effects i.e. interaction on the molecular level, between calcein and any compounds present, rather than degradation of the barrier. In general, a tendency towards lower calcein permeability was seen in the presence of SNEDDS and/or lipolysis as shown in Fig. 2B. The decrease in permeability was assumed to be a reflection of the different colloidal structures present in the medium. A schematic sketch of the conditions during the lipolysis is presented in Fig. 3.

The colloidal structures formed during lipolysis may affect the permeability of drug molecules. In the case of a lipophilic compound the incorporation of drug into the formed colloidal structures, such as micelles and mixed micelles may result in a decreased diffusion rate, due to a decreased concentration in molecularly dissolved drug. For hydrophilic markers, the decrease in permeability may probably not be attributed to incorporation into micelles. The decrease may rather be due to other interactions with the colloidal structures on the molecular level.

3.4. Permeation studies with cinnarizine

The permeability studies of CINN from a suspension in aqueous buffer and in a SNEDDS dispersion, respectively were carried out using both the biomimetic Permeapad® barrier system and the empty support sheet. Results in Fig. 4 show that the permeability of CINN in the aqueous buffer was slightly lower through the biomimetic barrier as compared to the empty support sheet. However, this difference was not significant. Further the results in Fig. 4 show that the permeability of CINN from the SNEDDS dispersion was significantly lower through the barrier when compared to the empty support sheet. These results indicate that the lipid layer in Permeapad® also in the present case adds to the function of a permeation barrier as has been described earlier [32].

Both for the support sheet and the biomimetic barrier, the apparent permeability coefficient (P_{app}) of CINN was lower when dispersed in the SNEDDS formulation, as compared to the aqueous

Table 3
Apparent permeability coefficient (P_{app}) obtained during the permeability validation studies and the explorative barrier functionality studies using calcein. Data presented as mean ± SD (n = 3–9).

Permeation studies	Duration	P_{app} (10^{-6} cm/s)	*P*-value	*n*
Calcein in aqueous solution (control)	5 h	3.36 (0.46)		3
Calcein in SNEDDS	5 h	3.19 (1.35)	0.83	9
Calcein + lipolysis	4 h	2.28 (0.81)	0.11	3
Calcein in SNEDDS + lipolysis	4 h	1.99 (0.09)	0.007*	3
Calcein in SNEDDS + lipolysis	24 h	2.89 (0.45)	0.28	3
Explorative barrier functionality studies				
Calcein in SNEDDS	4 h	4.11 (0.13)	0.052	3
Calcein + lipolysis	4 h	3.29 (0.32)	0.84	3
Calcein in SNEDDS + lipolysis (4 h)	4 h	2.86 (0.30)	0.19	3
Calcein in SNEDDS + lipolysis (24 h)	5 h	3.08 (0.36)	0.45	3

* *P*-value compared to control significant different < 0.05.

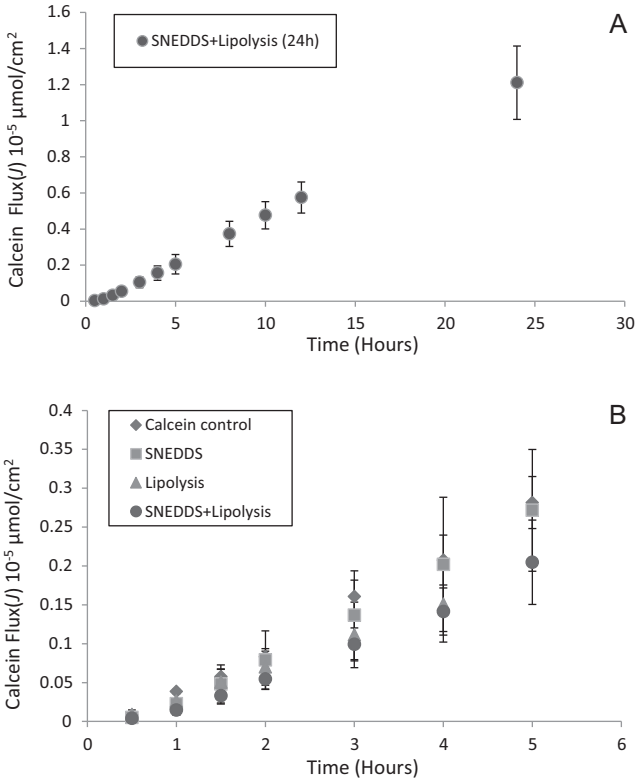

Fig. 2. The cumulative permeated calcein, a hydrophilic marker, (A) in the presence of SNEDDS formulation and lipolysis digestion medium over 24 h. Data presented as mean ± SD (n = 3) and (B) Calcein in aqueous solution, compared to calcein in the presence of SNEDDS formulation, lipolysis digestion medium and a combination of both, respectively. Data presented as mean ± SD (n = 3–9).

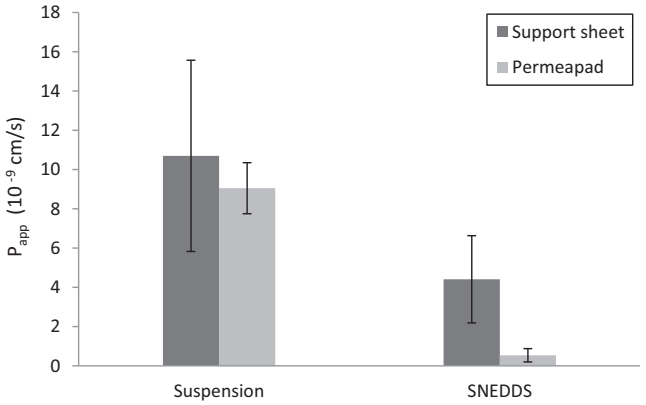

Fig. 4. The apparent permeability coefficient (P_{app}) of cinnarizine from an aqueous suspension in buffer pH 6.5 and from a SNEDDS dispersion through Permeapad® and the empty support sheet, respectively. Data presented as mean ± SD (n = 3).

saturated solution (suspension), as can be seen in Fig. 4. This was expected since CINN was solubilized inside the lipid droplets of the SNEDDS and hence, not freely available for permeation, as it was in the aqueous suspension. There is a distinct difference between solubilized drug in a lipid carrier and molecularly dissolved drug with respect to permeation, and it is widely accepted that only the molecularly dissolved drug molecules are able to permeate [4]. Even though the lipid formulation was able to increase the apparent solubility of CINN, the drug was maintained in the formulation when applied to the barrier; hence it was not able to permeate freely. Fong and coworkers [6] have shown that the increase in apparent solubility of a BCS class II drug in various lipid formulations did not result in an increase in *in vitro* permeability [6]. Therefore it is necessary to include physiological processes, i.e. lipid hydrolysis (lipolysis) as an essential step, in order to use permeability studies of lipid formulations for the prediction of their performance *in vivo*.

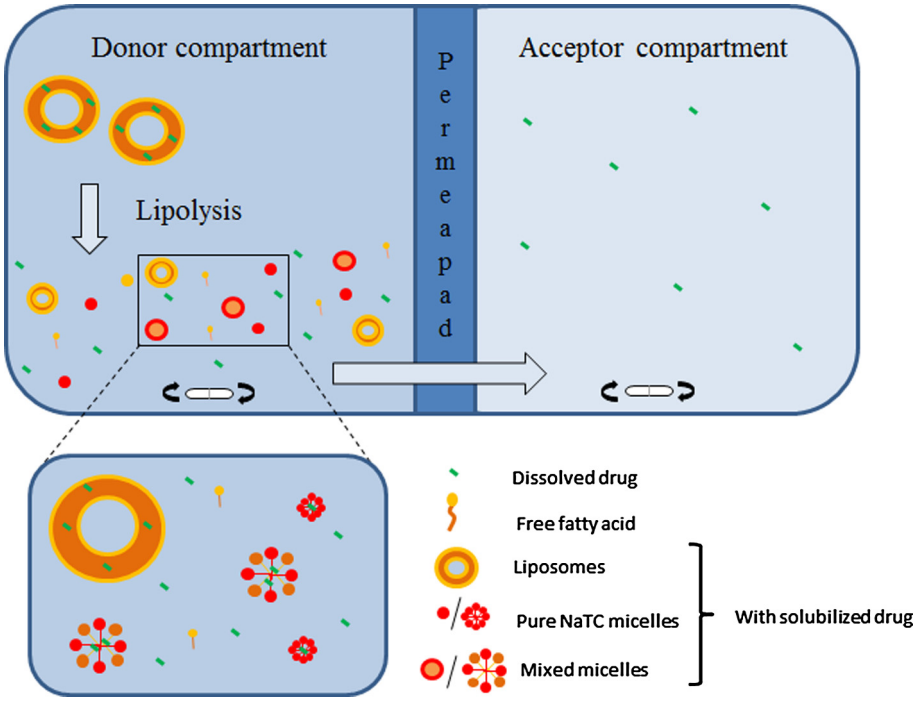

Fig. 3. A schematic overview of the different colloidal structures that may be formed during the lipolysis/permeation.

3.5. Lipolysis/permeation studies using SNEDDS and model drug cinnarizine, employing biomimetic barrier

Lipolysis/permeation studies on SNEDDS with the model drug CINN were carried out using the biomimetic barrier. To our knowledge this was the first successful attempt to combine lipolysis and permeation simultaneously in a single *in vitro* model. The permeability of CINN dissolved in the SNEDDS formulation in the proposed lipolysis/permeation model resulted in a flux (J) of 88.99 ± 16.95 μmol/cm^2·s, which was much higher than from the non-hydrolyzed SNEDDS dispersion, (J) = 1.83 ± 1.15 μmol/cm^2·s. The flux (J) was approx. 50 times higher when combining lipolysis with permeation; this indicated the importance of evaluating both elements in combination when working with lipid based formulations.

A similar lipolysis/permeation experiment was carried out using the empty support sheet without the lipid layer of the barrier, to determine the effect of the lipid layer in Permeapad®, in relation to lipolysis/permeation studies. The permeability of CINN in SNEDDS, when combined with a lipolysis step, was higher for the empty barrier support than if using the barrier. This indicated that the lipid layer in Permeapad® makes a significant contribution to the transport of the lipophilic drug. Fig. 5, depicts the cumulative permeation of CINN over time for both the empty support sheet and the biomimetic barrier.

The permeation of CINN across the biomimetic barrier was not in steady state during the entire time period of the experiment (5 h). It was observed that the rate of permeation, expressed in Fig. 5 as the slope of cumulative amount of permeated drug, decreased and flux reached a plateau after approx. 2 h. This phenomenon is seen once equilibrium between the acceptor and donor chambers is achieved. However, sink conditions were always maintained in this study, since 4 out of the 5 mL of the acceptor chamber volume was replaced with fresh buffer every 20 min, and the total amount of drug that had permeated during the 4 h study was only a very minor fraction (0.00012%) of the amount of drug in the donor chamber.

An important issue, which needs to be taken into account in order to understand this behavior, was the many factors contributing to the complexity in the model. Even though, the model was simplified compared to *in vivo* – the dynamic setup and formation of many different colloidal structures, added to the complexity. The decline in the flux after approx. 2 h may therefore be a consequence of the formation of micelles and other colloidal structures. If the micelles were slowly formed, and the increase in number of micelles interacts with the molecularly dissolved drug then, the drug will be solubilized inside the micelles and the amount of molecularly dissolved drug will decrease – which may have resulted in the low overall permeability observed.

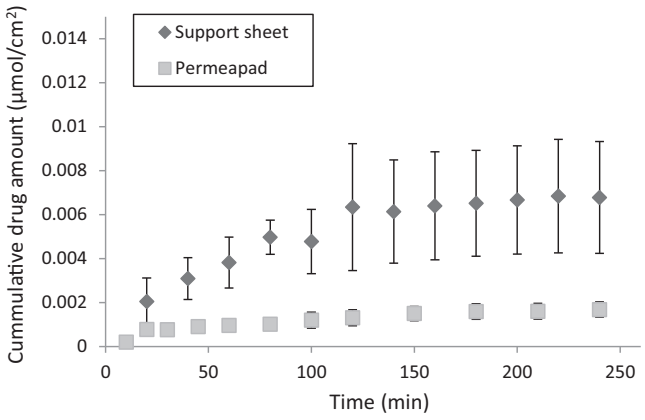

Fig. 5. The cumulative amount of permeated cinnarizine drug from a SNEDDS formulation across Permeapad® barrier and the support sheet, respectively. Data presented as mean ± SD (n = 3).

There were several factors in this proposed model that were simplified compared to the *in vivo* situation, including the removal of FFA by formation of calcium soaps. *In vivo* FFAs are absorbed, however, during the *in vitro* lipolysis; the FFAs may inhibit the enzymatic activity of pancreatic lipase, therefore calcium was added to form calcium soaps with the FFAs. It is currently unknown, if the presence of these calcium soaps can affect the drug absorption in the lipolysis/permeation setup.

The loading of CINN into the chosen SNEDDS formulation and CINN precipitation upon lipid digestion has previously been studied using *in vitro* lipolysis [14,31]. The same formulations have been studied *in vivo* in dogs and compared to the data obtained *in vitro* [14,15]. Results from these *in vivo* studies showed that the bioavailability of CINN in dogs was not affected by different drug loading concentrations in the SNEDDS. Although precipitation *in vitro* occurs, no significant decrease of bioavailability has been observed [14]. Therefore the *in vitro* lipolysis setup cannot be used as the only tool to predict the fate of LBDDS *in vivo*.

The data described in this study establish that the biomimetic barrier can be used for combining lipolysis/permeation studies using a known SNEDDS formulation and that an increase in CINN permeation could be observed, when the lipid formulation was digested. If this can be translated into a better *in vitro in vivo* correlation (*IVIVC*) remains to be determined, but the data in the present study provided a model, which can allow for the investigations to be performed. These data can then help to understand if the Permeapad® lipolysis/permeability approach is a predictive *in vitro* model for oral bioavailability for lipid based formulation.

4. Conclusion

The present study combines *in vitro* lipolysis and permeation in one simultaneous *in vitro* model. A semi-dynamic lipolysis/permeation setup was used and studies carried out using CINN in a SNEDDS formulation as a model of LBDDS system. Permeapad® was firstly validated with respect to the pancreatic enzymes and SNEDDS excipients using calcein as a marker. Results showed that the barrier maintained its integrity in the presence of the lipolysis medium. Results obtained from a model formulation of CINN in a SNEDDS showed significantly higher permeability of CINN, when lipolysis was combined with permeation. These findings once more stress the fact that for meaningful prediction of the *in vivo* performance of lipid based formulations, lipolysis needs to be considered.

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