Chapter-6

*In-vitro* lipid digestion study of SEDDS
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6.1 Introduction

While numerous techniques have been developed to address the increasing number of new drug molecules that demonstrate oral absorption limited by low aqueous solubility, methods that utilize drug pre-dissolved in a lipid vehicle remain popular (Williams et al. 2976-92). The basis for using lipids stems from a number of studies that noted improved absorption and bioavailability of a poorly water-soluble drug (PWS) following co-administration with a lipid-rich meal. In general, lipid-based drug delivery systems (LBDDS) therefore aim to harness the positive effect of dietary lipids on oral drug absorption (Hauss 667-76) (Porter, Trevaskis and Charman 231-48) by circumventing drug dissolution, which in the case of PWSD is often slow and potentially limits the rate and extent of drug absorption, and by increasing the solubilisation reservoir in the GI fluids (Devraj et al. 323-33).

In particular, when lipids are digested in the GI tract, lipolytic products combine with endogenous amphiphilic molecules (primarily bile salts and phospholipids) to form colloidal phases such as liquid crystals, vesicles and micelles (Dahan and Hoffman 96-105). The nature of the colloidal structure formed and the drug affinity for each colloidal species are critical determinants for enhancing the drug’s solubilisation and preventing the precipitation of the drug (Markopoulos et al. 3145-53). However, the precipitation of the drug during digestion could occur once the drug solubilisation capacity of the colloidal structure is exceeded (Bakala-N’Goma et al. 1279-87).

As LBDDS enter the small intestine, digestion of formulation components is inevitable and may significantly impact the subsequent formulation behaviour. Assessment of all types of LBDDS during in vitro digestion tests is therefore necessary for a complete understanding of formulation performance.

6.2. Materials and methods

6.2.1. Materials for preparing digestion buffer

Tris maleate, lipase, taurocholate were purchased from MP Biomedicals LLC, c/o Shreenath diagnostics and services, Ahmedabad, India. Sodium chloride was obtained from Finar Chemicals (India) Private Limited, Ahmedabad, India. Calcium chloride was obtained from High
purity laboratory chemicals, Mumbai, India. Lecithin soya, 30% was obtained from Himedia laboratories, Mumbai, India. All other reagents and chemicals were of laboratory grade and were used as received.

6.2.2. Design of *in-vitro* lipid digestion study

The procedure of lipolysis was followed as described in the literature with minor modifications (Dahan and Hoffman 96-105; Wasan et al. 76-84). The composition of lipolysis buffer is shown in table 6.1. The conditions were broadly representative of fasted state intestinal conditions. The selected formulations (0.5 ml, 1 ml and 1.5 ml) (as described in chapter 5) were added to the 35.5 ml of lipolysis buffer in a thermostated water bath with magnetic stirrer at 37°C. All the formulations were allowed to disperse prior to initiation of digestion. Thereafter, 1 mg of lipase enzyme (equivalent to 1000 IU/ml) was added to the lipolysis medium in order to start the digestion process. The pH was kept at 7.4 by continuously titrating the free fatty acids produced during the lipolysis against 0.2 M NaOH and monitoring with pH-meter. At different time (5, 15, 30, 45 and 60 min), 3 ml aliquots were sampled and immediately kept in the ice and then in the freezer (-20°C) to stop any further lipolysis (Fernandez et al. 3077-87). All the aliquots of the medium were centrifuged at 25,000 rpm for 1.5 h at 37°C. Then, the aqueous phase was decanted from the sediment. The separated aqueous phase was analyzed for LCH using UV-spectrophotometry (Ibrahim et al. 323-28). The same procedure was repeated for blank SEDDS formulations i.e. without drug, and the aqueous phases separated at the end were used as reference blank in UV spectrophotometry determination of the aqueous phase containing LCH. This step was taken to nullify the effect of digestion products on analytical estimation of LCH. The schematic set up is shown in the figure 6.1. In addition, the aliquots of 1 ml were withdrawn after 5, 10 and 30 min of adding lipase enzyme and kept frozen for structure determination of digestion products using Transmission Electron Microscopy (TEM). The morphology and colloidal structures were analyzed using electron microscope (Tecnai G2 20 TEM, Phillips Holland) operating at 70 kV capable of point-to-point resolution.
### Table 6.1. Composition for digestion buffer

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity (Molar Concentration)</th>
<th>Quantity in mg for 35.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Maleate</td>
<td>50 mM</td>
<td>215.02</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>150 mM</td>
<td>308.85</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>5 mM</td>
<td>26.09</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>5 mM</td>
<td>92.3</td>
</tr>
<tr>
<td>Lecithin</td>
<td>1.25 mM</td>
<td>336.185</td>
</tr>
</tbody>
</table>

![Figure 6.1. Schematic illustration of lipid digestion study setup](image)

### 6.3. Results and discussion

Theoretically, after centrifugation of the digestive medium three distinct phases should get separated i.e. (1) top most lipid layer (2) middle aqueous layer and (3) pellets at the bottom (Larsen, Sassene and Müllertz 245-55). In the present study, for all the formulations only aqueous and pellet phases were separated as shown in figure 6.2, which is believed to be due to presence of surfactants and co-surfactants along with lipids. After the digestion study there was no
undigested part of oil phase, which is the characteristic *in-vitro* behaviour of the SEDDS containing high amount of surfactants in presence of digestive enzymes (Cuiné et al. 995-1012). Upon estimation of LCH in aqueous phases derived after digestion of various formulations, useful information could be obtained. The results are depicted in table 6.2 and figure 6.3. The LC-SNEDDS which contained only 10% ricebran oil: GMO mixture (1:9) as oil phase, 60% tween 80 as surfactant and 30% propionic acid as co-surfactant released 78.38% of LCH at the end of 60 min. This amount of LCH in the aqueous phase can be attributed to digestion of surfactant tween 80 rather than oil phase. Studies suggest that high levels of digestible surfactant become determinants of drug dissolution as well as precipitation. Cuine and his co-workers reported, tween 80 to possess 54% extent of digestion in an in-vitro digestion experiments (Cuiné et al. 995-1012). Moreover the smaller globule size not only promotes efficient dispersion but digestion also, because research suggested that the exposed surface area of the formed globules represents the working sites for lipase enzyme (Porter and Charman S127-47). Therefore, increasing the specific surface area of the emulsified lipids enhances the action of the enzyme (Ibrahim et al. 323-28). LC-SNEDDS, by virtue of possessing high amount (60%) of digestible tween 80 and very fine globule size of 8-11 nm, successfully released highest amount of LCH into aqueous phase.

![Figure 6.2. Phases separated after centrifugation of the digestion medium](image)

Figure 6.2. Phases separated after centrifugation of the digestion medium
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The MC-SMEDDS which contained 40% of Capmul MCM as oil phase, 48% of Cremophor RH40 as surfactant and 12% of PEG 400 as co-surfactant released 64.77% of LCH. Several studies have demonstrated that Capmul MCM upon digestion can hold very less amount (approximately 30%) of the drug in the dissolution and allow the rest to be precipitated (Cuiné et al. 995-1012; Christopher J H. Porter et al. 1405-12; Reymond and Sucker 673-76; Christopher J. H. Porter et al. 1110-21). But in this formulation Cremophor RH 40 was also present which is reported to be the least digestible surfactant with only 7.5% of extent of digestion (Cuiné et al. 995-1012). Therefore, poor ability of Capmul MCM to keep the drug in dissolved form was offset by the presence of non-digestible surfactant along with the PEG 400 as co-surfactant. The reason being the incorporation of the co-surfactant and/or co-solvents into the surfactant layer formed at the water/surfactant interface, leading to increase in the flexibility and fluidity of the interfacial film. The flexibility provides stability to globules. Hence, appreciable amount of LCH was found in the aqueous phase. In addition, though LC-SNEDDS released highest amount after completion of digestion, upon initiation after 5 min MC-SMEDDS released highest amount of LCH in aqueous phase. This finding can be justified by the fact that Capmul MCM is mixture of medium chain mono- and di-glycerides and therefore can readily be converted into mono glycerides and free fatty acids, whereas ricebran oil:GMO mixture is long chain triglyceride which takes longer to digest (Ibrahim et al. 323-28).

The SC-SMEDDS, which contained 40% triacetin as oil phase and 60% Tween 80 as surfactant released 51.12% LCH in aqueous phase upon digestion. Triacetin, a short chain triglyceride which sometimes also behaves as co-solvent, possessed poor capacity to hold the drug in the dissolved form (McEvoy et al. 973-85).

Surprisingly, LC-4:6 and MC-4:6 showed similar capacity to release the drug in aqueous phase upon digestion. In case of MC-4:6, 58.86% drug was found in aqueous phase which could be due to Capmul MCM and Tween 80. Both being highly digestible and hydrophilic, have proven tendency to precipitate the drug. Ideally, LC-4:6 should have resulted into releasing high amount of LCH in aqueous phase because in several studies long chain triglycerides (if present in high amount in the formulation) are reported to solubilize the lipophilic drug upon extensive digestion (Christopher J. H. Porter et al. 1110-21) (C. J. Porter et al. 1405–12). However, in the present study, the result was not complying with the above finding. One reason can be thought as LC-4:6
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resulted into very course emulsion (500-600 nm) in different media upon dispersion providing less surface area for lipase to work.

LC-SNEDDS and MC-SMEDDS showed high amounts of LCH dissolved after digestion were again compared by repeating the digestion studies for extended time and increasing lipid load.

Table 6.2. Results of lipid digestion study

<table>
<thead>
<tr>
<th>Time in min</th>
<th>% Drug Available in aqueous phase</th>
</tr>
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<tbody>
<tr>
<td>LC-SNEDDS</td>
<td>LC-4:6</td>
</tr>
<tr>
<td>5</td>
<td>32.68</td>
</tr>
<tr>
<td>15</td>
<td>39.01</td>
</tr>
<tr>
<td>30</td>
<td>56.58</td>
</tr>
<tr>
<td>45</td>
<td>65.72</td>
</tr>
<tr>
<td>60</td>
<td>78.38</td>
</tr>
</tbody>
</table>

To identify the end point of digestion and to explore whether more amount of lipid excipients can dissolve more of LCH, the study was extended up to 80 min, increasing the amount of lipid gradually from 0.5 ml to 1 ml and 1.5 ml keeping the amount of drug constant at 10 mg. In case of LC-SNEDDS, there was no noticeable increase in drug amount in the aqueous phase after 60

Figure 6.3. Comparative chart for digestion study of all formulations

To identify the end point of digestion and to explore whether more amount of lipid excipients can dissolve more of LCH, the study was extended up to 80 min, increasing the amount of lipid gradually from 0.5 ml to 1 ml and 1.5 ml keeping the amount of drug constant at 10 mg. In case of LC-SNEDDS, there was no noticeable increase in drug amount in the aqueous phase after 60
min, so we can say that within 60 min the digestion was completed (Figure 6.4). With the increase in the lipid load % drug in the aqueous phase decreases. Upon dispersion of large amount of LC-SNEDDS, more time was required to equilibrate due to large amount of Tween 80 in the formulation. Moreover the resultant solution became cloudy due to viscous gel formation over pre-concentrate. Subsequently, the decreased amount of LCH dissolved in the aqueous phase was noted. This result can be attributed to the hypothesis that at higher lipid levels, especially long chain triglycerides i.e. ricebran oil and GMO, difficulty to dispersion leads to poor digestion by lipase.

Figure 6.4. Effect of lipid load on digestion of LC-SNEDDS
In case of MC-SMEDDS, at high lipid loads the digestion of Capmul MCM leads to the production of a highly solubilizing aqueous medium that becomes supersaturated with drug (Figure 6.5). In contrast to the results of LC-SNEDDS, lower loads of medium chain lipid provided poor solubilizing conditions for drug and a significant proportion of the drug precipitated. Thus increasing the lipid load of MC-SMEDDS lead to increase in the % drug in aqueous phase, though the difference was not significant.

6.3.1. TEM analysis of in-vitro digestion samples

The mechanism of lipolysis, as indicated by schematic diagram (Figure 6.6), describes the buildup of different kinds of polar lipids on the surface of the oil droplet as a result of the hydrolysis via the interfacial action of pancreatic lipase (Fatouros, Bergenstahl and Mullertz 85-94). This lipid material (mainly monoglycerides and fatty acids) produces multilamellar liquid crystalline phases on the surface of oil droplet which are gradually “detached” from the surface and produce either multi or unilamellar vesicles and finally upon further interaction with bile salts, to mixed micelles (Rigler, Honkanen and Patton 836-57). The schematic drawing in figure 6.6 shows the transformation of the oil droplets into their intermediate lipolysis products. Initially, only oil droplets and micelles were present in the medium; while at 2 and 5 min, unilamellar and bilamellar vesicles appeared together with micelles with the progress of lipolysis.
and at the end, there are more micelles, unilamellar vesicles, and very few oil droplets present in the system. (Fatouros, Bergenstahl and Mullertz 85-94).

Figure 6.6. Schematic mechanism of lipid digestion of SNEDDS as presented by Fatouros and his co-workers (Fatouros, Bergenstahl and Mullertz 85-94)

Similar results were found for LC-SNEDDS as shown below. At 5 min the formulation is dispersed well to produce fine size nanoemulsion (Figure 6.7A). At 10 min, little larger structures were seen, which can be due to formation of micelle because during digestion, triglycerides are converted to free fatty acids, monoglycerides, and diglycerides which combine with bile salt and phospholipids to form mixed micelle, unilamellar vesicles and multilamellar vesicular structures (Figure 6.7B) (Williams et al. 2976-92; Sek, Porter and Charman 651-61; Thomas et al. 860-71). At 30 min, the intercalated structures were seen which suggest the formation of more of mixed micelles (Figure 6.7C). The formation of micelles promotes the traffic of drug molecules across the gut membrane in an effective way. Hence better systemic concentrations can be expected. Microscopic examination results were in accordance with the findings of schematic diagram as well as LCH estimation in aqueous phase which gradually showed increasing amounts of LCH as the digestion proceeded.
6.4. Conclusion

The intermediate phases produced during lipid digestion can play an important role in the drug solubilisation and trafficking in the gastrointestinal tract, influencing the overall performance of the formulation. Therefore, characterization of the microstructure of such phases is important for understanding and possibly predicting the in vivo performance of lipid-based formulations (Fatouros, Bergenstahl and Mullertz).

The amount of the drug in the aqueous phase was found to be the highest in case of LC-SNEDDS. The droplet size was smallest and hence faster lipolysis was also expected. Moreover the TEM study revealed formation of mixed micelles and hence providing evidence of better trafficking of LCH across the GI wall. Therefore we can conclude from this study that LC-SNEDDS provides better release after digestion as compared to other tested formulations. Which was also reflected through in-vitro drug release study in biorelevant media. Hence the LC-SNEDDS is expected to perform better in-vivo as compared to MC-SMEDDS and SC-SMEDDS.
6.5. References


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Sek, Leab, Christopher J. H. Porter, and William N. Charman. "Characterisation and Quantification of Medium Chain and Long Chain Triglycerides and Their in Vitro

