DISSERTATION

submitted to the

Combined Faculties of the Natural Sciences and Mathematics

of the Ruperto-Carola-University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

Presented by

Atefeh Nemati

Apotekerin

Born in Marvdasht, Iran

Oral examination:

27.11.2018

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LIPOSOMES AS A LIPID-BASED FORMULATION TO INCREASE THE BIOAVAILABILITY OF POORLY WATER_SOLUBLE DRUG FENOFIBRATE

Referees:

Prof. Dr. Gert Fricker

Prof. Dr. Ulrich Hilgenfeldt

To my beloved husband, who always picked me up on time

and my family who encouraged me to go on every adventure

Affidavit for Dissertation

I hereby declare that this thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Atefeh Nemati

Date:

27.11.2018

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ABSTRACT:

This thesis investigates the biopharmaceutical knowledge of different liposomal formulations using two different phosphatidylcholines (EPC and S 90) in the presence and absence of gelatine. The study aimed to characterize the oral lipid-based formulations in terms of physiochemical features, X-ray diffraction, dissolution, *in vitro* lipolysis and *in vivo* pharmacokinetic studies.

The prepared liposomes were used to investigate the physical features of vesicular phospholipid gels (VPGs) and the light-scattering assessed the size and polydispersity of the liposomes. Fenofibrate-loaded liposomes had remarkably high entrapment efficiency (95%–99%). The dissolution behaviour of the samples was evaluated in a paddle model and the results showed a negative correlation between the rate of liposome release and gelatine concentration. The prepared liposomes were used in a dynamic *in vitro* lipolysis model to study the digestion and solubilisation of the drug formulations in biorelevant media simulating the gastrointestinal environment. The release profile of fenofibrate for all liposomal formulations showed a general increasing trend (regardless of the type of phosphatidylcholine and amount of gelatine) towards the release of fenofibrate from the formulations in the aqueous phase. For the *in vivo* studies, EPC and S 90 liposomes were administered orally to fasted rats for pharmacokinetic characterization. The *in vivo* studies demonstrated that Soluthin S 90 formulations, regardless of the presence or absence of gelatine, showed significantly higher ($p<0.01$) pharmacokinetic parameters in terms of AUC and C_{max}. All formulations showed a similar T_{max}, and the presence or absence of gelatine had no significant effect on AUC, C_{max} or T_{max} for both EPC- and Soluthin S 90-type liposomal formulations.

In this study, the absence and presence of gelatine in the *in vitro* rat lipolysis model correlated with the results of the *in vivo* pharmacokinetic studies, and the data from the NaOH titration, corresponding with the digestion and FA release, virtually correlated with the *in vivo* plasma concentration. However, in term of the drug solubilisation profile, no significant correlation was observed between the *in vitro* and *in vivo* studies, particularly in the case of the *in vitro* lipolysis model.

In conclusion, the addition of gelatine did not have a significant effect on pharmacokinetic parameters. The new phosphatidylcholine mixture, Soluthin S 90, strongly improved the absorption and bioavailability of fenofibrate-loaded liposomes. Further studies should be performed to investigate the effect of fenofibrate-loaded liposomes prepared by Soluthin S 90 on the intestinal permeability of the API and solubilisation capacity of the formulations in an *in vitro* lipolysis model combined with an absorption step. It is also recommended to combine the formulation with different surfactants to choose the optimal formulation. In addition, it is necessary to examine the bioavailability of different poorly water-soluble drugs (PWSDs) formulated in Soluthin S 90 liposomes to elucidate the pharmacokinetic effect of such a formulation.

ZUSAMMENFASSUNG

Diese Arbeit untersucht das biopharmazeutische Verhalten verschiedener liposomaler Formulierungen, die mit zwei verschiedenen Phosphatidylcholinen (EPC und S 90) in Gegenwart und Abwesenheit von Gelatine erhalten wurden. Ziel der Studie war es, die oralen Formulierungen auf Lipidbasis in Bezug auf physiochemische Eigenschaften, Röntgenbeugung, Auflösung, *in vitro* Lipolyse und *in vivo* Pharmakokinetik zu charakterisieren.

Die hergestellten Liposomen wurden verwendet, um die physikalischen Eigenschaften von vesikulären Phospholipidgelen (VPGs) zu untersuchen, wobei die Größe und Polydispersität der Liposomen mittels Lichtstreuung beurteilt wurden. Fenofibrat-beladene Liposomen hatten eine bemerkenswert hohe Einschlusswirksamkeit (95% -99%). Das Auflösungsverhalten der Proben wurde in einem Paddelmodell ausgewertet und die Ergebnisse zeigten eine negative Korrelation zwischen der Rate der Liposomfreisetzung und der Gelatinekonzentration. Die hergestellten Liposomen wurden in einem dynamischen *in vitro*-Lipolyse-Modell verwendet, um die Verdauung und Solubilisierung der Arzneimittelformulierungen in biorelevanten Medien zu untersuchen, die die gastrointestinale Umgebung simulieren. Das Freisetzungsprofil von Fenofibrat für alle liposomalen Formulierungen zeigte einen allgemeinen zunehmenden Trend (unabhängig von der Art des Phosphatidylcholins und der Gelatinemenge) gegenüber der Freisetzung von Fenofibrat aus den Formulierungen in der wässrigen Phase. Für die *in vivo*-Studien wurden EPC- und S 90 - Liposomen oral an nüchterne Ratten zur pharmakokinetischen Charakterisierung verabreicht. Die in vivo-Studien zeigten, dass Soluthin S 90 - Formulierungen unabhängig von der Anwesenheit oder Abwesenheit von Gelatine signifikant höhere ($p < 0,01$) pharmakokinetische Parameter in Bezug auf AUC und C_{max} zeigten. Alle Formulierungen zeigten eine ähnliche T_{max}, und die Anwesenheit oder Abwesenheit von Gelatine hatte keine signifikante Wirkung auf AUC, C_{max} oder T_{max} für sowohl EPC- als auch Soluthin S 90-liposomale Formulierungen.

In dieser Studie korrelierte das Fehlen und Vorhandensein von Gelatine im *in vitro*-Lipolyse-Modell mit den Ergebnissen der pharmakokinetischen in-vivo-Studien, und die Daten der NaOH-Titration, die der Verdauung und der FA-Freisetzung entsprachen, korrelierten praktisch mit denen *in vivo* Plasmakonzentration. Im Hinblick auf das Arzneimittelsolubilisierungsprofil wurde jedoch keine signifikante Korrelation zwischen den i*n vitro*- und *in vivo* Studien beobachtet, insbesondere im Falle des *in vitro* Lipolyse-Modells.

Zusammenfassend hat die Zugabe von Gelatine keinen signifikanten Effekt auf die pharmakokinetischen Parameter. Das Phosphatidylcholin-Gemisch, Soluthin S 90, verbesserte die Absorption und Bioverfügbarkeit von Fenofibrat-beladenen Liposomen stark. Weitere Studien sollten durchgeführt werden, um die Wirkung von Fenofibrat-beladenen Liposomen, hergestellt durch Soluthin S 90, auf die intestinale Permeabilität der API und die Solubilisierungskapazität der Formulierungen in einem *in vitro*-Lipolyse-Modell, kombiniert mit einem Absorptionsschritt, zu

untersuchen. Es wird auch empfohlen, die Formulierung mit verschiedenen Tensiden zu kombinieren, um die optimale Formulierung zu wählen. Darüber hinaus ist es notwendig, die Bioverfügbarkeit verschiedenen schlecht wasserlöslichen Arzneimitteln (PWSDs) zu untersuchen, die in in Soluthin S 90-Liposomen formulierter, um die Pharmakokinetik Effekt einer solchen Formulierung aufzuklären.

1. INTRODUCTION

1.1 Oral Drug Delivery

There are different routes for drug administration, for instance parenteral, nasal, inhalation and transdermal, but by far the most convenient route is the oral administration for systemic purposes. The very early question being asked in pharmaceutical companies after the discovery of an Active Pharmaceutical Ingredient (API) is whether or not it could be orally applied. Oral drug delivery (ODD), one of the oldest drug deliveries is applied for a large number of therapeutic drugs available in the market as it is low-cost, non-invasive, convenient to use, higher compliance and in terms of the dosage form is flexible, accurate and also measurable. Besides these advantages, for oral drug administrations the main issue is to overcome the harsh condition of the gastrointestinal and have a successful effect and this mainly is mainly influenced by on the nature of drug compound and the type of formulation.

About 40% of the new chemical entities (NCEs) from the industrial drug discovery processes exhibit poor water solubility and low bioavailability after oral administration (BCS II or IV drugs), which take time and cost a lot to pass the phases after drug discovery (clinical testing, development, and regulatory) (Hite, M., Turner, S., Federici 2003). Many of these new drugs fail in clinical assessments due to the problem of water solubility, even though they showed a very strong treating effect in pharmacological terms. Furthermore, many existing drugs available on the market still face the problem of poor water solubility. The solubility in water of a drug is critical as it shows the solubility in the aqueous gastrointestinal (GI) fluids. Poor water solubility Strongly decreases the absorption from GI tract resulting in a poor oral bioavailability (Rinaki et al. 2003).

More than finding an appropriate oral formulation for recently discovered APIs, most of the pharmaceutical companies have been encouraged to reformulate the drugs and improve their medical effects. The different novel technologies for oral drug delivery are challenging the market situation. The companies are paying a lot of attention to reformulations as there are many advanced technologies, which control the release rate and bioavailability (Kalepu & Nekkanti 2015). The new novel oral delivery technologies can improve the efficacy by showing a better pharmacological result with the same quantity of the drug, which decreases the cost of manufacturing as well as the

cost of therapy to the patient and, therefore, increase the patient compliance. These technologies provide benefits for both pharma companies and patients.

It is very important for pharmaceutical companies to increase the compliance of patients since many patients are not going to the treatment regime due to the difficulty of administration or the unpleasant taste of a drug. It is also very critical for companies to find a convenient administration for a drug. A study in the USA by the New England Healthcare Institute (NEHI) revealed a very noticeable result in terms of non-compliance of the patients. It has shown that the cost of non-compliance to medicines is about $290 billion just in the U.S. This hardly suggests pharma companies to increase the compliance rate in order to earn more financial benefits (Cutler & Everett 2010).

1.2 The Biopharmaceutics Classification System

The Biopharmaceutics Classification System (BCS) was first proposed by Amidon in 1995 (Amidon et al. 1995). The BCS is a complimentary classification guideline to differentiate drug compounds according to their aqueous solubility and intestinal permeability. The aim of the system is to improve, simplify, and speed up the drug development process. There are three important factors rolling in the BCS guidance; solubility, dissolution, and intestinal permeability, which define the rate and amount of drug absorption (F. and D. Administration 1997). In the process of drug development, the concept of BCS provides a basis to predict about the *in vivo* pharmacokinetics of oral drug products and possess relationship between drug release and the absorption process (Sachan et al. 2009). The BCS classification consisted of four individual classes (Figure 1):

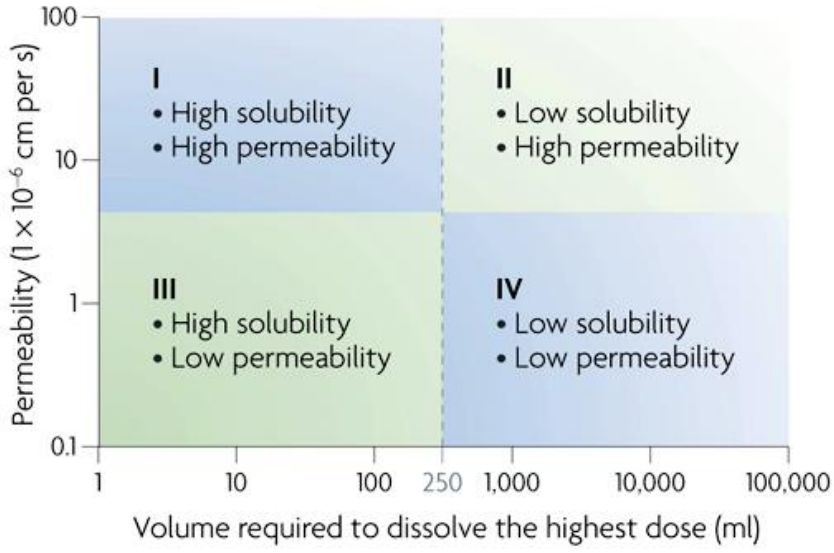


Figure 1. Biopharmaceutics Classification System classification (Rautio et al. 2008).

Class I is ideal for oral drug delivery systems with a high solubility as well as high permeability. Drugs belonging to this class dissolve easily in hydrophilic media and the absorption is expected to be high (Di & Kerns 2015). Class II contains drugs with a low solubility in water but a high intestinal permeability. In this category, the dissolution test is expected to be rate-limiting regarding absorption. Approximately 70% of new molecular entities are distributed in this class (Benet 2007). Drugs belonging to Class III possess high water solubility and a low permeability through the intestinal wall. The permeability test is commonly used to predict the bioavailability of this drug group. Class IV drugs have a low solubility as well as a low permeability, which makes the development of these compounds risky and really costly (Kerns & Di 2008).

Fenofibrate (FF), a highly lipophilic drug, is a BCS class-II drug and used widely as a hypolipidemic agent to lower the risk caused by atherosclerosis. Fenofibrate as a poorly water-soluble drug (PWSD) is mainly used to reduce cholesterol levels and like other fibrates, it reduces both low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels, as well as increasing high-density lipoprotein (HDL) levels while reducing triglyceride (TG) levels. Due to the low water solubility of fenofibrate, dissolution is slow and, therefore, the oral bioavailability is limited (Zuo et al. 2013). Currently, different oral drug delivery systems have been developed to increase bioavailability of FF, for instance, solid dispersion (Kawakami et al. 2013), a self-microemulsifying drug delivery system (Patel & Vavia 2007), lipid-based formulations (Tian et al.

2013), nanocrystals (Zuo et al. 2013), mesoporous carbon (Niu et al. 2013) and liposomes containing bile salts (Chen et al. 2009). The micronized fenofibrate formulation (Lipanthyl® capsules) available in the market possesses a significant improvement in dissolution behavior followed by increasing oral bioavailability.

1.3 Liposome formulation

After an API molecule is identified, it must be delivered to the target area of the body to have a pharmacological effect. Many new drug candidates possess poor water solubility and, therefore, low bioavailability after oral administration. In addition, non-specific distribution of drug molecules to another site of the body results in undesirable side effects, and should be avoided as much as possible. These are challenging issues for formulation scientists to overcome. To achieve an ideal effect for an API, one of the common choices is nano-technological drug delivery system. There are a wide variety of drug carrier forms branching into nano-technological drug delivery systems, including liposomes, nanoparticles, polymeric micelles, and dendrimers. Liposome formulation is a valuable tool which connects drug discovery and (pre)clinical application (Banerjee 2001) and this research is focused on liposome as a drug delivery system.

The word liposome comes from two Greek words: “Lipos” means fat and “Soma” means body. Liposome was first described in the mid-60s as a model of cellular membranes by a British hematologist named Alec Bangham at the Babraham Institute in Cambridge. Bangham and his colleague were testing the institute's new electron microscope when they made a noted observation about phospholipids forming closed multilamellar vesicle in an aqueous solution (Bangham et al. 1965). Since this discovery, the field of liposome research has progressed enormously, and the applications area expanded from drug and gene delivery to cosmetics, diagnostics or food and chemical industry (Vishvakrama & Sharma 2014).

A liposome is defined as a tiny phospholipid nanovesicles consisting of one or more concentric membranous lipid bilayers with central isolated aqueous spaces. Membranes are normally made of phospholipids with a hydrophilic head group and a lipophilic tail group consisted of long hydrocarbon chains. In an aqueous environment, the hydrophilic head has affinity towards water while, the hydrophobic tail is derived back by water to form a closed structure. Liposomes are the

drug carrier and are able to be loaded with a different variety of molecules, for instance, small drug molecules, proteins, nucleotides, and even plasmids. Nowadays, liposomal formulations of various therapeutic drugs are available in the market (Dua et al. 2012).

The liposome can be formulated and processed to differ in size, composition, charge, and lamellarity. The type of bilayer components determines the "fluidity" and the charge of the lipid bilayer. For example, unsaturated phosphatidylcholine species extracted from natural sources (such as egg phosphatidylcholine) help bilayers to be more permeable and less stable, although the saturated phospholipids with long acyl chains (such as dipalmitoylphosphatidylcholine) form a rigid, impermeable bilayer structure (Akbarzadeh et al. 2013). Phospholipids spontaneously create closed structure when they are exposed to aqueous solutions. More importantly, the unique ability of liposomal systems to entrap both hydrophilic and lipophilic compounds enable a diverse range of drugs to be encapsulated by these vesicles. Hydrophilic molecules are encapsulated in the interior aqueous compartments; whereas lipophilic drugs can be accommodated in the lipid phase (Laouini et al. 2012). The surface of liposomes can be modified by the incorporation and covalent linkage of glycoproteins or synthetic polymers (Banerjee 2001). Figure 2 schematically illustrate the structure of liposome and lipophilic and hydrophilic drug entrapment models. Liposomes are used as drug delivery systems due to their excellent characteristics such as flexibility, biocompatibility, biodegradability, increasing therapeutic index of drug and efficacy, increasing the stability of entrapped drug from the hospital environment, reducing side effects, providing sustained release and acting as a reservoir of drug (Kavrakovski et al. 2013).

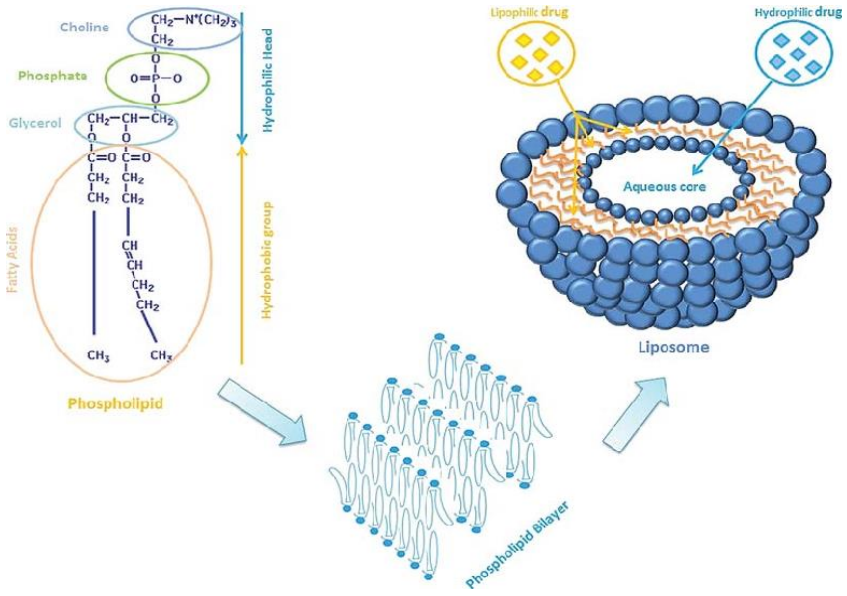


Figure 2. Liposome structure and lipophilic and hydrophilic entrapment positions (Laouini et al. 2012).

1.4 Light Scattering

The size of liposomes is normally determined by two methods; the electron microscopic examination, and dynamic light scattering (DLS) which sometimes referred as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS). Although electron microscopy is one of the most precise determination methods, it is very time-consuming and requires expensive equipment that may not be immediately in hand. In contrast, DLS is simple, automatized, and rapid and easy to perform. Light scattering is a consequence of random changes of the light after scattering from a solution or suspension. DLS is a non-invasive technique, which is most commonly used to measure the size of particles and molecules in suspension (Brittain 2003). DLS measures the 'Brownian motion' of particles, which is a random movement of microscopic particles suspended in the liquid due to collisions moving with molecules of the surrounding medium. It also records and analyses the intensity of absolute light scattering, which is represented as count rate in the unit of kilo counts per second (kcps).

The light scattering properties of particles depend on the particle size and number. The correlation between scattering intensity and particle number is linear in diluted samples, which

means each particle scatters light independently without being influenced by proximate particles (Hulst & van de Hulst 1957; Elsayed & Cevc 2011).

Shining a monochromatic light beam onto a suspension with random thermal moving molecules causes a Doppler Shift, which changes the wavelength of the light source. At that point, it is possible to calculate the size distribution and give a description of the particles motion (Brownian motion) in the suspension. The particle size is described as hydrodynamic radius and is modeled by the Stokes-Einstein relationship (Torchilin & Weissig 2003). The hydrodynamic radius is known as the diameter of a particle which has the same translational diffusion coefficient (symbol D). The Stokes-Einstein equation is described below:

$$D_h = \frac{k_B \mathrm{T}}{3\pi\eta D_t} \qquad Equation\ 1$$

Where:

D_h = hydrodynamic dimeter (the particle size)

D_t = the translational diffusion coefficient ($m^2 \cdot s^{-1}$)

k_B = Boltzmann's constant

T = thermodynamic temperature (K)

η = viscosity of suspending liquid ($N \cdot s \cdot m^{-2}$)

The Stokes-Einstein equation connects diffusion coefficient measured by dynamic light scattering to the particle size. Although the calculations are completely handled by instrument software, it is noticeable that the equation reminds a few important points as follows. It is necessary to keep a constant temperature during the measurement, as the viscosity is strongly temperature-dependent. The translational diffusion coefficient is reliant on the type of ions in medium, the size of particle core on the surface and also the concentration of the testing suspension (Meyers n.d.; Clark et al. 1970). The PCS instrument normally consists of six main components (Figure 3). Laser (1) provides the light source for illuminating the dynamic sample particles inside a cell (2). The

scattered light is measured by the detector (3), which is positioned at 173° or 90° (according to a different kind of instrument). The intensity of scattered light must be within a certain range; otherwise, the detector is not able to measure it correctly. The intensity of the laser source and the intensity of scattering are regulated by an attenuator (4). The scattering intensity signal is then passed to a correlator(5), which compares the scattering intensity at successive time intervals to derive it the rate at which intensity is varying. Data from correlator are sent to the computer (6). Special software analyses these data and calculates the size of particles in the sample (Instruments 2009). Finally, it should be noted that the particle size determined by dynamic light scattering is the hydrodynamic size that means, the determined particle size is the size of a sphere that diffuses the way as your particle. In this study, Zetasizer Nano ZS was used.

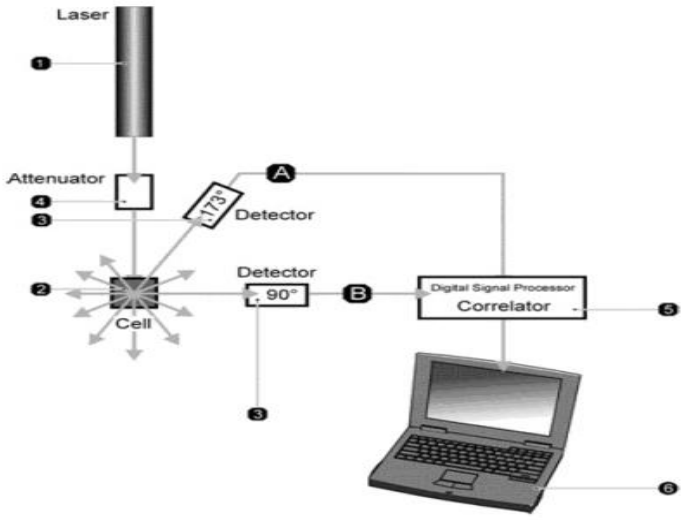


Figure 3. The photon correlation spectroscopy instrument and its six main components (Instruments 2012).

1.5 Dissolution

The official pharmacopoeial dissolution methods are the simplest methods, which have been used to estimate the oral API behavior, the rate of absorption and the bioavailability. By far, the dissolution tests are used to evaluate the release of desire drug from both solid and semisolid dosage forms for quality control (QC) and product development purposes. In case of QC, dissolution methods are used to control deviation of manufacturing, control the consistency of

products from batch to batch and ensure continuing performance and product quality. With respect to research and development purposes, the tests are widely used to guide the new DDS development by predicting the behavior and release of drug in gastrointestinal conditions (Services & Administration 1997).

In the dissolution process the drug molecules are transferred from a solid phase into an aqueous solution phase. In case of oral dosage forms the *in vitro* dissolution studies as the quality control testing are particularly important as they help the drug development scientists to estimate the function and effectiveness of the drug products after administrating them in patients (Sinko & AN 2005). Furthermore, *in vitro* dissolution testing could equivalence the *in vivo* animal studies. The Food and Drug Administration (FDA) describes the testing condition for BCS I drugs which *in vitro* dissolution testing can completely simulates the *in vivo* studies (Guidance 1997).

1.5.1 *In vitro* dissolution testing

Various dissolution apparatus has been described across several Pharmacopoeias including United States Pharmacopeia (USP) and European Pharmacopeia (Ph.Eur.) for dissolution testing of drug substances and pharmaceutical dosage forms. To predict *in vivo* drug release, *in vitro* dissolution tests should reassemble the *in vivo* conditions that the DDS will encounter while passing through the human GI tract. Figure 4 shows the four most popular dissolution apparatus for oral dosage forms; the basket (apparatus I), the paddle (apparatus II), the reciprocating cylinder (apparatus III) and the flow-through cell (apparatus IV).

The basket (USP I) and paddle (USP II) methods are by far used widely and are the chosen method for testing immediate release of oral solid dosage forms, as they are simple, easy to handle, well standardized and used world widely. In terms of flexibility, these methods can be used for evaluating the dissolution profile of a large variety of drugs and drug products, therefore, they are generally recommended by USP. The apparatus model consists of a rotating basket or paddle in a large volume vessel filled with 500 − 1000 mL of dissolution medium, but the typical volume is 900 mL (The United States Pharmacopeia and National Formulary 2014a; The European Directorate for the Quality of Medicines & Healthcare 2014). The dissolution media and the volumes are chosen to ensure sink conditions, enabling the release of entire dosage from

the formulation. Usually, samples are withdrawn and the undissolved materials are filtered off to spectroscopically quantify the dissolved fraction of the dose.

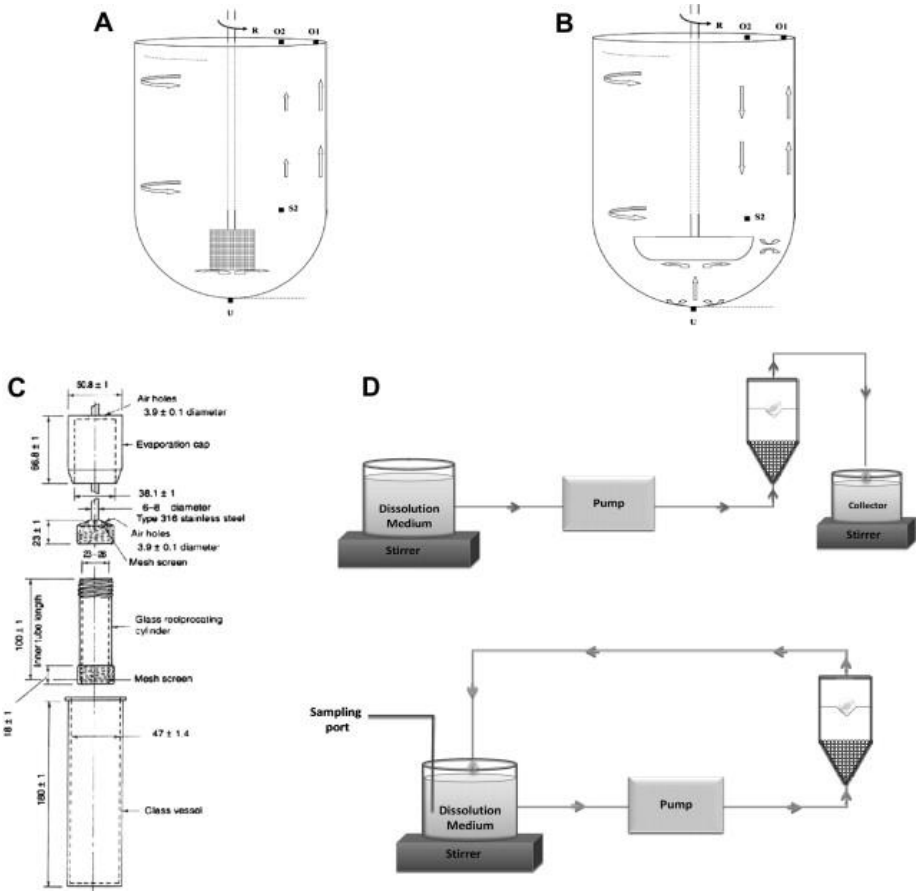


Figure 4. The four popular apparatus models; (A) the basket (apparatus I), (B) the paddle (apparatus II),(C) the reciprocating cylinder (apparatus III), and (D) the flow-through cell (apparatus IV) (Kostewicz et al. 2014).

The principle of the reciprocating cylinder (USP III) is similar to disintegration tester, with the dosage form retained in a cylinder. The cylinder can be moved between a series consist of different media, this could be an advantage over the apparatus I and II by offering the possibility of achieving a dissolution profile which covers the entire GI transit.

The flow-through cell (USP IV) evaluates formulation effects such as particle size and choice of excipients. The model is suitable to resemble both gastric and the intestinal fluids as they are easy

to vary the dissolution media compositions and the volume (small volume for small intestinal and larger volumes for sink conditions). The reciprocating cylinder (USP III) and the flow-through cell (USP IV) are alternative models. They can be operated as open or closed systems and are mostly used as modified-release systems (Sinko & AN 2005). Figure 4 summarizes the operation steps of the compendial/conventional dissolutions methods. During the past decades, dissolution systems increasingly used biorelevant media made of aqueous salt solutions or buffers with or without the addition of surfactants, more importantly containing bile salts and phospholipids. Fasted State-Simulated Intestinal Fluid (FaSSIF) was the first biorelevant media suggested by Dressman and co-workers in 1998 (Galia et al. 1998). Since then, a lot of studies have been conducted to characterize the GI luminal fluid, with the consequence of developing various media simulating luminal conditions in different parts of GI tract. Nowadays, biorelevant dissolution media like FaSSIF or fasted state-simulated gastric fluid (FaSSGF) are frequently used by research groups worldwide as well as development groups in pharmaceutical industries (Kleberg et al. 2010).

1.5.2 Theories of dissolution

The dissolution procedure contains two individual steps; interfacial and transport. The primary step is the interfacial reaction, which helps the drug molecule to release from the solid surface in to solid-liquid interface. In the second step the molecules are transport from the solid-liquid phase into the available solution (Lee et al. 2008).

The diffusion layer model is based on the Fick´s first law and states that the dissolution process is mostly controlled by diffusion of free molecules from the diffusion layer (liquid film layer surrounding the particle) into solution. The dissolution model was first developed by Noyes and Whitney and was finally modified by Nernst and Brunner (Noyes & Whitney 1897; Nernst 1904). This model is controlled by the affinity of the drug molecules towards the solvent and described below.

$$\frac{dM}{dt} = \frac{DA}{h}(Cs - Ct) \qquad \textbf{Equation 1}$$

Where M is the mass of solute dissolved in time t, dM/dt is the dissolution rate, A is the

effective surface area of the drug particles exposed to the solution, *D* is the diffusion coefficient of the drug, *h* is the thickness of the diffusion layer around each drug particle, *Cs* is the saturation solubility of the drug in the dissolution medium and *Ct* is the concentration of the drug at a time t in the bulk solution. According to this model, the effective surface area (A) and the boundary layer thickness (h) is held constant during dissolution. Moreover, the mentioned factors, the physicochemical properties of the drugs are also affected by the conditions in the gastrointestinal tract, for instance, phospholipids and bile salt concentrations bile, volume and hydrodynamics of the contents in the gastrointestinal tract (Dressman et al. 1998). Thus, for having a better prediction of the *in vivo* conditions, it is substantial to consider these mentioned parameters accordingly.

1.6 Lipid digestion, drug solubilisation, and absorption

Digestion and absorption of dietary lipids, as well as lipid-based formulations, occur throughout the gastrointestinal tract (GIT). The BCS class II drugs are poor oral absorption compounds and generally have a slow drug dissolution within the GIT (MacGregor et al. 1997; Dressman & Reppas 2000). It has been proven that the absorption of these groups of molecules could be improved after formulating in a lipid-based construction (MacGregor et al. 1997; Birru et al. 2017). Following ingestion, Lipid digestion starts from the activity of lingual lipase, a lipolytic enzyme secreted from the lingual serous glands on the surface of the tongue (Cuiné et al. 2007; Tran, Siqueira, Amenitsch, Müllertz, et al. 2017). In the stomach, the gastric lipase as well as mechanical mixing transfer the dispersion of the lipids into an emulsion (Porter et al. 2007) and afterwards, the emulsified lipids in the stomach enter the small intestinal. The gastric emptying time could be different according to the size of the droplets, where the long chain fatty acids (FAs) strongly delay this timing (Armand et al. 1996).

The gastric lipases, subclasses of esterase, are responsible for hydrolysis of lipids by breaking the ester bond between the glycerol backbone and FAs (Cuine et al. 2008), and the chief cells in the fundic mucosa of the stomach are responsible for producing these type of enzymes (Porter et al. 2007; Moreau et al. 1988). Human gastric lipase is generally active in a pH range of 2-7 and the optimal pH range between 4.5 and 5.5 (Larsen et al. 2011), whereas the pH-dependent pancreatic lipase shows the optimal activity between pH 6.5 and 8.0 (Armand et al. 1992). In humans, the lipid digestion in the stomach is responsible for about 10-30% of the hydrolysis of dietary fat while the

majority (more than 98%) of the hydrolysis of lipids occurs in the intestine with the help of pancreatic lipase (Cuiné et al. 2007).

After entering the small intestine, pancreatic lipase helps the dispersion to get hydrolysed and absorbed (Embleton & Pouton 1997). The enterocytes in the intestine are the main site of GIT for absorption and consist of a glycerol backbone and three FAs chains linked by ester bonds. Pancreatic triacylglyceride lipase hydrolyses triacylglycerides at 1 and 3 positions and produces 2-monoglyceride and two FAs from a triacylglyceride molecule it hydrolyses (Figure 5).

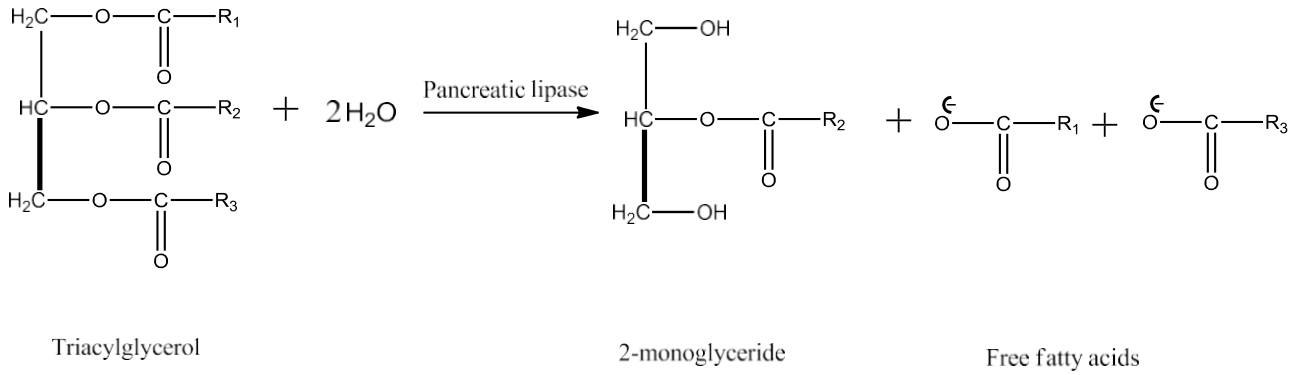


Figure 5. Hydrolysis of triacylglycerols by pancreatic lipase (Vijayalakshmi et al. 2008).

The natural existence of lipids in the intestine as well as the digested lipids stimulates secretion of bile from the gall bladder. The bile mixture consisted of bile salts, phospholipids, and cholesterol (Carey & Small 1978). The bile salt with a polar and nonpolar face has surfactant properties and emulsifies the existing lipids into a micelle form and resulted in solubilizing the lipophilic drug in the aqueous phase of intestinal fluid. The bile mixture together with the products of lipid digestion form colloidal structures. These colloidal structures facilitate the drug solubilisation by removing adsorbed water-soluble proteins including lipase from the surface and help to remove the restraining force for further digestion. The colloidal structures also facilitate the absorption by acting as a transportation vehicle in the lumen of intestine (Holm et al. 2013; Tran, Siqueira, Amenitsch, Rades, et al. 2017; Hernell et al. 1990). Figure 6 shows an overview of the lipid digestion process and the fate of the lipid-based drugs.

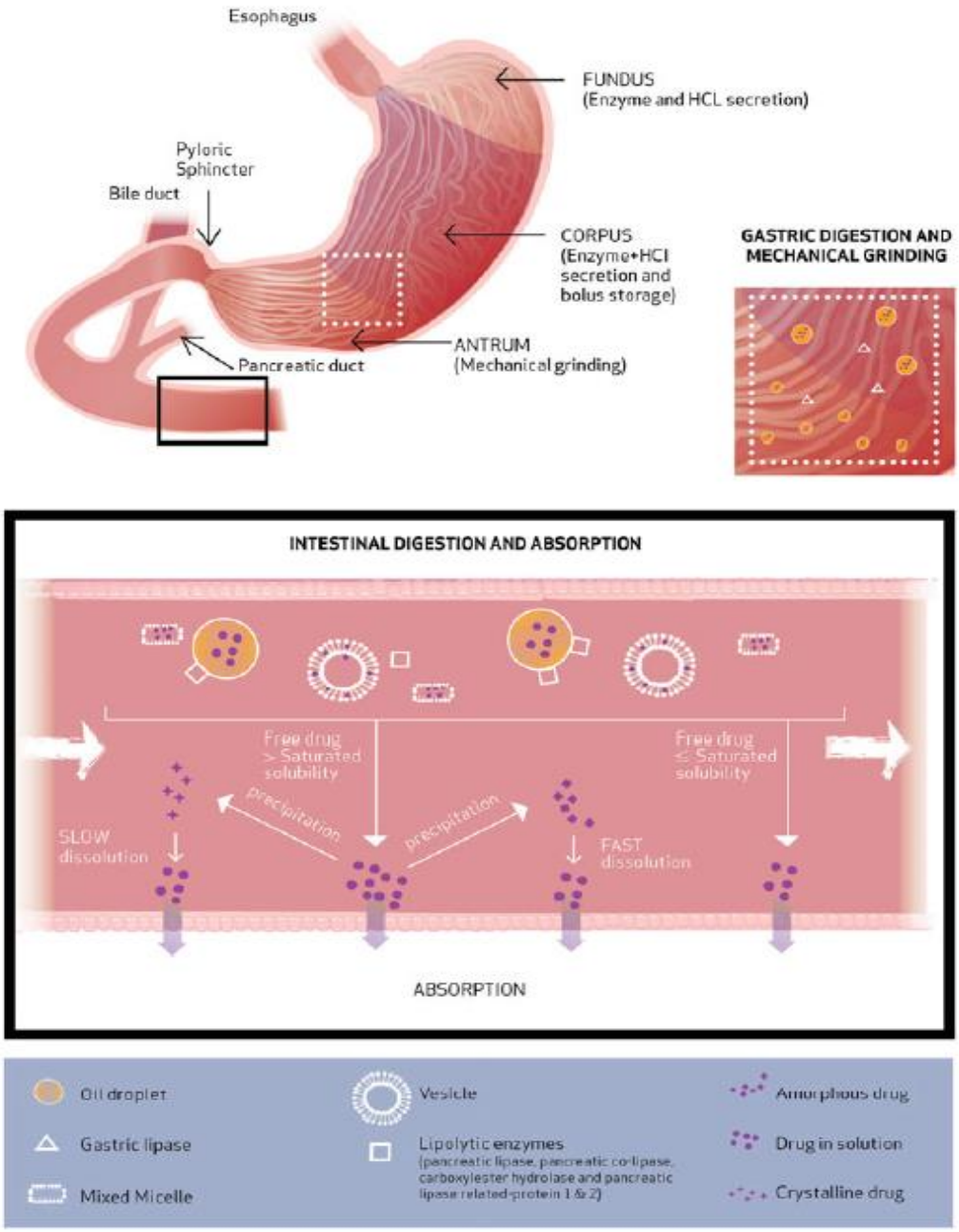


Figure 6. lipid digestion and drug solubilisation of a lipid-based formulation after oral administration (Müllertz et al. 2016).

It has proven that fatty acids with more 12 carbon atoms are absorbed into the lymphatic circulation while fatty acids with chain length lower than 12 are primarily absorbed into the portal blood (Porter et al. 2007; Kiyasu et al. 1952). Molecules with log P higher than 5 and a lipid

solubility of a minimum of 50 mg/mL are expected to transport to lymphatic system (Porter & Charman 1997). The GI tract is very rich in terms of blood and lymphatic vessels, which makes the absorption of lipid digestion products and drug possible. However, the preferred absorption is in the blood as blood flow is significantly higher (500 times) than lymph flow (Reymond & Sucker 1988).

1.7 Physiology of gastrointestinal tract of humans and rats

Although the drug products produced for human-being purposes should be ideally tested in humans, which is quite impossible for the early screening stages of drug discovery, such as toxicity studies and drug efficacy. The rats are widely used in early drug development as they are low cost, small, easy to handle, reproducible, and most importantly have a very similar metabolism to humans (Do et al. 2011). Apart from similarities, there are important physiological differences between rats and the human body, which should be noted during developing *in vitro* lipolysis models (DeSesso & Jacobson 2001). The pH in the stomach of rats is within range of 3.5 to 4 while in humans it is from 1.5 to 3 (Kalantzi et al. 2006). In rats, the source of lipolytic activity in the stomach is from the lingual lipase (Hamosh & Scow 1973) while in humans, the lipase is from the gastric mucosa (Bernbäck et al. 1989). Moreover, the lipase activity in humans is more than rats (Anby et al. 2014). The concentrations of bile salts in humans is from 2-5 mM in the fasted state (Pedersen et al. 2000; Persson et al. 2005) and from 8-15 mM in the fed state (Hernell et al. 1990; Persson et al. 2005), while Tanaka et al. (2012) describe the rats bile concentration around 50 mM in the fasted state (Tanaka et al. 2012) and some other studies showing a range between 12 and 20 mM, which are still higher than humans (Hagio et al. 2009; Vonk et al. 1978).

Figure 7 illustrates the gastrointestinal tract (GIT) of human and rats. The rats have the same overall GIT organization as humans but there are a few important differences. The length of the human intestinal tract is just 5.5 times the length of the rat intestinal tract, in spite of the much larger body size of the human (70kg) compared with the rat (0.25kg). The relative length of the small intestine differs from the human that the jejunum makes up nearly the entire small intestine. The cecum of the rat is very large and does not have a vermiform appendix (Amidon et al. 1995). Furthermore, it has been proven that the administration of high volumes of liquid in humans increases the rate of gastric emptying (Sunesen et al. 2005), while in case of rats, this effect is still unclear (Gärtner 2002; Borowitz et al. 1971).

Nevertheless, it is generally assumed that the data from rat studies can be considered for humans (Lin 1995), however, the physiological differences should be taken into account, especially in case of comparing the results from the rat's studies to *in vitro* models simulating human conditions.

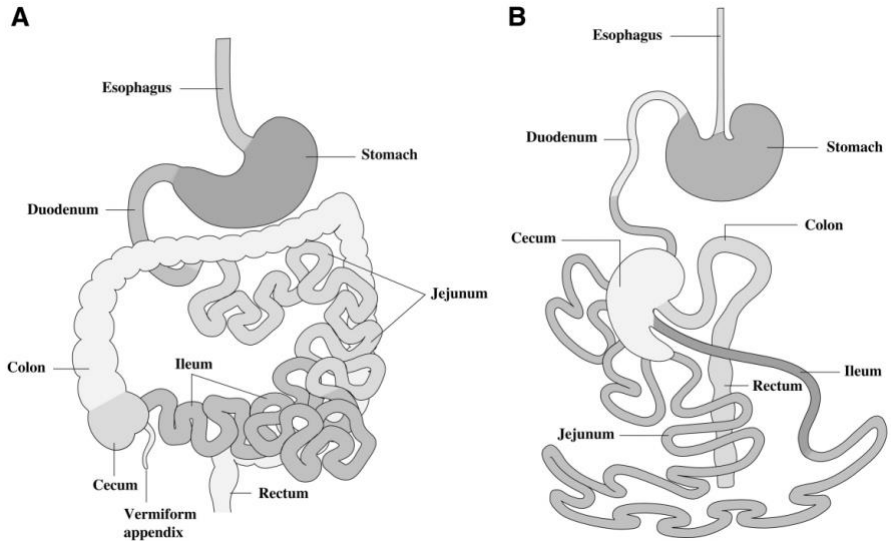


Figure 7. Overview of the Gastrointestinal tract in a Human (A) and in Rat (B) (Amidon et al. 1995).

1.8 *In vitro* lipolysis model and its parameters

The gastrointestinal tract of a human being is a complicated organ involves enzymes, bile secretion, salts, pH, temperature, and agitation and although a lot of studies were carried out to simulate the *in vivo* condition of human GIT, still there is no unique *in vitro* model to evaluate the fate of different APIs. The *in vitro* assessment of lipid-based formulations should be different from the other solid dosage forms. In this case, since the lipid-based formulations incorporate lipids, there is always the possibility of formation micelles and moreover due to hydrolysis of the ester bonds of lipids by lipase enzyme available in GIT, monoglycerides and fatty acids will be released. Thus, the available lipid in LBF will affect the physiochemical processes occurring in the gastrointestinal tract (Cuine et al. 2008; Cuiné et al. 2007).

The dynamic *in vitro* lipolysis model was first proposed by Zangenberg et al. in 2001 to provide an understanding of the rate and extent of lipolysis by simulating intestinal lipid digestion of

LbDDS (Zangenberg et al. 2001a; Zangenberg et al. 2001b). Furthermore, it gives the opportunity to determine the drug release by determining the drug content of solubilized or precipitated part during the lipid digestion (Dahan & Hoffman 2008; Vithani et al. 2017).

An *in vitro* lipolysis model simulates the *in vivo* conditions of human GIT by using biorelevant dissolution media (Dressman & Reppas 2000). This *in vitro* model helps LbDDS to assess lipid digestion, the drug release as well as *in vitro–in vivo* correlations of water-soluble drugs (Dahan & Hoffman 2008). The human *in vitro* lipolysis model simulates the human GIT conditions and this may cause some troubles to correlate the *in vivo* results from the rat's experiments. Siqueira and coworkers simulated an *in vitro* lipolysis model to predict the *in vivo* performance of the fasted state in rats (Siqueira et al. 2017). The lipid digestion was followed in this model by conditioning the rat gastric and intestinal tract. In this particular model, the gastric compartment is connected to intestinal part by a peristaltic pump. Moreover, according to the physiology of rats, some adjustments occurred in case of concentrations of bile salt and phospholipid, enzyme activity and importantly pH (Christensen et al. 2004). The main objective of an *in vitro* lipolysis model is to minimize the animal experiments due to ethical purposes. This will be possible by offering an inexpensive and fast way to predict the performance of a drug load lipid formulation in animals.

The *in vitro* lipolysis model based on rat physiology consisted of the gastric compartment with pH 4 and intestinal part. The pH value is compromised between the pKa of the free fatty acids, pH range in the stomach and small intestinal especially the pH of duodenum (5 – 5.5) the main site of bile and pancreas enzyme secretion (Rune & Viskum 1969) , and the optimum pH for the pancreatin activity (Müllertz et al. 2010; Williams et al. 2012). *In vitro* lipolysis models is a pH-controlled model which uses a biorelevant medium containing lipase, bile salts and phospholipids are used to simulate the lipolysis conditions in the stomach and small intestine (Crum et al. 2017; Kuentz 2012). Each compartment (gastric and intestinal) consisted of two titration devices (Ca^{2+} and NaOH), a thermostated double-wall reaction vessel, either magnetic or paddle stirrer to produce agitation, a pH electrode and a computer with software for evaluating titration and pH. A peristaltic pump transfers the gastric content into the intestinal compartment. Figure 8 schematically illustrates the compartments of the dynamic *in vitro* lipolysis. The rate of lipid digestion was controlled by addition of calcium ion into the lipolysis system. Titration of calcium could be either a fix addition at the beginning of the experiment or in a continuous manner during the experiment (Siqueira et al. 2017).

As the lipolysis process goes on, the concentration of free fatty acids increase and this could affect the rate of lipolysis by inhibiting the pancreatic lipase due to the by binding to free lipids interface (Alvarez & Stella 1989; Borel et al. 1994). Nevertheless, at the opposite point bile salts and especially calcium form insoluble calcium fatty acids soaps involving one divalent calcium ion (Ca^{+2}) and two FAs which facilitates the fatty acids elimination from the triglycerides by removing the oil/water interface (Larsen et al. 2011). Therefore, calcium is referred to be a salient factor to control the speed of lipolysis process (Armand et al. 1992).

The lipid digestion process initiates by pancreatic lipase which porcine pancreatin is the most common enzyme source used as a replacement for human pancreatic lipase (Zangenberg et al. 2001a). Pure bile salts or a mixture of crude porcine or bovine bile extract and phosphatidylcholine are used to represent and mimic the composition of human bile salts and phospholipids (Kuentz 2012; Griffin et al. 2014). It should be mentioned that the condition of the GIT (fed or fasted state) and the type and amount of the dietary affects the concentration of bile salts and phospholipids (Kalantzi et al. 2006; Jantratid et al. 2008). *In vivo* studies reported a wide range of ratios between the phospholipids and bile salts from 1 to 38.9, but the commonly employed ratio is ranged from 2 to 6. The most used ratio in *in vitro* lipolysis models is 1:4, which is also used in this study (Kleberg et al. 2010).

The release of FAs during the lipolysis causes a noticeable decrease in pH which is effectively determined by a pH electrode embedded in the system (Kuentz 2012). To maintain the pH constant from the beginning of lipolysis, the *in vitro* model automatically titrate the free FA available in the experiment environment by addition of sodium hydroxide (NaOH) which provides an indirect indication of the kinetics of lipid digestion (Williams et al. 2012; MacGregor et al. 1997).

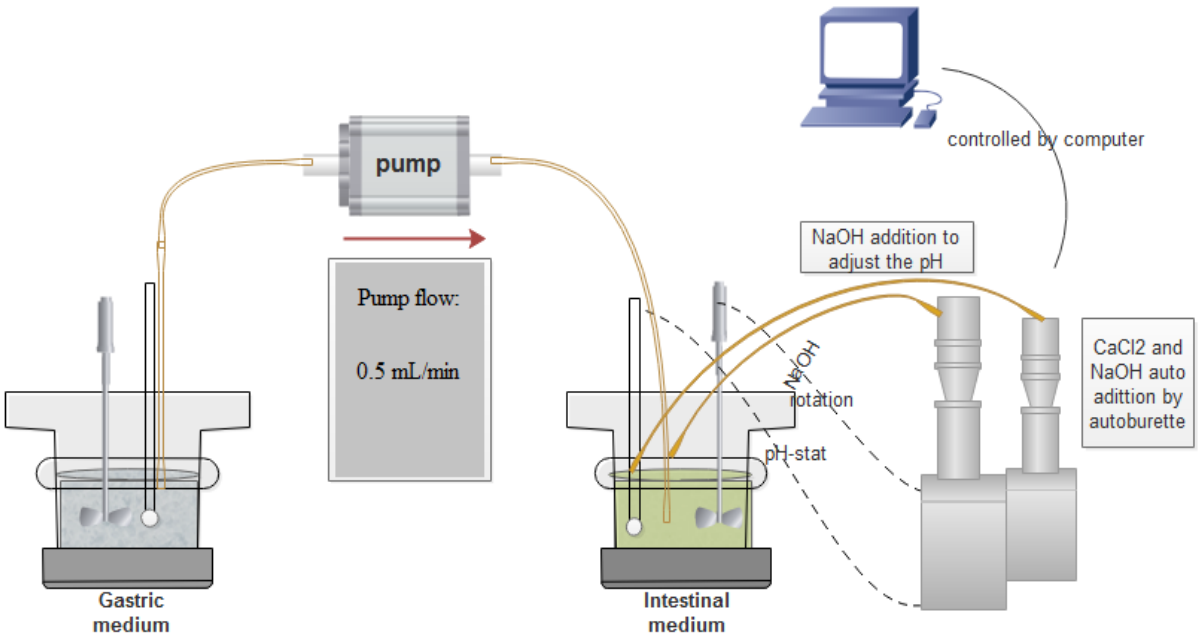


Figure 8. Schematic illustration of i*n vitro* lipolysis model and its different components containing gastric and intestinal steps and a peristaltic pump which transfers the dispersion of samples and gastric medium to the intestinal compartment.

1.9 The relation between *in vivo* and *in vitro* data

During early drug development, it is very vital to find out the right concentration of a drug to have an effective function at the site of action (Do et al. 2011; Klein 2010). This needs knowledge of dissolution and absorption occurring in GIT. The food and drug administration (FDA) describes a predictive mathematical model which provides information about the relationship between *in vitro* and *in vivo* response. This relationship is called *In vitro-in vivo* correlation (IVIVC) and help scientists to predict dissolution and absorption accurately (U. S. F. and D. Administration 1997). Therefore, the numbers of *in vivo* studies (in a human or animal body) reduce and resulted shortening the drug development time (Emami 2006). It is very often to build the animal studies according to the outcome of *in vitro* tests (Do et al. 2011). In case of BCS class II drugs, due to low water solubility dissolution is a time-limiting step for absorption and consequently, IVIVC is a useful support (Dressman & Reppas 2000).

There are some *in vitro* digestion models available to facilitate drug development by providing a platform to predict the *in vivo* behavior (Reymond & Sucker 1988; Dahan & Hoffman 2006). *In vitro* studies are usually carried out to characterize the GIT parameters affecting the drug absorption. Although some *in vitro* models showed good correlations for *in vivo* and *in vitro* results, still there is no specific and unique model to cover IVIVC of a broad range of lipid-based formulations(Elgart et al. 2013; Kaukonen et al. 2004). This is because of the dynamic and complicated condition of human GIT, which reacts differently facing various *in vivo* conditions. Moreover, in many previous studies, the *in vivo* results of rat studies are compared to the *in vitro* models simulating the human bodies (Thomas et al. 2014; Tran, Siqueira, Amenitsch, Müllertz, et al. 2017; Larsen et al. 2011). Due to the physiological difference between the GIT of the rats and human, it is important to improve the IVIVC by building an *in vitro* lipolysis model which simulates the rat GIT. These physiological differences applied to the enzyme activity, the phospholipids and bile salts concentrations and the volume of the relevant media (Di Maio & Carrier 2011).

FDA guidance for industry classifies IVIVC into 3 individual levels based on the ability of the correlation to reveal the plasma drug concentration-time profile; 1) Level A correlation; the highest level of correlation shows a linear relationship between *in vitro* dissolution and the *in vivo* pharmacokinetics. This correlation level assessed by deconvolution procedure which compares the fractions of drug absorbed in the *in vivo* study to the fraction of drug dissolved in the *in vitro* model. Deconvolution could act as a guideline to assess the *in vivo* release or absorption (Emami 2006). 2) Level B correlation does not uniquely replicate the exact *in vivo* plasma level curve, since different *in vivo* curves could produce same mean residence time values. This means that the average *in vitro* dissolution time is compared either to the mean residence time or to the mean *in vivo* dissolution time. 3) Level C correlation shows a single point relationship between dissolution factors (e.g., C_{max}, T_{max}) and does not give a complete overview of plasma concentration-time curve which is a critical factor for prediction of drug performance (U. S. F. and D. Administration 1997).

However, there are some examples that a unique IVIVC level was not capable to cover the IVIVC results (Dahan & Hoffman 2008). In these cases, the best way to show the correlation and similarity of trends could be by illustrating *in vitro* dissolution curves and *in vitro* dissolution profiles.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Model compounds

This study uses fenofibrate (FF) as a well-known, poorly water-soluble model compound in BCS Class II (Granero et al. 2005). Compounds within a BCS class behave in a similar manner in terms of solubility. Hence, fenofibrate can be considered as a representative of BCS Class II drugs, which have very low aqueous solubility of less than 0.3 mg/L. Furthermore, fenofibrate has a log P value of 5.24, which also indicates that it is a lipophilic drug (Hu et al. 2011). Fenofibrate is a prodrug, as it is a synthetic phenoxy-isobutyric acid derivative which is hydrolysed *in vivo* to yield fenofibric acid (Figure 9). This lowers plasma lipid levels by inhibiting triglyceride synthesis (Sharpe et al. 2002). Fenofibrate has a distribution volume of about 60 L and shows approximately high level of plasma protein binding (> 95%) to albumin (Miller & Spence 1998). The physicochemical properties of fenofibrate are listed in Table 1.

After oral administration, fenofibrate is rapidly converted to fenofibric acid through hydrolysis of its ester bond. Hydrolysis is catalysed by both tissue and plasma esterases and initiates with absorption. After an oral administration, only the fenofibrate's active metabolite fenofibric acid could be identified in the blood (Keating and Croom 2007; Chapman 1987; Yun et al. 2006; Buch 2010). The chemical structures of fenofibrate and its active metabolite fenofibric acid (FFA) are given in Figure 9.

Fibric acid derivatives (fibrates) are used for different metabolic disorders and mostly as hypolipidemic agents and they have significant treatment effects against the hypercholesterolaemia, mixed dyslipidaemia, hypertriglyceridaemia and diabetic dyslipidaemia. Fenofibrate is the isopropyl ester prodrug of fenofibric acid (2-[4-(4-chloro-benzoyl) phenoxy]-2-methylpropanoic acid) and modifies the lipid level by activating peroxisome proliferator-activated receptor α receptor (Gavzan et al. 2018).

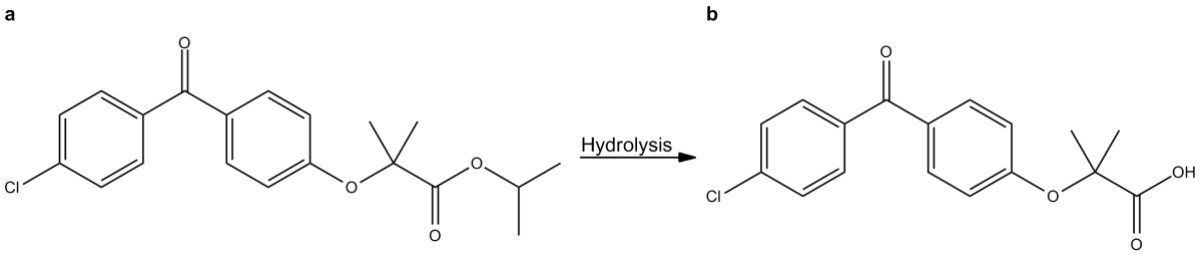


Figure 9. Molecular structures of fenofibrate (a) and its active metabolite fenofibric acid (b).

After oral delivery of fenofibrate, dissolution in the GI fluid is a critical precondition to estimate the occurrence in the GIT. As fenofibrate has low-water solubility, the dissolution step is the rate-limiting step for the further steps. Fenofibrate comparing to the other fibrate derivatives has the lowest bioavailability, therefore, one of the successfully suggested approaches to improving solubilisation in the GI is focusing on the type of the formulation which is designed to carry the desire API. It is reported from the previous studies that after administration of micronized fenofibrate to human being, due to the size reduction, high water solubility was observed (Miller & Spence 1998). Therefore, to increase the water solubility, dissolution rate and consequently bioavailability is critical to design and select an appropriate formulation approach is critical for BCS Class II drugs, and especially fenofibrate.

Table 1. Physicochemical properties of poorly water-soluble drug fenofibrate.

	Fenofibrate	**Fenofibric acid**
Molecular formula	$C_{20}H_{21}ClO_4$	$C_{17}H_{15}ClO_4$
Molecular weight [g/mol]	360.8	318.8
Solubility in water [µg/ml]	< 0.3 [a]	70 [b]
log P	5.24 [c]	3.99 [b]
pKa	-	3.1 [b]
Melting point T_m [°C]	80 – 81 [c]	179 – 183 [b]
Density [g/cm^3]	1.18 ± 0.06 [c]	-

logP: octanol-water partition coefficient.

[a] (Granero et al. 2005)

[b] (Linn et al. 2012)

[c] (Karmarkar et al. 2009) retrieved from: http://www.worldofchemicals.com/chemicals/chemical-properties/fenofibrate.html

2.1.2 Chemicals and reagents

Egg-phosphatidylcholine E80 (E-PC), Soluthin S 90 (S 90) and soybean phospholipids (Lipoid S PC), 99% was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was obtained from Sigma–Aldrich (St. Louis, MO, USA), Fenofibrate was from Smruthi Organics Ltd (Solapur, India), Dulbecco's phosphate buffered saline (PBS) was obtained from Gibco Life Technologies (Carlsbad, California, USA). Lime Bone Gelatine was from Gelita AG (Eberbach, Germany). Triton 1% (pure Triton X-100) was yield from Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium chloride, ≥ 99.5%, calcium chloride dihydrate, 99.0-102.0%, were purchased from Merck (Damstadt, Germany). Maleic acid, ≥ 99%, 4-bromobenzeneboronic acid, ≥ 95, pancreatin from porcine pancreas ≥ 3·U.S.P and bovine bile extract were purchased from (Sigma Aldrich). Tris (hydroxymethyl) aminomethane, ≥99% was purchased from Ohio (ICN Biomedicals). Ethanol (Ph. Eur. Grade) and Soybean oil (S-3547). Methanol ≥99%, acetonitrile, ≥ 99.9% were purchased from (Sigma, Aldrich). All other chemicals were obtained in the highest purity from the usual commercial sources.

2.2 Liposome Preparation

In this study, liposomes were prepared by the conventional thin film hydration method (Bangham *et al.*, 1965) to produce multilamellar vesicles (MLVs). Different type of phosphatidylcholins including egg phosphatidylcholins (EPC), soy bean phosphatidylcholins (SPC) and Soluthin S90, together with cholesterol and different concentrations of fenofibrate were separately dissolved in a mixture of chloroform and methanol in a ratio of 9 to 1 respectively to a final concentration of 100 mM. To prepare different liposomal formulations (as listed in Table 2. List of liposome compositions.), specific quantities of dissolved materials were mixed according to the desired final concentrations. The mixture was transferred into a round-bottomed flask in a 40 °C water bath and the solvent was removed using a rotatory evaporator to leave a thin, smooth lipid film around the flask. The flask was kept under vacuum conditions for 1 hour to ensure that all solvent was removed from the mixture and that a fully dried lipid mixture had formed. The lipid film was then hydrated using phosphate buffered saline (PBS) or a solution of gelatine (Gel) dissolved in PBS at different concentrations. Finally, vesicular phospholipid gels (VPGs) were formed using either dual asymmetric centrifugation or a probe sonicator, as described in sections 2.2.1 and 2.2.2.

Table 2. List of liposome compositions.

Samples	Phosphatidylcholin concentration	Cholesterol concentration	Fenofibrate concentration	Gelatine Concentration
Blank_0%Gel	75 mg	25 mg	0 mg	0 µg in 1 mL PBS
Blank_15%Gel	75 mg	25 mg	0 mg	150 µg in 1 mL PBS
Blank_20%Gel	75 mg	25 mg	0 mg	200 µg in 1 mL PBS
2.5FF_0%Gel	75 mg	22.5 mg	2.5 mg	0 µg in 1 mL PBS
2.5FF_15%Gel	75 mg	22.5 mg	2.5 mg	150 µg in 1 mL PBS
2.5FF_20%Gel	75 mg	22.5 mg	2.5 mg	200 µg in 1 mL PBS
4FF_0%Gel	75 mg	21 mg	4 mg	0 µg in 1 mL PBS
4FF_15%Gel	75 mg	21 mg	4 mg	150 µg in 1 mL PBS
4FF_20%Gel	75 mg	21 mg	4 mg	200 µg in 1 mL PBS

Table 2 provides information about the type and amount of each component of the formulations. For instance, to prepare 4FF_20% Gel formulations in PBS or 20% gelatine, a specific volume of chloroform/methanol-dissolved solution containing 4 mg of Fenofibrate, 21 mg of cholesterol and 75 mg of the intended PC was transferred into the rotatory evaporator. Depending on the purpose of the study and the type of experiment, different concentrations of liposomes are required. In case of this example, in the rehydration step, an aqueous solution containing 200 µg of gelatine dispersed in 1 mL PBS was added to the thin layer of lipid film.

2.2.1 Dual asymmetric centrifugation

Dual asymmetric centrifugation (DAC) was established in the 1970s as a rapid and useful technology for mixing viscous components using apparatus known as a speedmixer (Wang et al. 2002). Massing and co-workers were the first to use a speedmixer to produce multilamellar liposomes (Massing et al. 2008). DAC is a specific centrifugation method which turns the vials around the main rotation axis at a specific angle and distinct speed and distance. While the vials are spinning around the centre of the centrifuge in the DAC process, they simultaneously rotate around their own individual centre (vertical axis) with the normal centrifugation process. These two overlying movements of the sample materials in the centrifugation vial result in shear forces that help the viscus materials to mix completely. The normal rotation of the centrifugation vial around

the main axis forces material outwards while the smaller rotation around the vertical centre constantly pushes the material in the opposite direction (see Figure 10 and Figure 11).

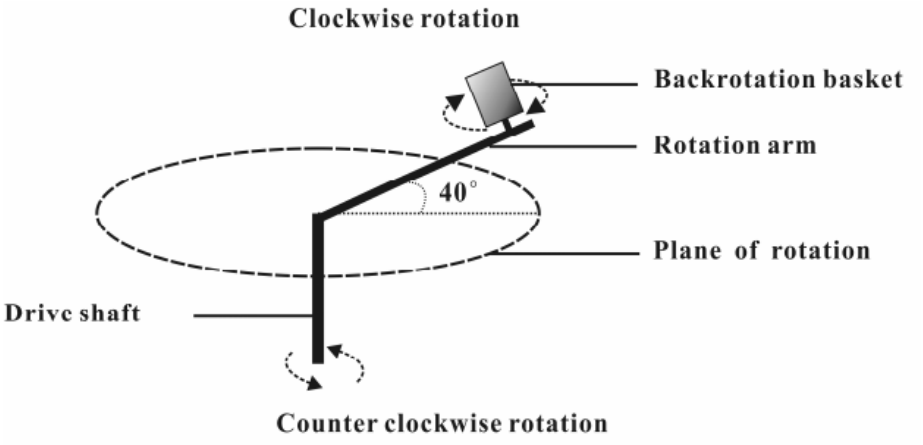


Figure 10. Schematic illustration of dual asymmetric centrifuging (Huang et al. 2014).

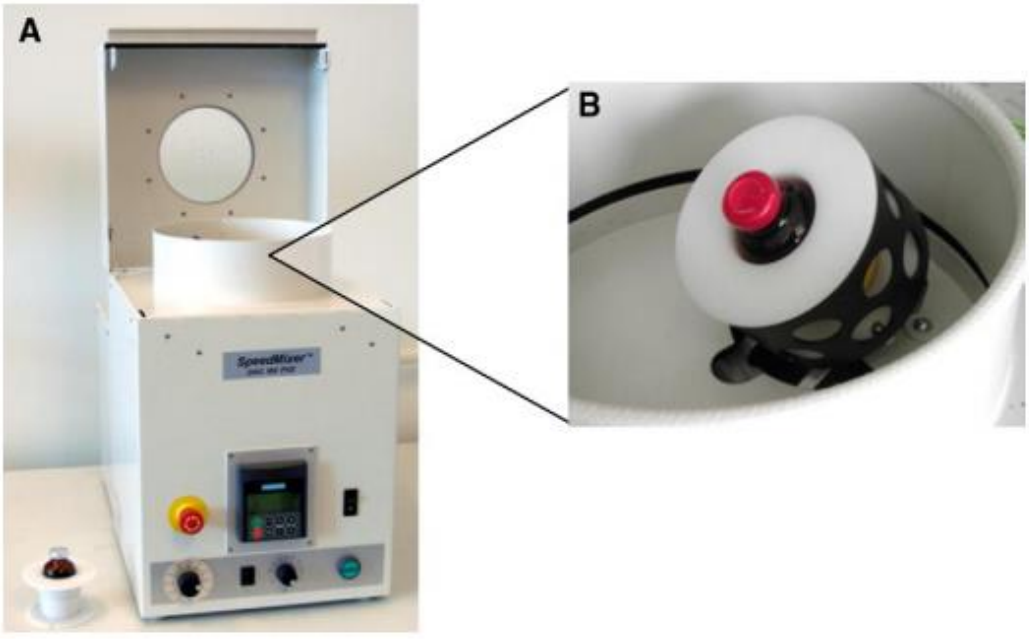


Figure 11.Dual asymmetric centrifuge (DAC) with open lid view (A) and the rotation chamber (B). (Massing et al. 2008).

In addition to mixing the viscous materials, the shear forces arising from the centrifugal movement can be used for other purposes. These constant, strong shear forces can facilitate homogenization of the material, and this process and method of preparation can therefore be proposed as a fast and effective method to produce viscous, uniquely sized liposomes. The maximum speed at which DAC can be used is 3540 rpm, which is the speed that was primarily used in this study (Massing et al. 2008).

To achieve a homogenized product using DAC, the lipid concentration should be within the range of 350–450 mg/ml. In this study, the dried lipid film mass was added to the aqueous solution (PBS with and without gelatine) at a ratio of 2:3, but for glass beads (ø 0.75–1 mm), a ratio of 2:5 was maintained. The addition of glass beads, or indeed any kind of bead (e.g. stainless beads), is highly recommended as it helps the mixture to form small, homogenized and highly reproducible liposomes. The mixture of lipid film, aqueous solution and glass beads was centrifuged and homogenized for 30 minutes at a maximum speed of 3540 rpm. Notably, some factors can affect the liposome size such as the lipid concentration, DAC speed, homogenization time and addition of glass beads. One advantage of this preparation method over other methods used to prepare liposomes is that, since the final product is highly viscous, it is possible to dilute the product to any desired final concentrations for different experiment purposes.

2.2.2 Probe sonication

Sonication (using either a bath or a probe tip sonicator) is used widely for the preparation of unilamellar and multilamellar liposomes (Maulucci et al. 2005). Probe tip sonicators transfer high energy to the lipid dispersion, which can result in overheating and subsequent degradation of the lipid suspension. Moreover, the sonicator tips can release titanium particles into the suspension. Due to these disadvantages, the best way to use the sonication to prepare liposomes is to combine the probe tip sonicator and the water bath methods. Using this approach, the sonicator tip is placed in a sample tube containing the dispersion, which is placed in the bath sonicator, and the sonication process is typically run for 5–10 minutes to produce a hazy and non-clear solution which scatters light. The size and dispersity of the solution can be affected by different factors including the type and concentration of the mixture, temperature, power, volume and duration of sonication. The liposomal formulation should not be stored for a long period of time as the liposomes are naturally unstable and could fuse spontaneously.

Depending on the desired final concentration, the mass of dried lipid film containing different concentrations of lipid and drug (Table 2) was dispersed into 10 mL of aqueous solution containing various concentrations of gelatine in PBS. The mixture was then applied to probe sonicator with an ultrasonic probe (50/60 KHz, 230V, Chemical Instruments AB, Sweden) at 80% ultrasonic power amplitude for 10 min to form the fenofibrate-loaded lipid vesicles. The probe, with a tip diameter of 6 mm, was dipped about 15 mm in the liquid. The ultrasound burst was set to 3 s with a pause of 7 s between two ultrasound bursts. The sample vial was kept in a temperature-controlled water bath to prevent heat damage (4°C).

2.3 Size and Size Distribution

Size determinations including hydrodynamic size (Z average) and polydispersity index (PDI) were determined using methods based on dynamic light scattering, which measures the intensity of the scattered light produced on the movement of the existing particles in the sample. The light-scattering method can be applied to particles with a diameter ranging from 0.3 nm to about 10 μm (Malvern Instruments Limited 2003). Of note, the desired method does not calculate the particle size directly; it measures the size indirectly according to the fluctuation effects detected by the scattering device. Therefore, it may give unreliable results for particles that are not spherical in shape (Müller et al. 2000). In this study, liposome dispersions were measured at 25 ˚C by photon correlation spectroscopy on a Malvern Zetasizer Nano® instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) and all measurements were repeated tree times. The Z average was used to determine the hydrodynamic size (diameter) of particles where PDI indicates the width of size distribution and the homogeneity of the particles in the system. Dispersions with a PDI of less than 0.2–0.3 can be considered as a homogenous system, where PDI 1 shows a heterogeneous dispersion (Schubert & Müller-Goymann 2005).

2.4 Light-Scattering Measurements

In this study, a Zetasizer instrument using software version 6.20 was used for purposes other than the determination of particle size. The instrument was also used to measure absolute light scattering intensities, which gives an arithmetic mean of count rate and is reported as mean

count rate in kilo counts per second (kcps). Liposome samples were diluted with water to concentrations of 20−200 µg/ml. At this range of concentration, stable particle size is ensured by increasing the solubility and preventing particle dissolution (Brittain 2003). The size measurements were conducted at position 4.65 mm, with automated attenuator settings at a temperature of 25 °C. The Zetasizer instrument was used with a He-Ne laser (wavelength 633 nm, 4.0 mW) and a photodiode detector at a detection angle of 173°. The incident laser light could be set from 0 (total laser block) to 11 (full laser power), but the ideal concentrations are reached when the attenuation factors are set from 7 to 9. In this study, all determinations were performed at attenuator settings between 7 and 9 for absolute light scattering measurements. The measurement position, which is the distance from the cuvette wall, was always at 4.65 mm to ensure a constant scattering volume. Since attenuator and position settings can influence the intensity of scattered light, they were both fixed and set to give scattering intensity values in the range of 100−2000 kcps. All measurements were performed in disposable semi-micro polystyrene cuvettes with lids at a temperature of 25 °C, and were repeated three times.

2.5 Cryogenic Electron Microscopy

Cryogenic electron microscopy (Cryo_EM) is a type of transmission electron microscopy (TEM) which uses samples at cryogenic temperatures (generally liquid nitrogen temperature) to study the structures of different biological molecules (Kühlbrandt 2014). Cryo_EM allows the observation of specimens which are not fixed or even stained, and therefore allows samples to be examined in their natural environment. This can be considered as one of the advantages of this biological method over other structural methods such as X-ray crystallography, in which specimens need to be crystalized and consequently can undergo irrelevant conformational changes (Callaway 2015).

The working principle of TEM is the same as for normal light microscopy, except that the source of the light is small-wavelength electrons instead of the normal light. TEMs generally provide electrons from two different sources, including field emission guns and thermionic electron guns.

To verify the structure of liposomes as well as determine the effect of gelatine on liposome integrity, cryo_transmission electron microscopy images were prepared. Liposome dispersions containing 4% (w/w) of fenofibrate, prepared in either PBS (as a control) or in a solution of gelatine

dissolved in PBS, were diluted in PBS to a concentration of 10 mg/mL and applied to a glow-discharged Quantifoil sample support grid. The grids (2/2) were glow discharged for 10 s in a mixture of H_2 and O_2 gas and the dispersion was blotted from one side at 4 °C with 100% humidity for 8–10 s using a Vitrobot (FEI, Hillsboro, Oregon). Directly after blotting, the grid was dipped into liquid high heat-capacity ethane. To provide clear images, it is essential that the freezing process is as quick as possible to prevent ice formation around the specimens. The grids were viewed under a Titan Krios microscope (FEI, Hillsboro, Oregon) equipped with a Quantum 963 SE energy filter operated at 200 kV and liquid nitrogen (LN) temperature. Zero-loss filtered images were taken at 64,000× magnification (pixel size 2.2 A) using a 2k Ultrascan Quantum CCD camera.

2.6 Drug Entrapment Efficiency

The drug entrapment (encapsulation) efficiency, which corresponded to the amount of drug that could be incorporated in the liposomes, was determined by directly measuring the concentration of the loaded drug in the lipophilic layer of the liposomes. The free and unloaded drug in suspension was separated from the lipid vesicular particles using Sephadex G-50 chromatography. About 1.5 g Sephadex G-50 was packed into a 10-cm glass column with an inner diameter of 1 cm. After draining the gravity column with running buffer, a suspension of fenofibrate-loaded liposomes was applied and the free drug was separated from the drug-loaded liposomes according to size and gravity. The remaining eluent containing liposomes was treated with 1% Triton-X100 to disrupt the vesicular structure and release the loaded fenofibrate. The content of fenofibrate was determined using an Agilent 1100 HPLC system (UltiMate3000, Dionex, Dreieich, Germany).

The entrapment efficiency (%EE) was calculated as:

$$\%EE = Wc/Wt \times 100$$

where Wc and Wt denote the drug content in liposomes and total drug in the dispersion, respectively.

2.6.1 High-performance liquid chromatography

Fenofibrate and fenofibric acid concentrations were determined by high-performance liquid

chromatography (HPLC). All samples were diluted in HPLC vials, as samples containing gelatine may interfere with HPLC performance by precipitating the determination column and blocking the running flow in the HPLC. For each sample, three independent dilutions were prepared and two injections were performed from each vial. To evaluate the accuracy of the system, a complete calibration of the method was repeated every 3 months, and for each sequence, new control standards were run. Calibration standards were prepared in extraction medium at concentrations of 1−500 µg/ml. The chromatograms were analyzed using Chromeleon software. The HPLC parameters are given in Table 3.

Table 3. An overview on high pressure liquid chromatography (HPLC) method parameters for quantification of fenofibrate.

Mobile phase	
Eluent A	H_2O/AcN: 95/5 + 0.01% TFA
Eluent B	H_2O/AcN: 5/95 + 0.01% TFA
Extraction medium	H_2O/AcN: 20/80
Column	Zorbax Eclipse Plus C18 4.6 x 50mm 3.5µm
Column temperature	30 ˚C
Flow	2 ml/min
Gradient	0 min A/B 100/0 3 min A/B 80/20 6.8 min A/B 30/70 8.3 min A/B 0/100 11 min A/B 0/100 12 min A/B 100/0
Injection volume	20 µl
Sample temperature	20 ˚C
Detection wavelength	288 nm

in coefficients of determination $R^2 > 0.995$.

2.7 X-Ray Powder Diffraction

Recent studies have shown that, during *in vitro* lipolysis, amorphous precipitations have a faster dissolution rate than the crystalline phase (Xiao et al. 2016). The existence of crystals in the formulation facilitates precipitation and, therefore, fewer drugs become solubilized and absorbed. One effective method to distinguish between the presence of crystal (long-range order) and amorphous forms in the samples and to evaluate the crystal structure of the API incorporated in the liposomes is by the analysis of X-ray powder diffraction (XRPD) patterns obtained from X-rays scattered by the sample. The X-ray amorphous form is obtained from a solid powder that contains no crystalline or long-range order pattern. The XRPD patterns act as a unique fingerprint for crystalline substances and provide information about the length of the short and long spacing of the lipid structure of the liposomal formulation (Mehnert & Mäder 2012). The sets and positions of the picks can be compared with the crystallographic unit cells available in the database in order to identify the crystalline substances. To obtain perfect X-ray picks, it is important to obtain patterns with a high resolution and low background.

There are some parameters which can affect the crystallization behaviour of the testing materials; molecular structure, type of the solvent, pressure, temperature, rate of crystallization, and presence of impurities. The lipids are typically known to be polymorphisms and they crystallize in different positions and arrangements: α, β' and β modification. (Sato 2001). These factors strongly affect the drug incorporation and release rates.

X-ray powder diffractograms were measured at ambient conditions on a PANalytical X'Pert Pro diffractometer equipped with a PIXcel detector (PANalaytical B.V., Almelo, the Netherlands). Samples were placed on a stainless-steel disk for measurement, and a continuous 2θ scan was performed in the range of 2–40° using Cu Kα radiation (λ = 1.54187 Å). Kβ radiation was eliminated using a nickel filter. The applied voltage and current were 45 kV and 40 mA respectively. Each measurement was performed with a step size of 0.0260°2θ and a speed of 0.0336°/s. To avoid the effect of preferred orientation, the samples were rotating during the experiment. Data were collected using X'Pert data collector version 2.2, and were analyzed using X'Pert high score plus version 2.2.4 (both from PANalaytical B.V.).

2.8 Dissolution Behaviour

The early stage of oral absorption of a pharmaceutical API is the dissolution of drug in the physiological environment of the GI tract. Drug particles first need to be dissolved in the GI fluid to permeate the mucosa at the absorptive sites in the GI tract (1); therefore, the solubility of the drug and its dissolution rate are critical for determining the *in vivo* behaviour (Klein 2010).

To evaluate the dissolution behaviour of solidified VPGs, a paddle dissolution apparatus with two kinds of dissolution media were used. The first model used the simulated intestinal fluid (SIF) to assess the release of liposomes from the semi-solid formulation, while the second model used two different biorelevant media containing fasted state simulated intestinal fluid (FaSSIF) and fasted state simulated gastric fluid (FaSSGF) to determine drug release from the desire formulation. The composition of used dissolution media is given in Table 4.

Table 4. The chemical composition and properties of different tested intestinal fluids; simulated intestinal fluid (SIF) (Gray & Dressman 1996), fasted state simulated intestinal fluid (FaSSIF) and fasted state simulated gastric fluid (FaSSGF) (Klein 2010).

	SIF	FaSSIF	FaSSGF
Sodium taurocholate	-	3 mM	80 µM
Lecithin	-	0.75 mM	20 µM
Pepsin	-	-	0.1 g
NaCl	-	6.186 g	34.2 mM
NaOH	0.896 g	qs. pH 6.5	-
NaH2PO4.H2O	-	3.438 g	-
KH2PO4	6.805 g	-	-
Deionized water qs.	1000 mL	1000 mL	1000 mL
pH	6.8	6.5	1.6
Osmolality (mOsmol/kg)	113	270	120.7 ± 2.5
Surface tension (mN/m)	Not available	54	42.6

2.8.1 Release of liposomes

The initial dissolution tests were carried out by using USP Apparatus II to evaluate the release of liposomes from the semi-solid formulation. In each dissolution experiment, 900 mL of simulated intestinal fluid (pH 6.8) was used as a testing medium and all experiments were conducted at 37 ± 0.5℃ with a paddle rotation speed of 75 rpm over 2 h. Sampling aliquots of 1 ml were withdrawn at 5, 20, 40, 60, 80, 100 and 120 min, and were replaced with an equal volume of fresh medium maintained at the same temperature. Each dissolution test was performed in triplicate. Where liposome gel was not completely dissolved within 2 hours, an additional sample was taken at 150 and 180 min. After sampling, the solutions were filtered through a 0.22 μm syringe filter and analyzed by PCS using a Zetasizer Nano ZS. The derived count rate, which gives the arithmetic mean of the scattering signal, was determined to show the number of particles converted from the semi-solid formulation into free liposomes. Therefore, the derived count rate was standardized to show the percent release of liposomes. To determine the liposome quality, the liposome size and size distribution were determined.

2.8.2 Drug release in different biorelevant media

The use of biorelevant media such as FaSSIF and FaSSGF has appeared in the past decade and has developed to be uses as a standard medium in pharmaceutical dissolution testing, particularly if *in vitro–in vivo* correlation is intended (Galia et al. 1998; Lee et al. 2008). The lipid-based formulations were evaluated for their stability in different biorelevant media: FaSSIF (pH 6.5) and FaSSGF (pH 1.6), prepared according to the USP.

The dissolution experiments were conducted as described in Section 2.7.1 to determine the release of fenofibrate from the respective formulation. A volume of 900 mL of each biorelevant media was applied to the glass vessel of the USP II paddle apparatus at 37 ± 0.5 ℃ with a speed of 75 rpm, according to the FDA instructions. Samples were collected at 5, 10, 20, 45, 60, 90, 120 and 180 min. For each sampling, 2 mL of the experimental solution was collected and replaced with fresh medium at the same temperature. After filtration through a 0.22 μm syringe filter, samples were divided into two separated stocks. The first stock was used to determine the release of fenofibrate from the liposomes. The released fenofibrate was separated from the liposomes using a Sephadex® G50 gel exclusion chromatographic column eluted with PBS (pH 7.4). The eluent containing

liposomes was treated with 1% Triton-X100 to disrupt the vesicular structure to release fenofibrate. The content of fenofibrate was determined by an Agilent 1100 HPLC system (UltiMate 3000, Dionex, Dreieich, Germany). The percent of drug release (% release) in different media at the given timepoint was calculated from the equation:

$$\mathrm{EE\%} = \frac{\mathrm{Wt} - \mathrm{Wc}}{\mathrm{Wt}} \times 100$$

Where W_c is the drug content incorporated into liposomes at different time points and W_t is the total drug content in the dispersion. The second stock was used in parallel to evaluate the stability of liposomes in terms of particle size and also dispersity using a Zetasizer Nano ZS.

2.9 Storage Stability

In the development of an API, it is vital to evaluate the stability of the formulation under storage conditions. Testing should cover the physical and thermal stability, sensitivity to moisture and potential to lose solvent. Different environmental factors can affect the physical stability of liposomes by changing their size and distribution, integrity and saturation of fatty acid groups. During storage, liposomal vesicles are at risk of aggregation, fusion and leakage of the contained API, and the lipid component of liposomes and type of incorporated drug substance plays an important role in these processes (Administration 2015).

Conventional and matrix liposomes were assessed for storage stability over a period of 3 months according to ICH guidelines, with minor changes (Jain et al. 2012). Briefly, samples containing fenofibrate with and without gelatine were stored at 4 °C or room temperature (25°C) at relative humidity ≤55%. The liposomal suspensions were observed each month for changes in physical appearance, such as the size and size distribution, as indicators of kinetic stability.

2.10 *In Vitro* Lipolysis Model

The intestinal *in vitro* lipolysis model developed by Zangenberg et al., with some modifications, was used to investigate the lipid digestion and solubilisation of the drug in the bio-relevant media. An *in vitro* lipolysis study simulating the lipid fate in the human GIT was performed as described below. All media and required solutions were prepared in advance.

2.10.1 Media and solution preparation

For each experiment, the *in vitro* lipolysis model used freshly prepared solutions and bio-relevant media. The media and solutions were prepared as detailed as follows.

a) Preparation of bio-relevant media

To prepare the gastric and intestinal bio-relevant media, all excipients were weighed out separately and transferred into a 500 mL glass beaker to which 200 mL purified water was added. The solution was mixed with a spoon and transferred to a 500 mL volumetric flask. The solution was protected from light and stirred overnight with a magnetic stirring bar, and then the pH was adjusted to 6.5 for intestinal media and pH 4 for gastric media before the volume was adjusted to 500 mL with purified water. The prepared solution was used within the period of 24–48 hours. Table 5 shows the composition of the bio-relevant media used in the *in vitro* lipid digestion.

Table 5. The composition of digestion media used in *in vitro* human lipolysis and *in vitro* rat lipolysis models

	Human		Rat
	Intestinal medium	Gastric medium	Intestinal medium
Total volume (mL)	30-40	50	100
Bile bovine (mM)	2.95	0.08	15.00 [1]
Phosphatidyl choline (mM)	0.26	0.02	1.65 [2]
Sodium chloride (mM)	73.0	34.2	70.0
Calcium chloride (mM)	1.4	-	-
Maleic acid	2.0	2.0	2.0
Tris maleate (mM)	2.0	2.0	2.0
pH	6.5 ± 0.1	4.0 ± 0.5 [3]	6.5 ± 0.1 [3]
Lipase source	*Porcine pancreatin*	*Rhizopus oryzae*	*Porcine pancreatin*
Lipase activity	660 USP units/mL	12.0 TBU*/mL [4]	800 USP*/mL [5]

* TBU: tributyrin unit; USP: United States pharmacopeia.

1 (Hagio et al. 2009)

2 (Holm et al. 2013)

3 (Kararli 1995)

4 (Sassene et al. 2016)

5 (Mosgaard et al. 2017)

b) Preparation of pancreatic and gastric lipase solution

The precise mechanism of rat pancreatin activity remains unclear by now (Anby et al. 2014; Tønsberg et al. 2011), although current *in vitro* lipolysis models simulating the human conditions primarily use pancreatin enzymes with activity between 500–800 USP/mL (Mosgaard et al. 2017; Larsen et al. 2013). The enzyme activity used in this model was 800 USP/mL, as used by (Christophersen et al. 2014). Lipolysis is initiated by the addition of a certain amount of pancreatic lipase solution and, after sample collection; lipid digestion is inhibited by the addition of 4-bromobenzeneboronic acid (4-BBBA) solution at a concentration of 1 M.

Pancreatic and gastric lipase solutions were prepared immediately prior to use to prevent loss of activity. Briefly, 78.65 mg of the pancreatin was suspended in 5 mL water to obtain an activity of 800 USP/mL. In previous experiments, pancreatin was added to the intestinal medium, but to avoid initiating the digestion of lipids existing in the medium, the enzymatic solution was added to water. The suspensions were centrifuged for 7 min at 4000 rpm at 4°C in a Heraeus Megafuge 16R centrifuge (Langensenbold, Germany) and 4 mL of the resulting supernatant was added to the lipid digestion vessel to initiate the digestion process. For gastric enzymes, 8.33 mL of *Rhyzopus* enzyme was added to 3 mL of gastric medium.

c) Preparation of calcium chloride and sodium hydroxide solutions

A solution of calcium chloride and sodium hydroxide were prepared according to Table 6. The mixtures were prepared from the desire chemical resources and after dissolving in Milli-Q water, they stirred overnight at room temperature to produce clear and homogenous solutions.

Table 6. Chemical properties of calcium chloride and sodium hydroxide solutions

	Concentration (M)	**Final volume (L)**	**Chemical resource**
NaOH	0.5	1	$CaCl_2 \cdot 2H_2O$
$CaCl_2$	0.5	1	NaOH pellets

2.10.2 Liposome samples

The liposomal formulations were prepared using the probe sonication method from various phosphatidylcholine compositions, namely E-PC, S-PC or Soluthin S 90, both with and without gelatine as listed in Table 7. For this specific experiment, liposomes were prepared at a concentration of 0.625% (0.625 g/100 mL) and subsequently monitored for size and polydispersity with the help of a Zetasizer Nano ZS. In each lipolysis run, 2 mL of samples containing 125 mg of the formulation (5 mg FF + 45 mg cholesterol + 75 mg of the desired lipid) was used.

Table 7. The chemical properties of the liposome samples tested in *in vitro* lipolysis.

Samples	PC type	Fenofibrate W/W (%)	Gelatine concentration in PBS	Concentration W/V (%)
4%FF_0%Gel_EPC	EPC	4%	0 µg in 1 mL PBS	0.625%
4%FF_20%Gel_EPC	EPC	4%	20 µg in 1 mL PBS	0.625%
4%FF_0%Gel_SPC	SPC	4%	0 µg in 1 mL PBS	0.625%
4%FF_20%Gel_SPC	SPC	4%	20 µg in 1 mL PBS	0.625%
4%FF_0%Gel_S 90	Soluthin S 90	4%	0 µg in 1 mL PBS	0.625%
4%FF_20%Gel_S 90	Soluthin S 90	4%	20 µg in 1 mL PBS	0.625%

2.10.3 Dynamic *in vitro* lipolysis assay

The *in vitro* study was performed using 5 mL of gastric medium (pH 4) and 20 mL of intestinal medium (pH 6.5) added to the gastric and intestinal compartments, respectively. The pH was set at 6.5 by potentiometric titration using 809 Titrando titrators combined with an Ecotrode Plus pH electrode. There were two Dosino dosing units which were connected; one was filled with 0.5 M sodium hydroxide (NaOH) and the second was filled with 0.5 M calcium chloride ($CaCl_2$). The flow rate for the $CaCl_2$unit was set to 0.01 mL/min. A stirrer with a propeller was immersed in the bio-relevant media located in a thermostatic glass vessel (KGW-Isotherm, Karlsruhe, Germany). The titrator was connected to a computer and controlled by Tiamo software versions 1.1 and 2.0. The whole lipolysis system (except the glass vessel) was obtained from Metrohm AG (Herisau, Switzerland). The temperature in the glass vessel was kept constant at 37±1°C using a Julabo water bath (Buch & Holm, Herlev, Denmark). The lipolysis apparatus is illustrated in Figure 12.

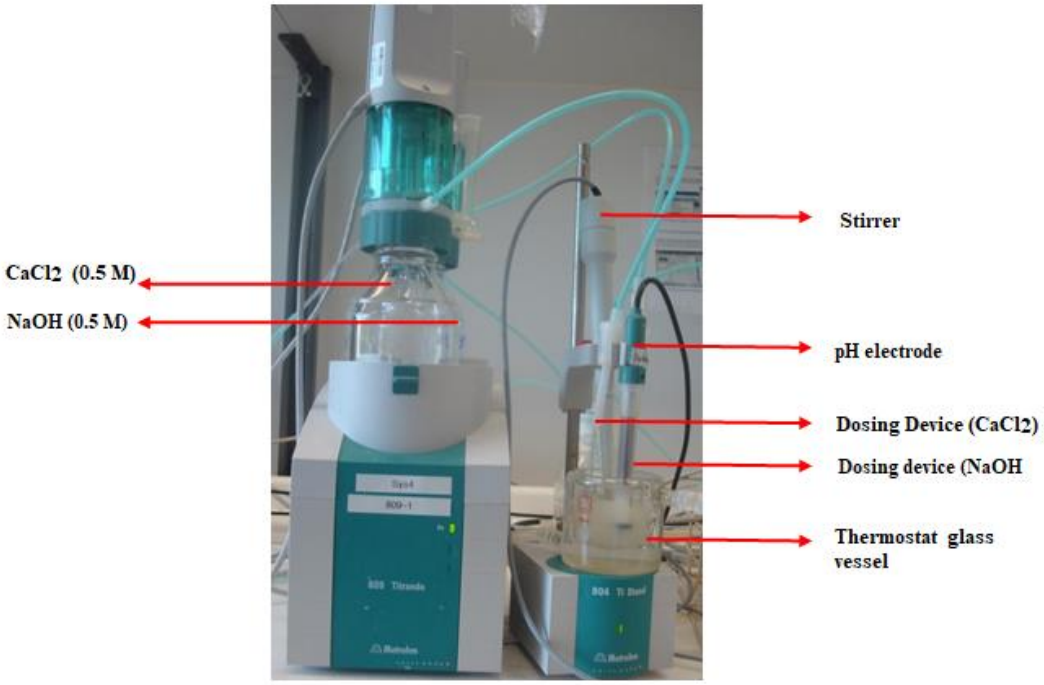


Figure 12. The experimental *in vitro* lipolysis compartment.

The experiment workup started when 2 mL of samples containing 125 mg of liposomes suspended in PBS was added to 2 mL of gastric medium, and 3 mL of gastric lipase enzyme was then added to the gastric medium in the thermostated vessel (gastric vessel) with stirring. The pH of the gastric medium was monitored and manually adjusted by adding a few drops of 0.05 M HCL after the addition of samples. The next step was to prepare the pump for transferring the gastric suspension into the intestinal compartment, which was the main environment for lipolysis testing.

The system was activated after adding 5 mL of pancreatin lipase solution to 20 mL of intestinal medium while simultaneously turning on the transfer pump. The pump transferred a specific volume from the gastric to the intestinal compartment at a flow rate of 0.5 mL/min. A computer-controlled pump automatically added $CaCl_2$ at a constant rate of 0.01 mL/min into the intestinal compartment. The pH of the intestinal compartment was set at 6.5 by titrating the required amount of NaOH. The gastric vessel was empty after 20 minutes. The continuous steps of the rat *in vitro* lipolysis model are shown schematically in Figure 13.

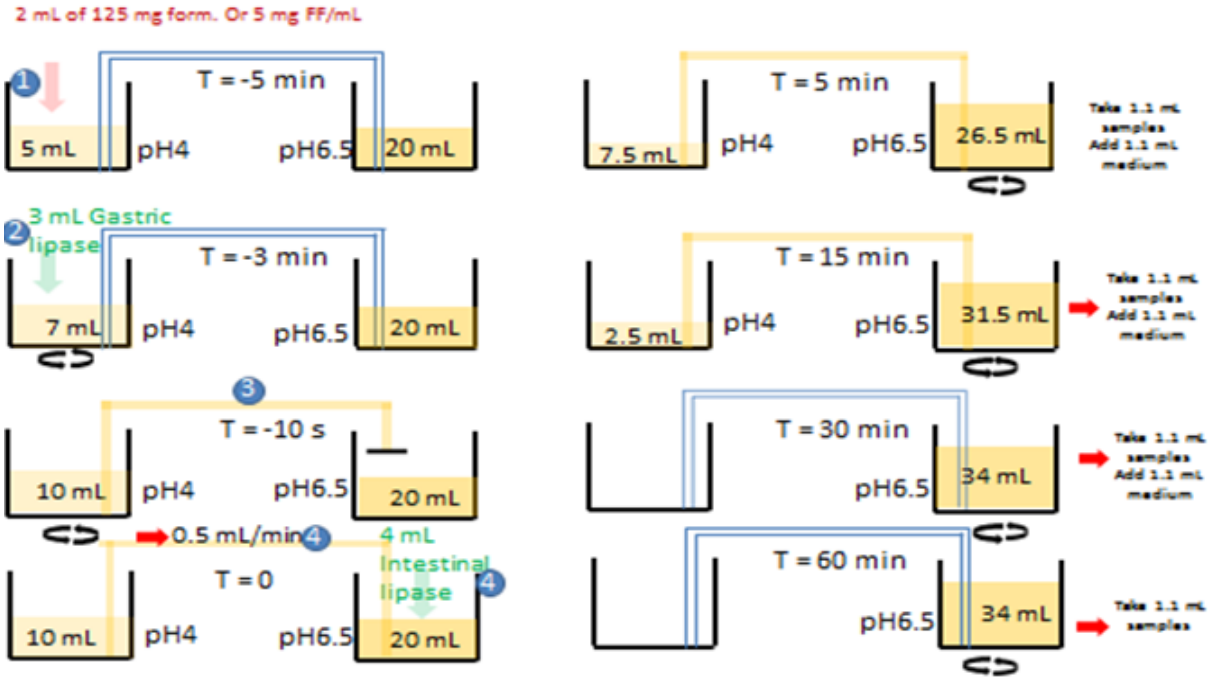


Figure 13. Schematic illustration of dynamic *in vitro* lipolysis model over the time.

Samples were taken at 5, 15, 30 and 60 min, at different volumes (100 µL and 1 mL) simultaneously. Each time a sample was taken, the sample volume was replaced with fresh intestinal medium. The 100 µL sample was quickly diluted with 900 µL of methanol and centrifuged in an Eppendorf Centrifuge 5417R (Germany) at 16,000 rpm for 20 min at 37°C, and the resulting supernatant was collected for HPLC analysis. These samples contained total fenofibrate concentrations (T-samples). The 1 mL sample was used to determine the concentration of fenofibrate in the different phases. Briefly, 7 µl of 1.0 M 4-BPBA (4-Bromo-phenylboronic acid) solution was added to the 1 ml sample to inhibit lipase activity. The mixture was centrifuged in Eppendorf Centrifuge 5417R (Germany) at the speed of 13.3 rpm for 15 min at 37 °C and the aqueous (supernatant) and pellet phases were separated. The supernatant phase was termed the aqueous phase and the precipitate was the pellet phase. Next, 200 µl of supernatant from the middle of the aqueous phase was diluted with 800 µl methanol and, after centrifugation at the same speed and temperature for 10 min, the supernatant was transferred to a HPLC vial. Samples from the supernatant of the aqueous phase contained the concentration of the desired API released from the formulation at a specific time (A sample). The pellet phase was redispersed in 500 µl methanol after mixing and sonication for 20 min and the suspension was subsequently centrifuged at the same

speed and temperature for 10 min. The supernatant was analysed as the P sample, which represented the amount of the fenofibrate remaining in the liposomal formulation. The recovery of Fenofibrate in the collected samples was quantified using HPLC. The chromatograms were analysed using Chromeleon software. Immediately after each sampling for A and P samples, lipase activity was inhibited with a 4-bromobenzeneboronic acid solution (Müllertz et al. 2010).

2.11 *In vivo* pharmacokinetics

The impact of different liposomal formulations on the bioavailability of Fenofibrate was investigated. Pharmacokinetic (PK) studies on rats were performed for orally (p.o.) administered suspensions of fenofibrate with different types of phosphatidylcholine (EPC and Soluthin S 90). The probe sonication method was used to form the lipid vesicles. A pulsed duty cycle of 3 min on/7 min off was used with a power delivery of 80% for 10 min. The sample vial was kept in a temperature-controlled water bath (4°C) to prevent heat damage. Subsequently, after liposome preparation, the size and dispersity of samples were evaluated using a Zetasizer. Table 8 shows the composition of the different liposomal formulations used for rat studies.

Table 8. The composition of the different liposomal formulations prepared by probe sonication method for the *in vivo* pharmacokinetic purposes.

Samples	Type of PC	Fenofibrate %(W/W)	Gelatine
S_90_0%	Soluthin S 90	4%	0 μg in 1 mL PBS
S_90_20%	Soluthin S 90	4%	200 μg in 1 mL PBS
EPC_0%	Egg PC	4%	0 μg in 1 mL PBS
EPC_20%	Egg PC	4%	200 μg in 1 mL PBS

Healthy male Wistar rats of approximately 300 g in weight (Charles River, Sulzfeld, Germany) were housed in cages without food and free access to water, but the water was removed 12 hours prior to administration and replaced 3 hours after dosing. The holding and study areas had automatically controlled light cycles (12 h light/12 h dark), temperature (23 ± 1◦C) and humidity (60 ± 10%). Animals were weighted prior to administration and body weights of 240−320 g were registered.

The oral formulations were administered by gastric gavage. The dose of fenofibrate was 2.1 mg/kg (0.006 mmol/kg) in accordance with the clinical dose (145 mg for a 70 kg adult) (Buch et al. 2009; Zhu et al. 2010). To apply 2.1 mg/kg of fenofibrate per rat by gavage, 1 mL of the formulation was administered to each animal. Therefore, 0.63 mg of fenofibrate or 15.75 mg of the 4% fenofibrate formulation was prepared in 1 mL of PBS with or without gelatine.

Each study group consisted of two animals and received one of the formulations. At seven time points, blood samples were taken from each animal. The sampling times were 0, 0.5, 1, 2, 3, 6 and 9 hours. Blood samples were collected from the retro-orbital venous plexus. The blood samples were collected in heparin-coated blood collection tubes (Microvette§R 200). Blood cells were separated by centrifugation at 1000 × g for 10 min in an Eppendorf centrifuge 5413C (Eppendorf, Hamburg, Germany) and the resulting plasma samples were stored at −80°C and analysed for fenofibric acid by HPLC (Labor Limbach, Heidelberg). After the last sampling time, the rats were sacrificed by CO_2 asphyxia.

2.12 Data and statistical analysis

The achieved results are provided as arithmetic means ± standard deviation unless otherwise indicated. The statistical significance was checked through the two-tailed Student's t-test in Microsoft Excel software.

3. RESULTS AND DISCUSSIONS

3.1 Preparation Method

Four different production methods were used to prepare the liposomal formulation. The preparation methods were assessed by determining the range of the size and polydispersity of the produced particles. Schubert refers to a PDI of less than 0.2–0.3 as a homogenous system, which was applicable for this study (Schubert & Müller-Goymann 2005). The characteristics of the liposomal particles are briefly summarized in Table 9.

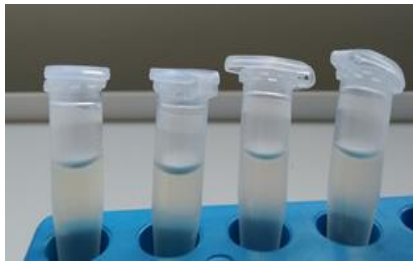
Table 9. Preparation methods and characterization of size and size distribution (n=3, mean ± SD).

Production method	Size (nm)	PDI
Ball milling	**157.4± 49.5**	**0.323± 0.096**
Extruder	**119.6± 2.7**	**0.057± 0.009**
Probe sonication [a]	**159.4± 27.7**	**0.305± 0.063**
DAC [b]	**132.2± 12.6**	**0.125± 0.02**

[a] Probe sonication for 10 min, 80% sonication intensity, 3s pulse on/7s off

[b] DAC for 30 min, speed of 3540 rpm

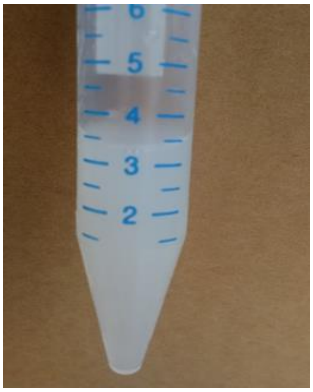
The liposome samples were prepared by milling the mixture for 30 min at a frequency of 30 Hz in 10-mL grinding jars containing ten 2-mm stainless steel balls in an oscillatory ball mill (Mixer Mill MM400, Retch GmbH & Co., Haan, Germany) located in a cold area (+6 °C). Although the produced liposomes were within an acceptable range in terms of size and size distribution, the final product was rejected as a grey layer of dirt had formed on the top of the samples after dilution of the final product. This interfering layer was formed due to interactions between the metal beads and the oily compositions, despite the beads being thoroughly washed using methanol.



a) b) c)

Figure 14. Ball milling machine(a), liposome produced by ball milling (b), and diluted liposomes (c).

The lipid extrusion technique was used to form the liposomes. The lipid suspension (concentration of 0.65% (W/V)) was passed through a polycarbonate filter (1.0 µm pore size) for 10 cycles under a nitrogen gas flow. The resulting liposomes were of ideal size and dispersity, but in addition to being highly time consuming, the main limitation of this method was that the final suspension was overly diluted as a result of a lack of lipid after filtration, rendering the output unsuitable for further experiments (particularly the animal experiments). Therefore, extrusion was also removed from the list of preparation methods.

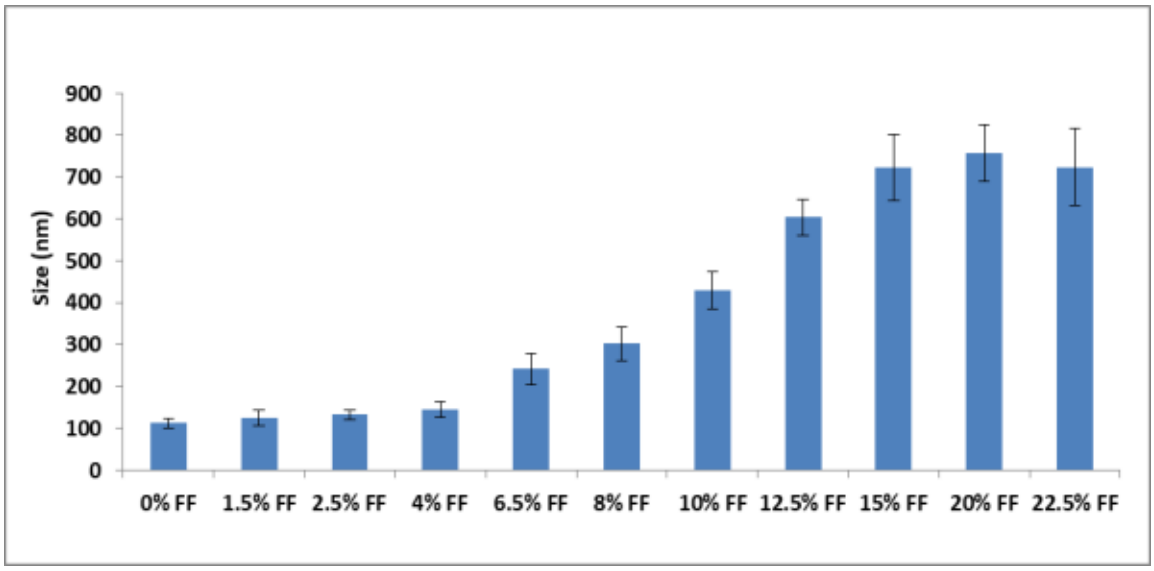


a) b)

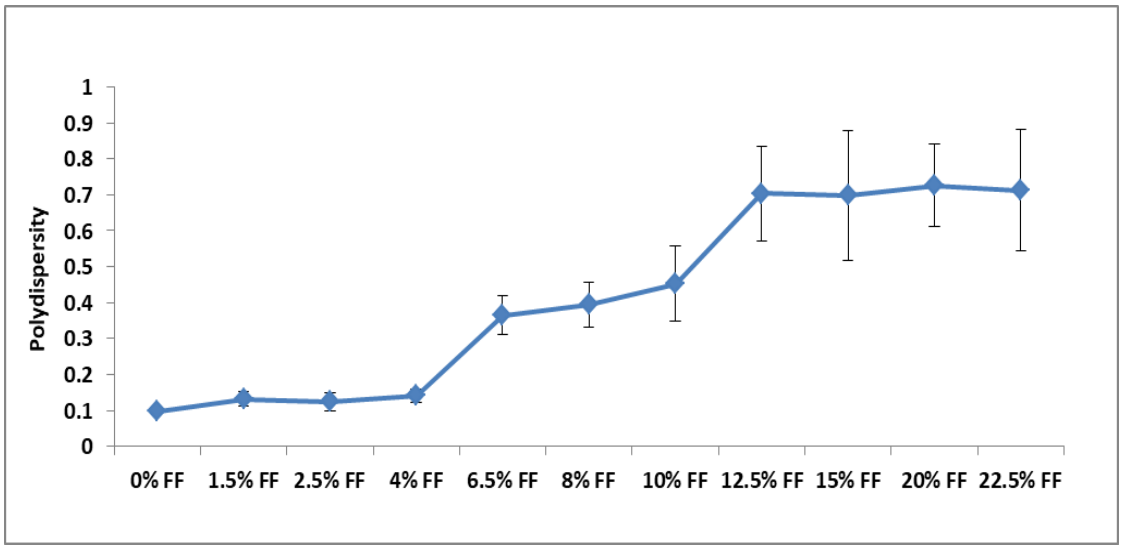
Figure 15. Extrusion machine (a) Liposome suspension produced by extruder (b).

Based on the diameter size and PDI described on Table 9, DAC was considered the optimal method for preparing liposomal samples, but the speedmixer device was not always accessible and due to this access restriction, the probe sonication method was also a suitable and alternative method to prepare samples as it produced liposomes within the normal range of size and distribution.

Additionally, an evaluation method was used to determine the threshold level of liposomes for loading the poorly water-soluble drug, fenofibrate. To achieve this, different concentrations of fenofibrate-loaded liposomes were prepared by DAC to determine the optimal concentration. The concentrations ranged from 0% FF (0 g fenofibrate, 40 g cholesterol, 60 g EPC) to 1.5% FF (1.5 g fenofibrate: 38.5 g cholesterol: 60 g EPC) to 22.5% FF concentration (22.5 g fenofibrate: 17.5 g cholesterol: 60 g EPC). Figure 16 illustrates the size (diameter) and size distribution characterization of liposomes at different concentrations. The figure shows a clear increase in size and polydispersity after 6.5% FF, which indicates that the 4% FF concentration may be considered as the optimal concentration due to its size and dispersity range.



a)



b)

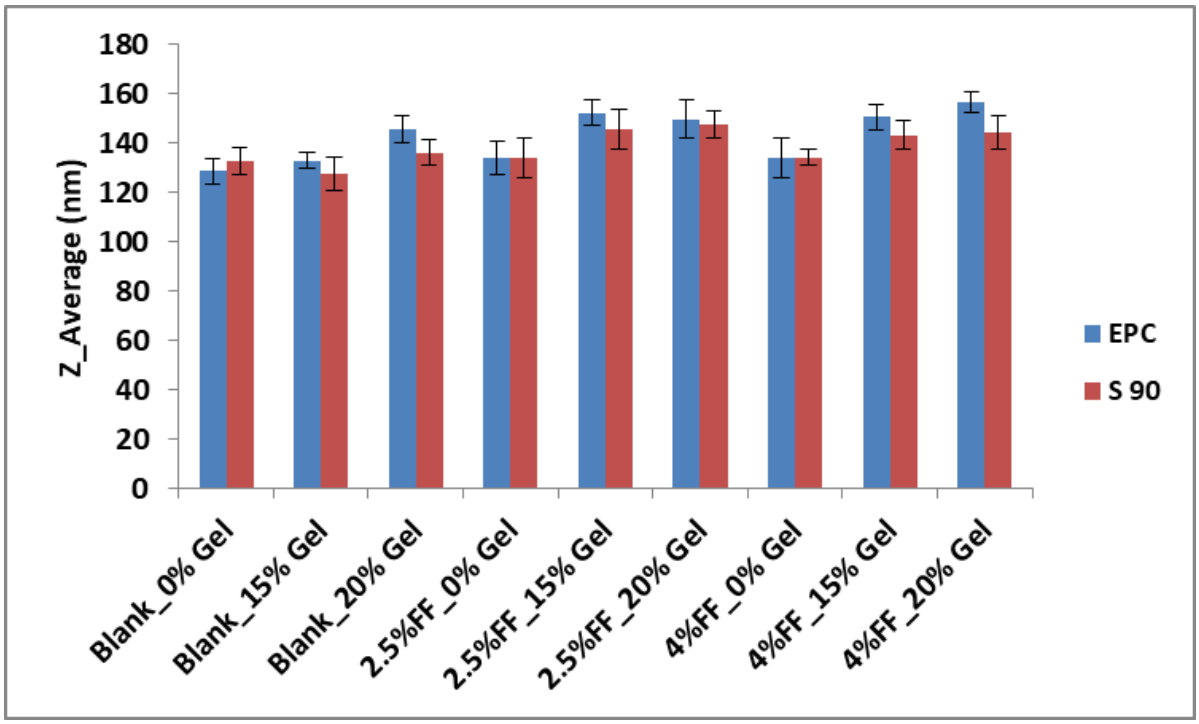
Figure 16. Characterization of liposomal particles in terms of a) size (diameter) and b) size distribution (n=3, mean ± SD).

3.2 Particle Size and Morphology

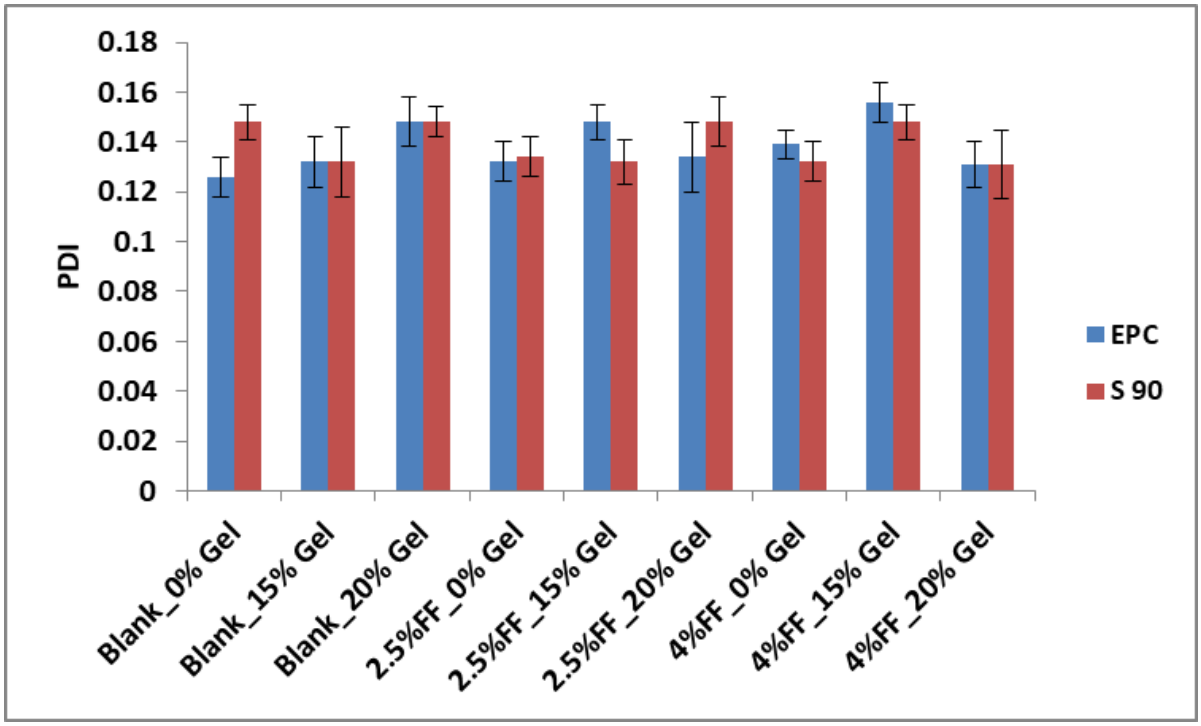
Liposomes were prepared using DAC according to Table 10. The samples were divided into two groups: the first group used EPC as the phosphatidylcholine while the second group used Soluthin S 90. Some samples contained gelatine (which it is believed to increase the viscosity outside and inside the liposomes) to evaluate its effect on the stability of liposomes. In the preparation method, different concentrations of gelatine were added to the PBS buffer. The size and size distribution of the prepared liposomes were characterized using a Nano ZS Zetasizer. These characterized formulations were also used to determine the entrapment efficiency, Cryo_EM, in the *in vitro* lipolysis study.

Table 10. The list of the prepared liposomes contents

Samples	EPC/S90 Concentration (gr)	Cholesterol Concentration(gr)	Fenofibrate Concentration(gr)	Gelatine In D-PBS
Blank_0% Gel	60	40	0	0 µg/1 mL PBS
Blank_15% Gel	60	40	0	150 µg/1 mL PBS
Blank_20% Gel	60	40	0	200 µg/1 mL PBS
2.5FF_0% Gel	60	37.5	2.5	0 µg/1 mL PBS
2.5FF_15% Gel	60	37.5	2.5	150 µg/1 mL PBS
2.5FF_20% Gel	60	37.5	2.5	200 µg/1 mL PBS
4FF_0% Gel	60	36	4	0 µg/1 mL PBS
4FF_15% Gel	60	36	4	150 µg/1 mL PBS
4FF_20% Gel	60	36	4	200 µg/1 mL PBS



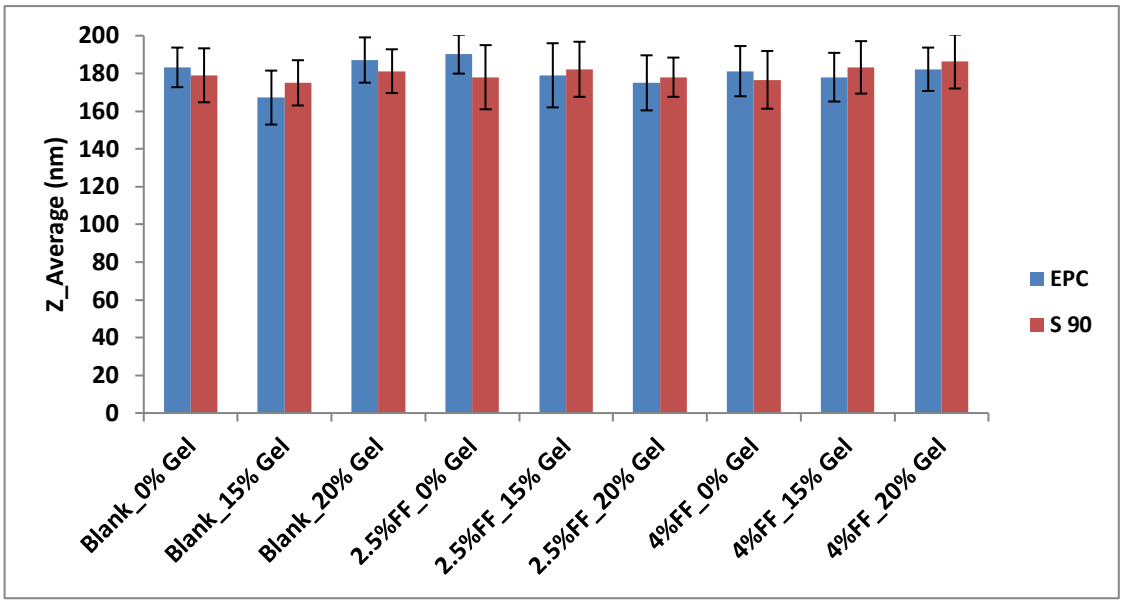
a)



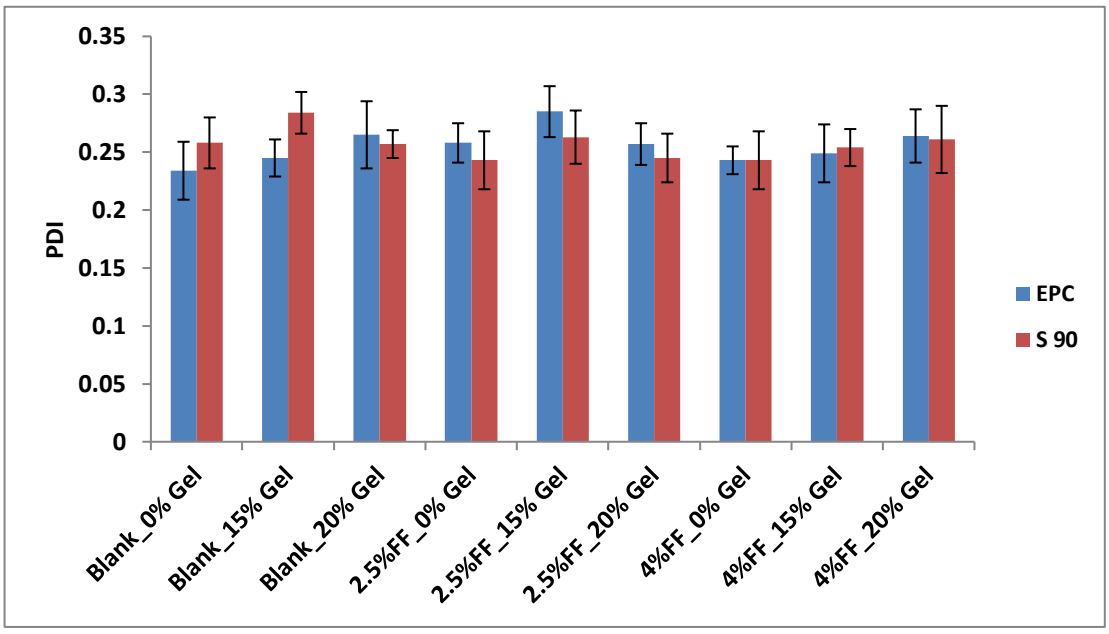
b)

Figure 17. The size (a) and PDI (b) of liposomes prepared by DAC (n=3, mean ± SD).

Figure 17 shows an acceptable size range of 127–156 nm of liposomes produced using either EPC or S 90. In the case of polydispersity, the resulting liposomes indicate a homogenous system as the reported PDI is always less than 0.156 (Schubert & Müller-Goymann 2005). It should be noted that both EPC and S 90 liposomes had a relatively similar range of size and distribution (considering the standard deviation). In a parallel study, probe-sonicated liposomes were characterized in terms of size and size distribution. Figure 18 illustrates the range of size and PDI of liposomes prepared by sonication method that was considered acceptable (167–190 nm for size and 0.234–0.285 for PDI) for further use.



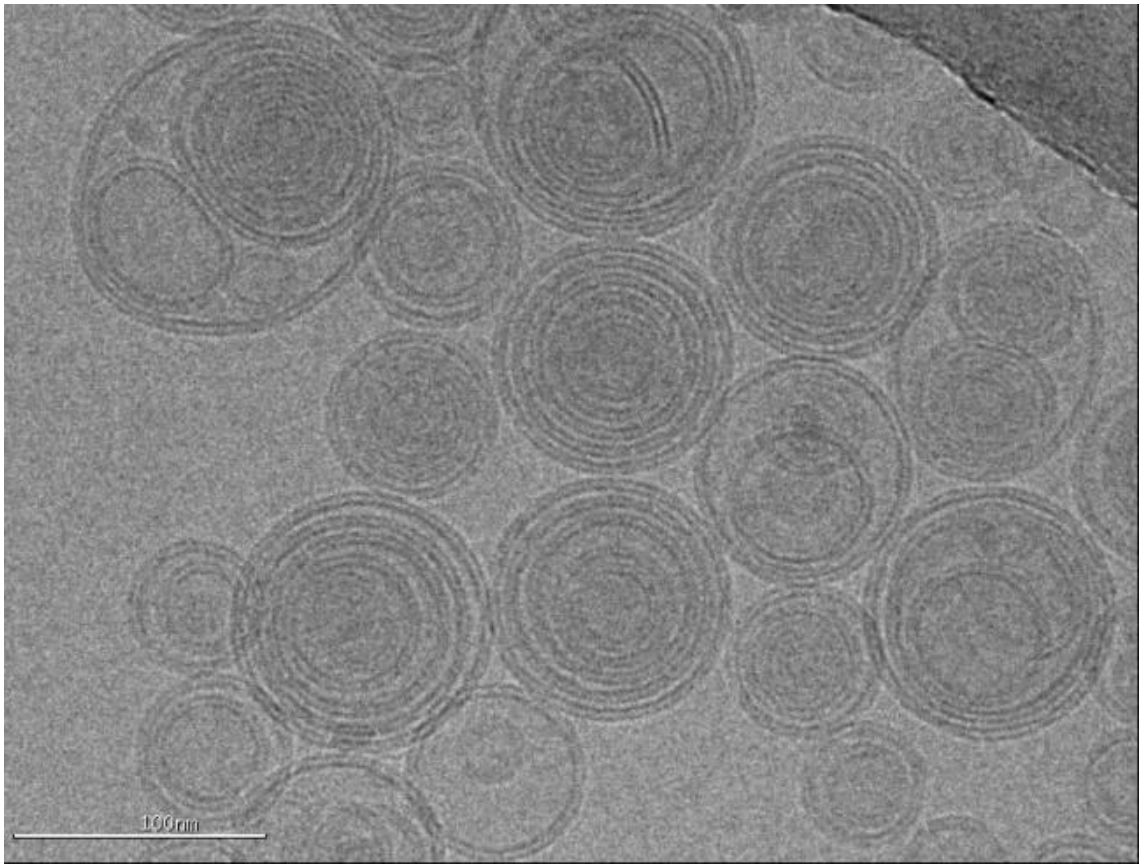
a)



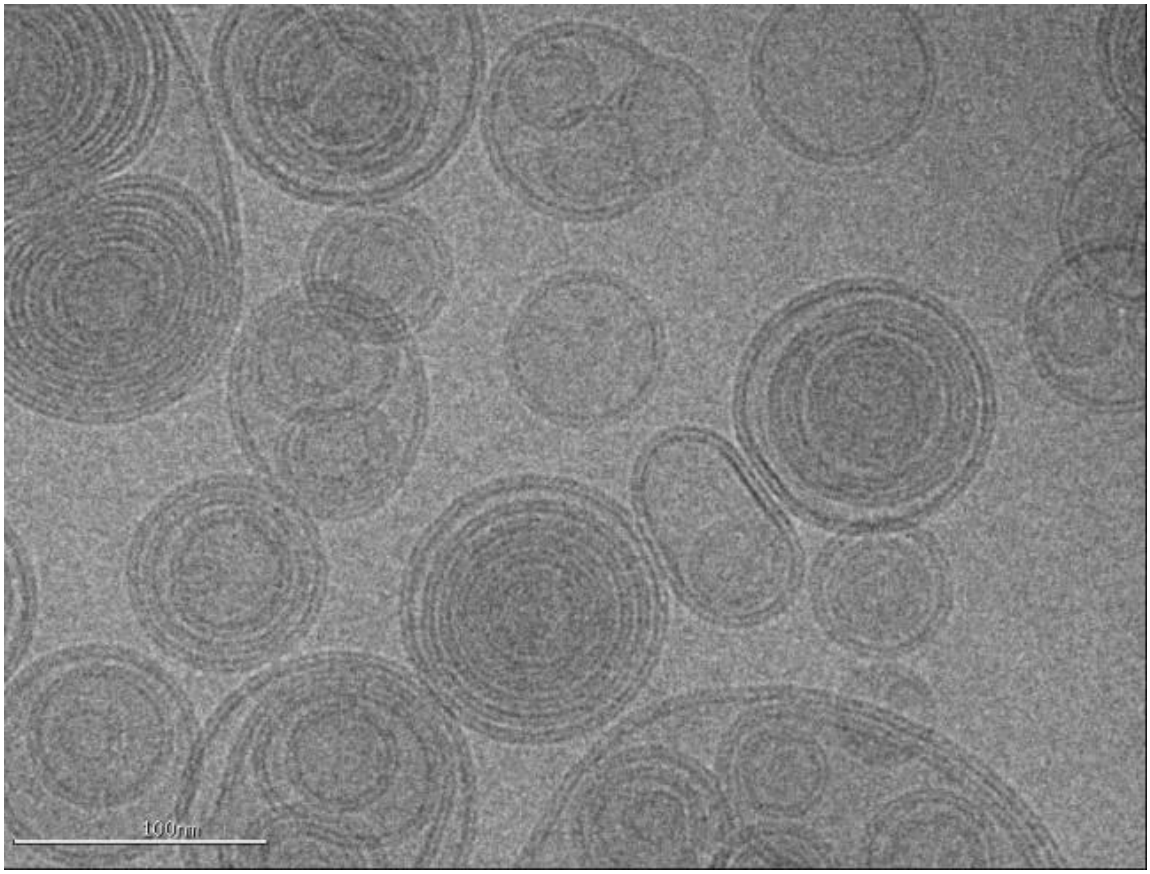
b)

Figure 18. The size and PDI liposomes prepared by probe sonication (n=3, mean ± SD).

Based on these results, DAC was selected as the optimal preparation method for liposomes, with probe sonication designated as an alternative method in case of speedmixer access restriction. The characterization of the physical features of liposomes was followed by Cryo-EM experiments to obtain more precise morphological information on the stability of liposomes formed in the presence and absence of gelatine.



a)



b)

Figure 19. Cryo_EM images of the liposomal samples a) without gelatine, b) matrix liposomes containing gelatine.

Cryo-EM was employed to observe the morphology of liposomes prepared in either PBS (control) or gelatine dissolved in PBS as shown in Figure 19. Cryo-electron micrographs showed that the liposomes were mainly multilammellar and similar in size and shape, independent of whether they were prepared in the presence or absence of gelatine. Therefore, according to the images obtained by Cryo-EM, it was concluded that gelatine did not have an effect on the size or shape of vesicular liposomes and, consequently, that it might not have a strong effect on the stability or bioavailability of the samples. However, Cryo-EM might not have been the most suitable approach to determine the effect of gelatine on liposomal formulation. Further *in vitro* experiments followed by *in vivo*

pharmacokinetic studies were therefore needed to evaluate the Cryo-EM images for supplementary experimental results.

3.3 Determination of Entrapment Efficiency

Entrapment efficiency (%) of the sonicated liposomes was determined (Table 11) for liposomes loaded either with EPC or S 90.

Table 11. Entrapment efficiency (%) and loading capacity (%) (n=3, mean ± SD).

Sample	%EE EPC_liposomes	%EE Soluthin S 90_liposomes
2.5% w/w FF_ 0% Gelatine	97.42± 1.07	98.43± 1.76
2.5% w/w FF_ 15% Gelatine	97.21± 2.11	96.42± 1.98
2.5% w/w FF_ 20% Gelatine	95.35± 1.72	95.98± 2.32
4% w/w FF_ 0% Gelatine	98.12± 1.9	99.04± 1.03
4% w/w FF_ 15% Gelatine	95.15± 2.05	97.32± 1.93
4% w/w FF_ 20% Gelatine	96.41± 2.14	97.06± 2.06

As shown in Table 11, the %EE decreased as the concentration of gelatine in the liposome matrix increased, however, this did not result in significant difference in entrapment efficiency between the liposomes produced from EPC and S 90 (considering the standard deviations). The entrapment efficiencies were markedly increased, representing a high level of fenofibrate encapsulation in the lipid layer. Fenofibrate has a very high affinity towards the lipid phase because of its high lipophilicity and this was expected for the poorly water-soluble drug e.g., fenofibrate. During liposome preparation, fenofibrate is incorporated within the lipid film, and subsequently in the lipid layer of liposomes, resulting in a very low amount of fenofibrate in the aqueous phase. The high affinity towards the lipid phase has also been observed for other lipophilic drugs (Sharma et al. 2010; Ekambaram & Sathali 2011).

3.4 X-ray Powder Diffraction

An assessment of the physical states of prepared liposomes was carried out XRPD to determine crystal formation. An XRPD peak indicates the crystalline nature at a particular value in the 2θ range. The XRPD patterns of crystalline fenofibrate and of blank and fenofibrate-loaded liposomes (various concentrations) are shown in Figure 20. In this study, the pure drug fenofibrate showed several characteristic reflections of crystalline fenofibrate. The size and sharpness of XRPD peaks are primarily dependent on crystal size. The pattern of crystalline fenofibrate was in accordance with that reported previously (de Waard et al. 2010). For liposomes, no crystal pattern was detected, even at high concentrations.

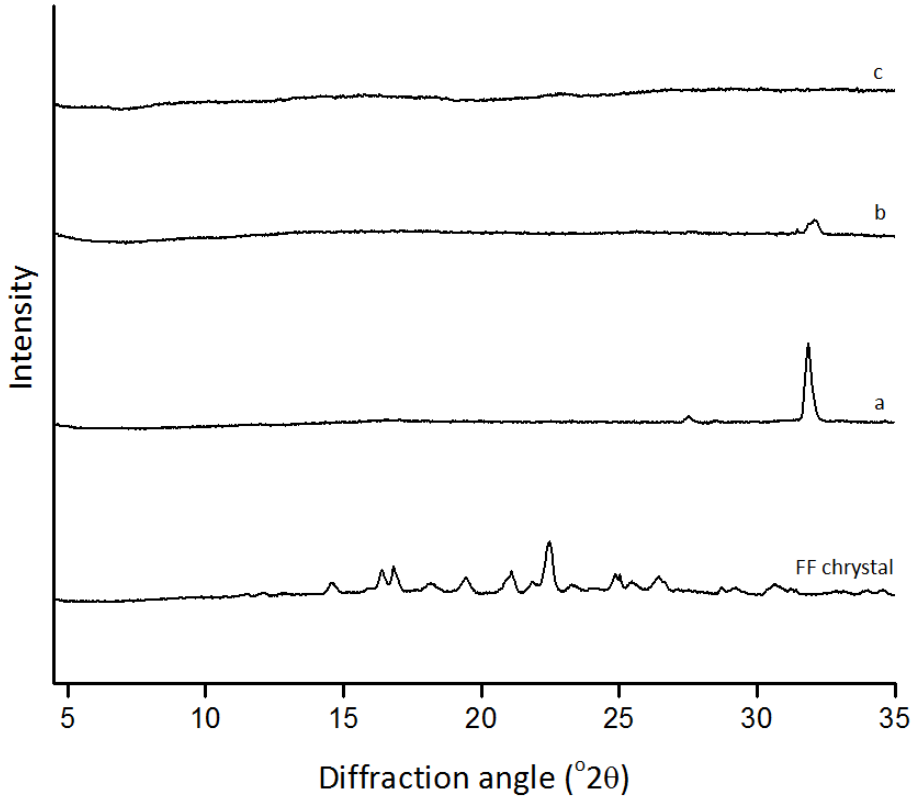


Figure 20. X-ray powder diffraction patterns of fenofibrate loaded liposomes containing a) 0% fenofibrate (Blank sample), b) 4% fenofibrate, c) 20% fenofibrate, and crystalline fenofibrate.

This indicates the existence of the amorphous form of fenofibrate or even molecular dispersion, which has faster dissolution than the crystalline form (Khan et al. 2016). As per liposomes with high concentration of fenofibrate, no crystalline was formed to reduce the rate of drug dissolution. Nevertheless, taking the results of Section 3.1 (Figure 16) into consideration, it was not possible for liposome preparation to go higher than 4% concentration due to the appearance of deformation (bigger size and higher PDI) after the desired concentration.

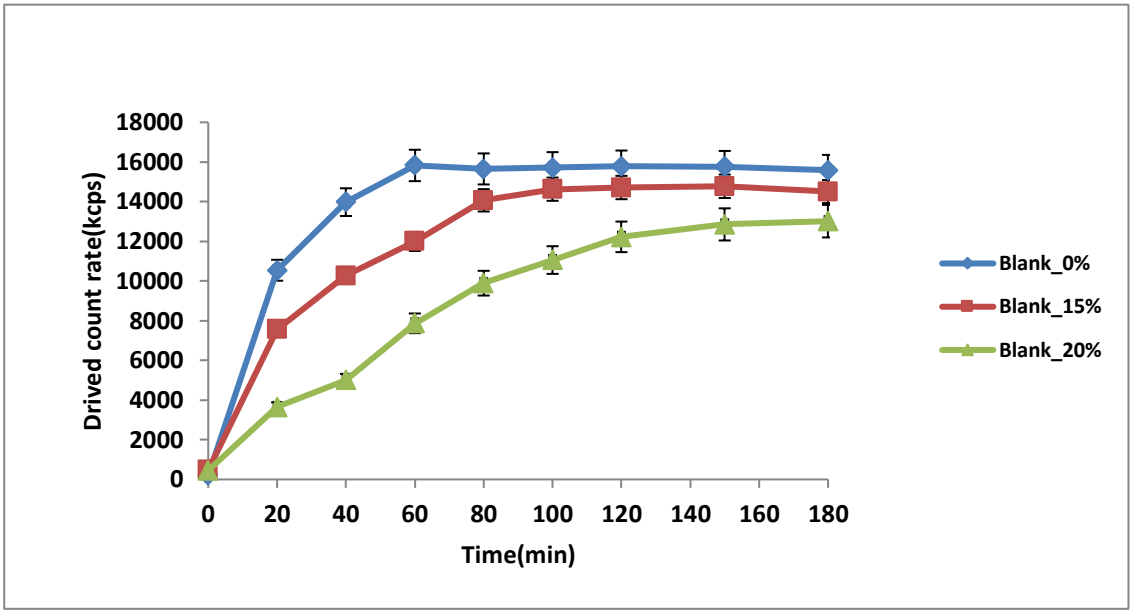
3.5 Dissolution

In most research settings, the dissolution apparatus is widely used to model *in vitro* drug digestion and release. The dissolution profiles of EPC-liposomes prepared by DAC were evaluated, and are summarized in this section. For this purpose, the USP II (paddle) apparatus was used as a compendial method for dissolution testing, following the protocols from the pharmacopoeias (The United States Pharmacopeia and National Formulary 2014b). To simulate the gastrointestinal environment, three types of media with different properties were used: Simulated Intestinal Fluid (SIF), Fasted-State Simulated Intestinal (FaSSIF, pH 6.5) and Fasted-State Simulated Gastric Fluid (FaSSGF). The techniques used to evaluate the quality of liposomes were different, and were selected according to the objective of each experiment. Furthermore, the light-scattering method was considered the optimal approach to assess the physical states of particles in term of size (diameter) and polydispersity.

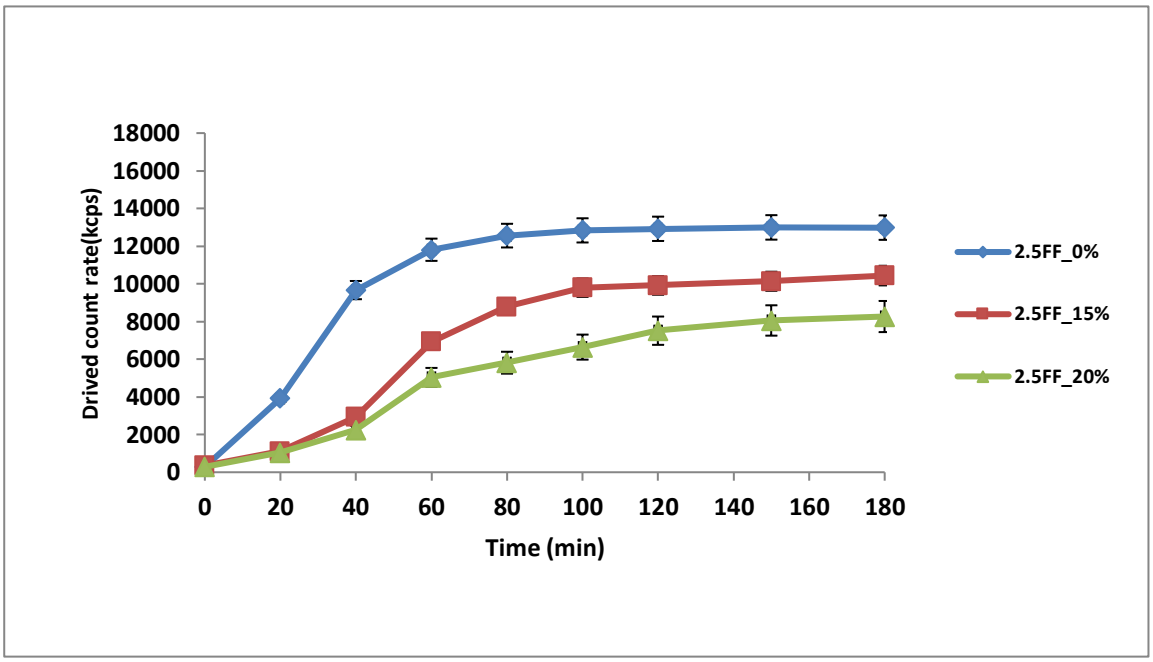
3.5.1 Liposome release

The dissolution behaviour of 100 mg of solidified vesicular phospholipid gels (VPGs) from DAC was evaluated using a paddle dissolution apparatus. For this purpose, SIF was used as the dissolution medium and EPC was the phosphatidylcholine used to prepare the liposomes. The physical states of collected samples were assessed by DLS using a Zetasizer Nano ZS. The derived count rate among the Malvern parameters is an effective parameter to track the concentration of liposomes converting from the semisolid formulation into free liposomes. Since the amount of scattered light is related to the size and number of particles present in a sample, the derived count rates or absolute light scattering in kilo counts per second (kcps) may represent the presence of

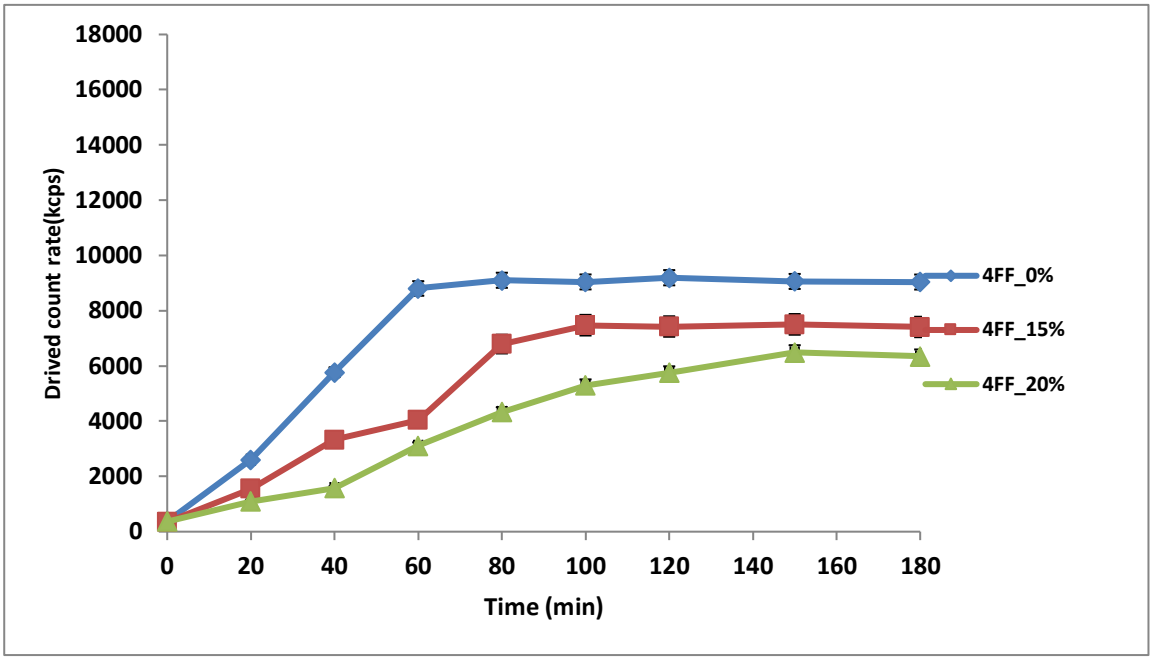
nanoparticles in the sample (Wallace et al. 2012). Figure 21 illustrates the release of liposomes from different concentration of the semisolid formulation.



a)



b)



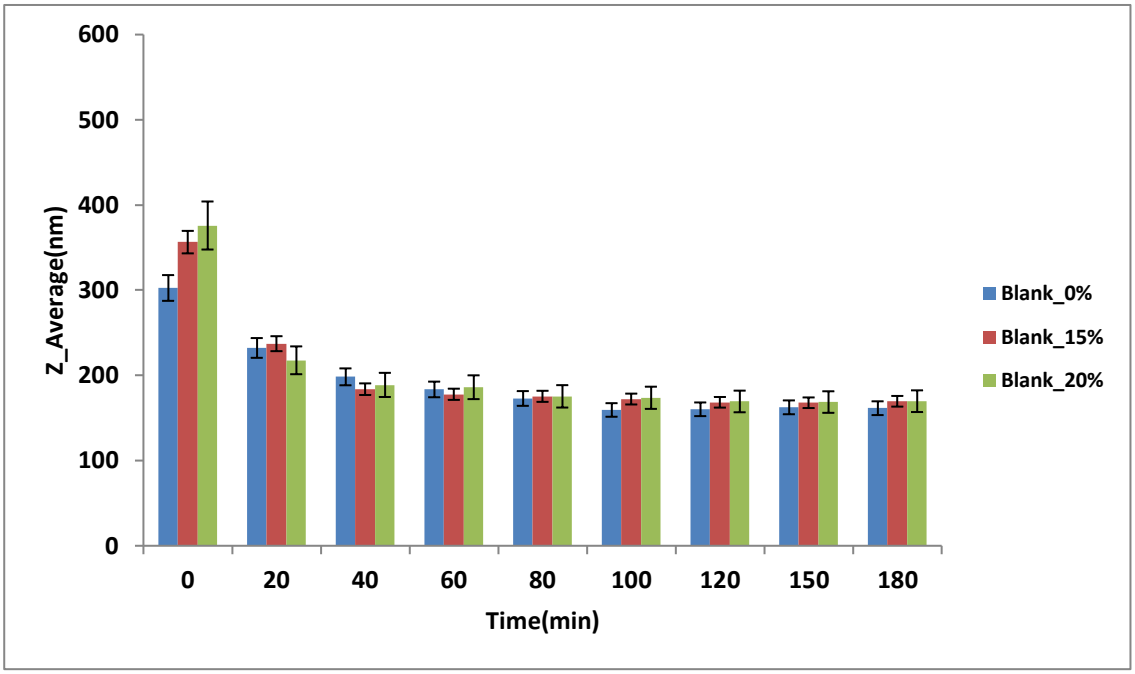
c)

Figure 21. Dissolution behaviour of EPC- liposomes containing different concentration of gelatine in simulated intestinal fluid pH 6.8 and 37 °C for blank sample (a), 2.5%FF (b), and 4%FF (c). Scattering intensities (in a correlation with particle concentration) were measured with a Zetasizer instrument and are expressed as derived count rate (kcps). Each data point is the mean ± SD of three independent experiments.

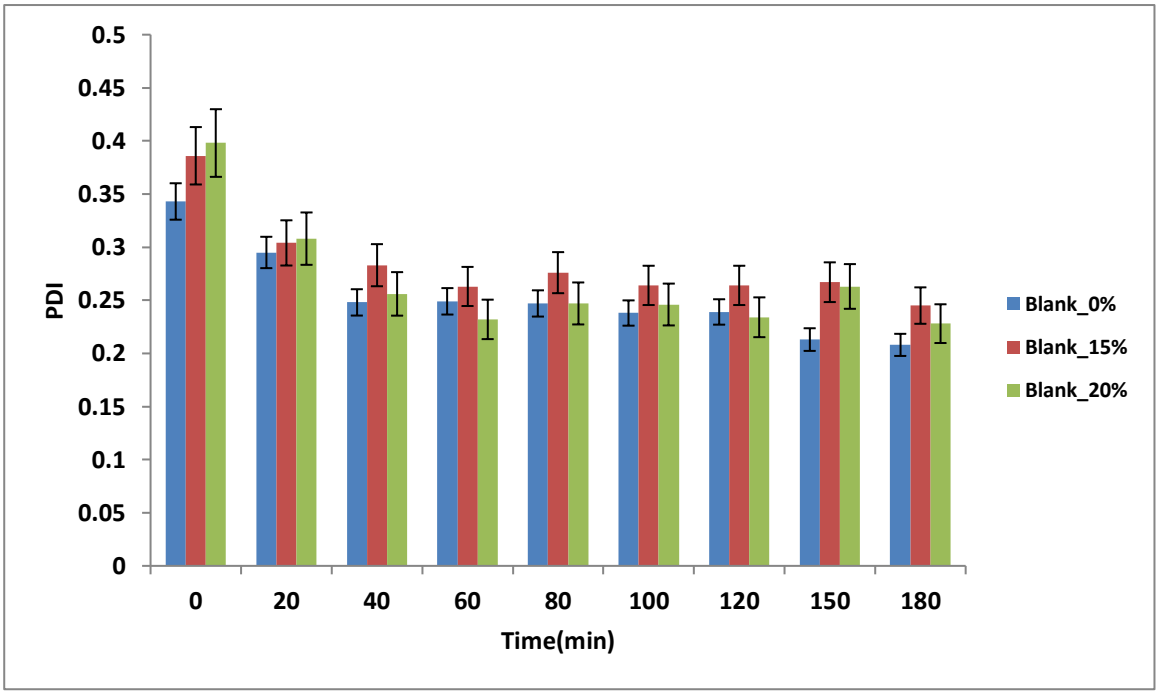
As shown in Figure 21, the amount of liposome released from the solidified VPGs increased over time. A negative correlation was detected between the rate of liposome release and gelatine concentration. The more gelatine that was incorporated into the liposomes, the lower the speed of dissolution a specific time point.

Liposomes prepared without gelatine were completely dissolved in the media, while for formulations containing 15% gelatine, a constant quantity of liposomes was observed after 100 min, indicating the capacity of the desired semi-solid formulation to release liposomes. The solidified formulation containing 20% gelatine consistently dissolved in SIF within 150 minutes, and thus had the potential for use as a sustained-release formulation. By comparing the level of the derived count rate for different concentrations of gelatine from Figure 21, gelatine can be taken as occupying some space in the solidified matrix liposomes as the initial concentration was identical for all prepared samples. In addition, the size (diameter) and polydispersity index of liposomes released

from the solidified dosage form were evaluated. Figure 22 illustrates the physical states of the examined liposomes.



a)



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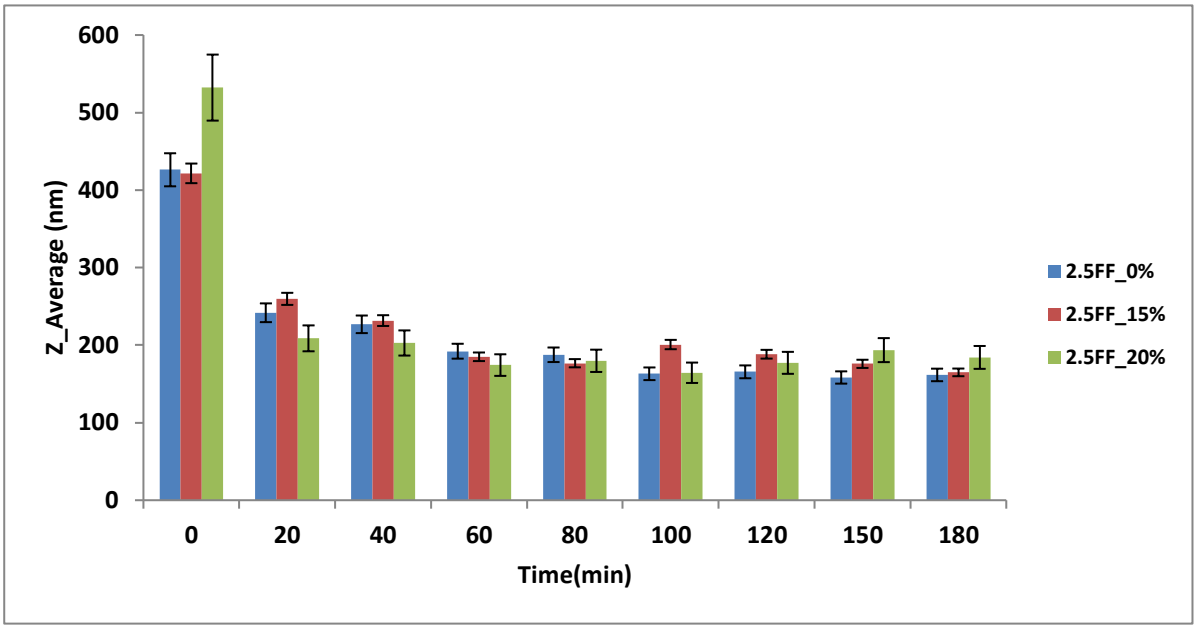
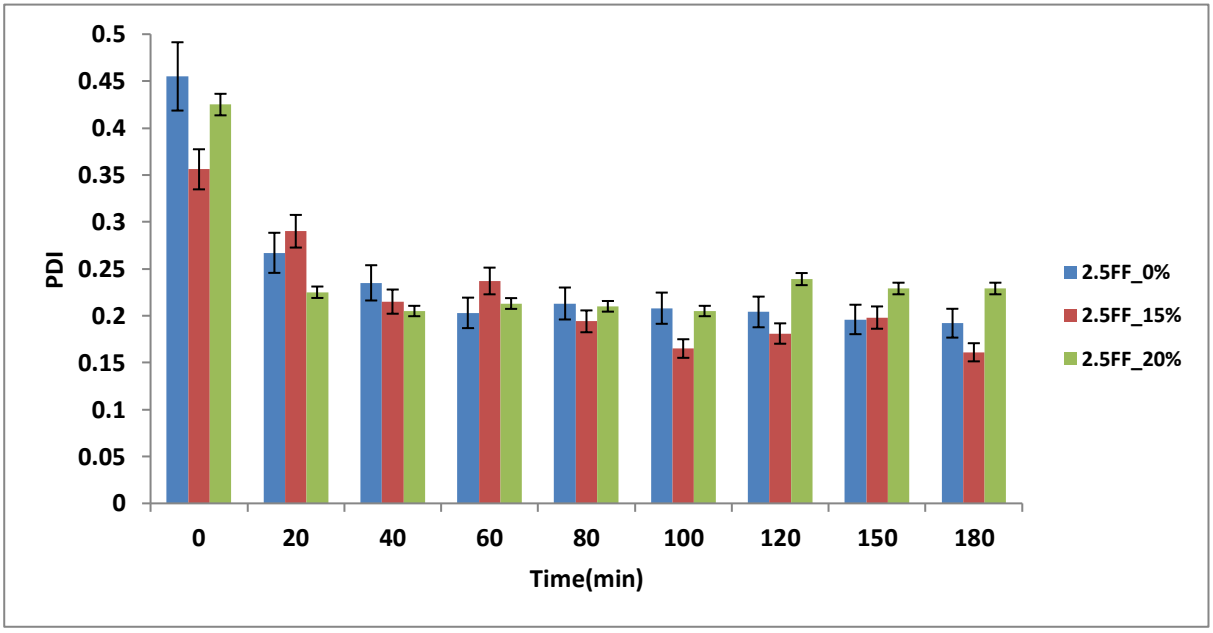

600
500
400
300
200
100
0
Z_Average (nm)
0
20
40
60
80
100
120
150
180
Time(min)
2.5FF_0%
2.5FF_15%
2.5FF_20%

c)


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PDI
0
20
40
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80
100
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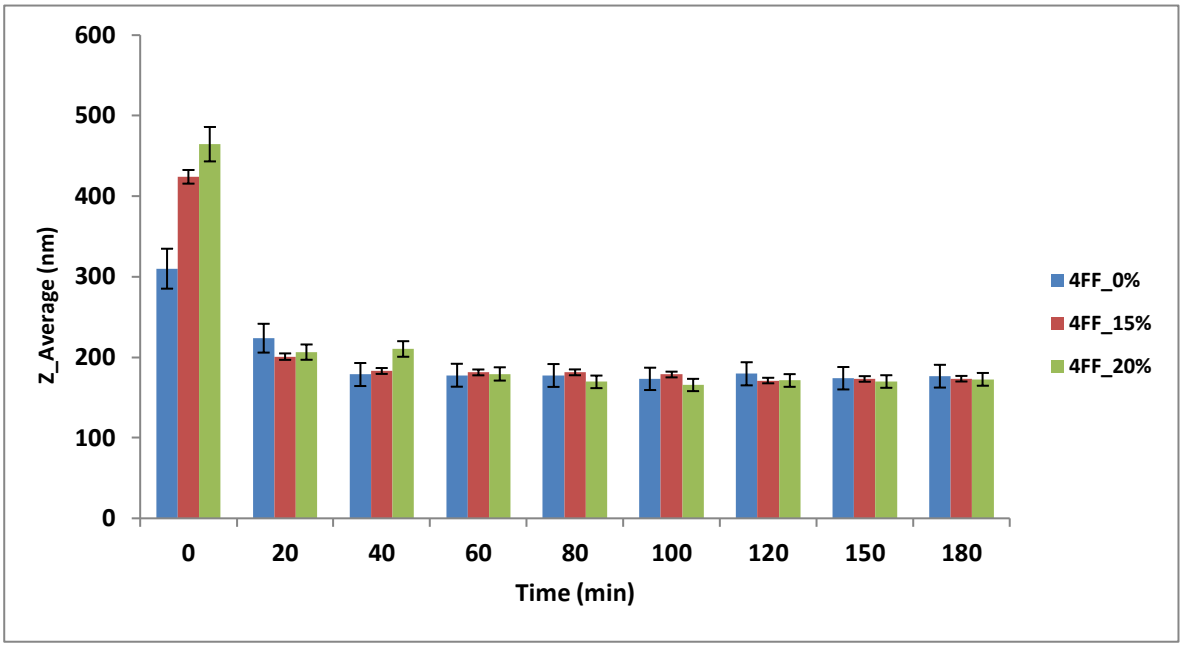
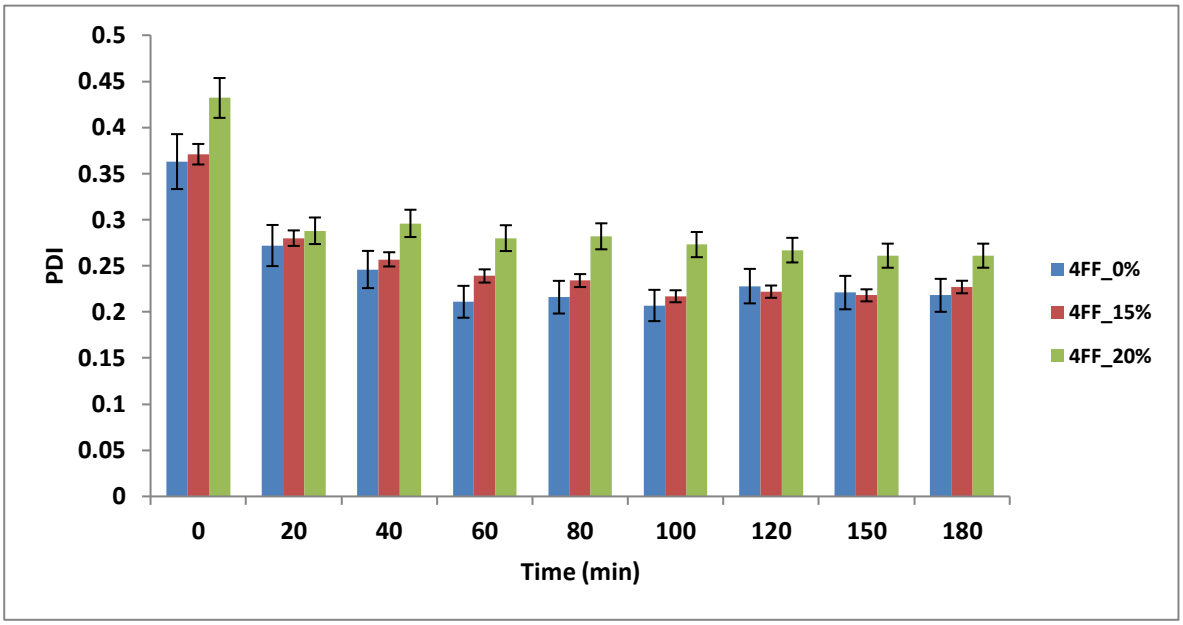
Time(min)
2.5FF_0%
2.5FF_15%
2.5FF_20%

d)



e)



f)

Figure 22. Size and poly dispersity index (PDI) of EPC-liposomes containing different concentration of gelatine in simulated intestinal fluid pH 6.8 and 37 °C for blank sample (a, b), 2.5%FF (c, d), and 4%FF (e, f). (n=3, mean ± SD).

The size of particles detected by DLS decreased over time and reached approximately 200 nm in the first 20 minutes. After 40 minutes of dissolution, samples were stable in size, ranging from 169–210 nm. In the case of polydispersity, samples were also stable after 40 minutes and were consistently lower than 0.3, indicating homogenous and within-range liposomal vesicles.

3.5.2 Drug release

A stability study of fenofibrate-loaded EPC liposomes prepared by DAC was carried out in FaSSIF (pH 6.5) and FaSSGF (pH 1.6) biorelevant media. FaSSIF medium was used to simulate upper small intestinal conditions while FaSSGF medium was used to represent fasting conditions in the stomach.

Dissolution profiles obtained in FaSSIF using the USP paddle apparatus are shown in Figure 23. The percentage of fenofibrate released from the formulation is marked by dots in different colours. Each set of experiments was run for 3 hours, and the quantity of drug released from liposomes containing 145 mg fenofibrate was determined.

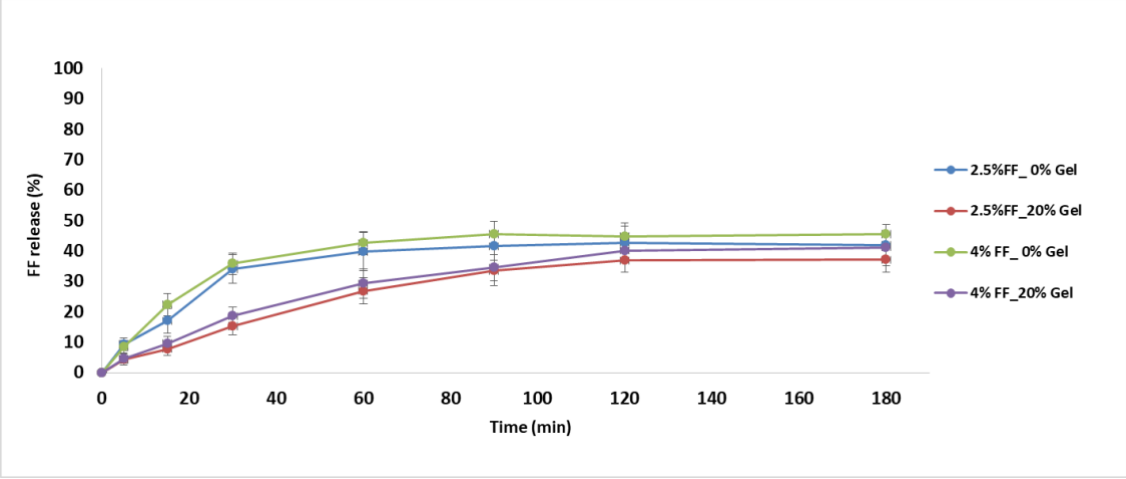
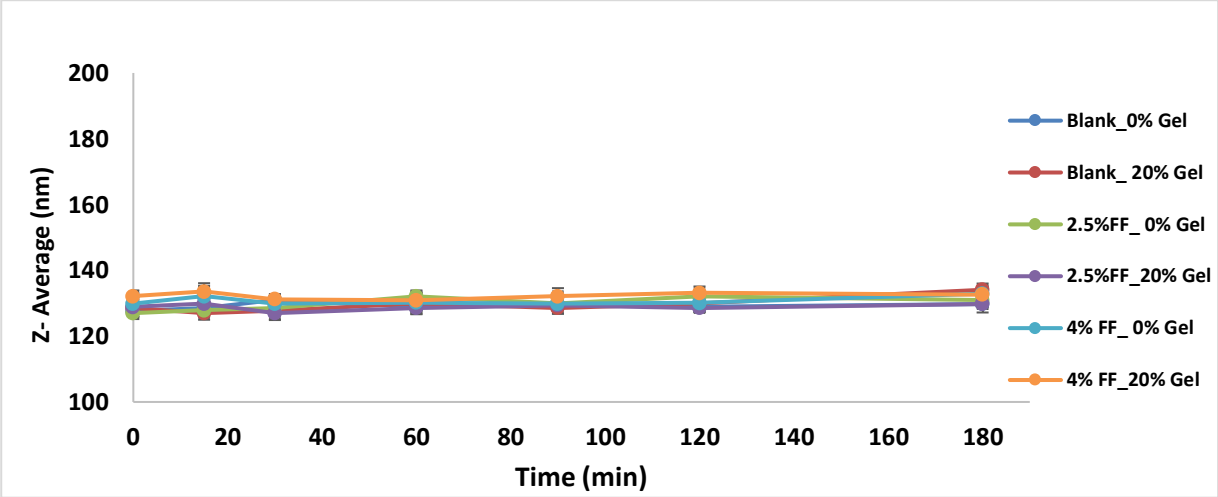


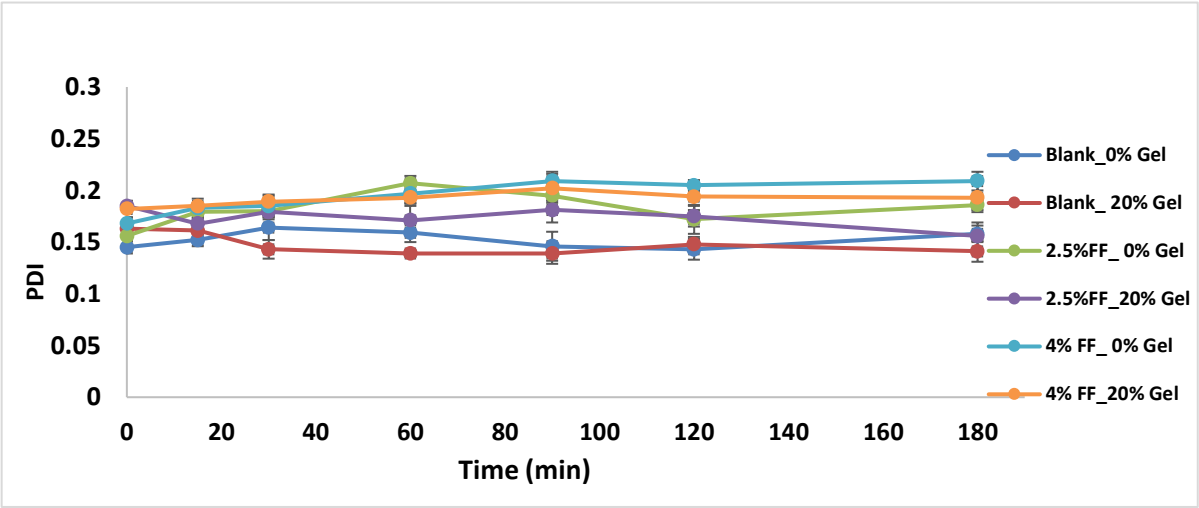
Figure 23. Mean dissolution profiles ± SD (n=3) of different concentrations of EPC-liposomes in Fasted-State Simulated Intestinal Fluid (FaSSIF, pH 6.5), using the USP paddle apparatus.

The results show clear differences in the dissolution profiles obtained for samples prepared in the absence or presence of gelatine. It was observed that around 40% of fenofibrate was released from EPC liposomes with different concentrations after 180 min. Figure 23 clearly depicts a steeper slope for the release of drug from liposomes without gelatine (regardless of the loading concentration), while liposomes containing gelatine showed linear dissolution profiles, representing a slower rate of release. The dissolution profile for non-gelatine-containing formulations was faster, with more than 80% of drug dissolved within the first 30 min. A statistically significant difference ($p < 0.05$) was observed between the amounts of fenofibrate dissolved after 30 min from gelatine-based liposomal formulations compared with non-gelatine formulations, although all had released the same amount of drug after 2 hours. Liposomes containing the same quantity of gelatine showed the same drug release profile over time.

Furthermore, the physical states of formulations were evaluated to obtain a better understanding of the fate of liposomes in the intestinal. The size and polydispersity of the tested formulations is shown in Figure 24.



a)



b)

Figure 24. Size (diameter) (a) and polydispersity index (b) of different concentrations of EPC-liposomes in Fasted-State Simulated Intestinal Fluid (FaSSIF, pH 6.5), using the USP paddle apparatus. (n=3, mean ± SD).

When the physical states of fenofibrate-loaded liposomes were measured in FaSSIF (pH 6.5) biorelevant medium, the formulations clearly showed relatively stability with respect to size, regardless of the loading concentration. This part of the study on dynamic particle size variation showed a PDI of less than 0.22, which implies to be rather in range and stable size distribution. These results represent the ability of formulations to maintain their integrity in simulated conditions of the upper small intestinal.

The harsh, acidic environment of the stomach was simulated by exposing the prepared formulations to FaSSGF (pH 1.6) in a paddle apparatus model. The dissolution profiles of the tested samples are illustrated in Figure 25.

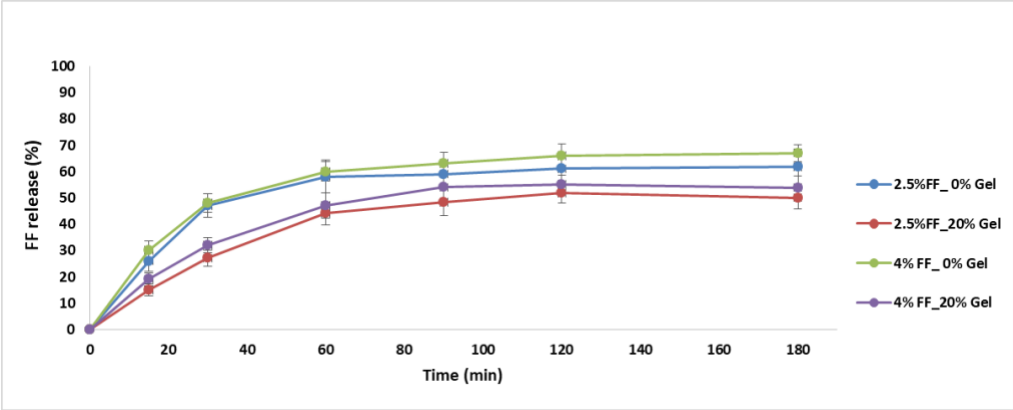
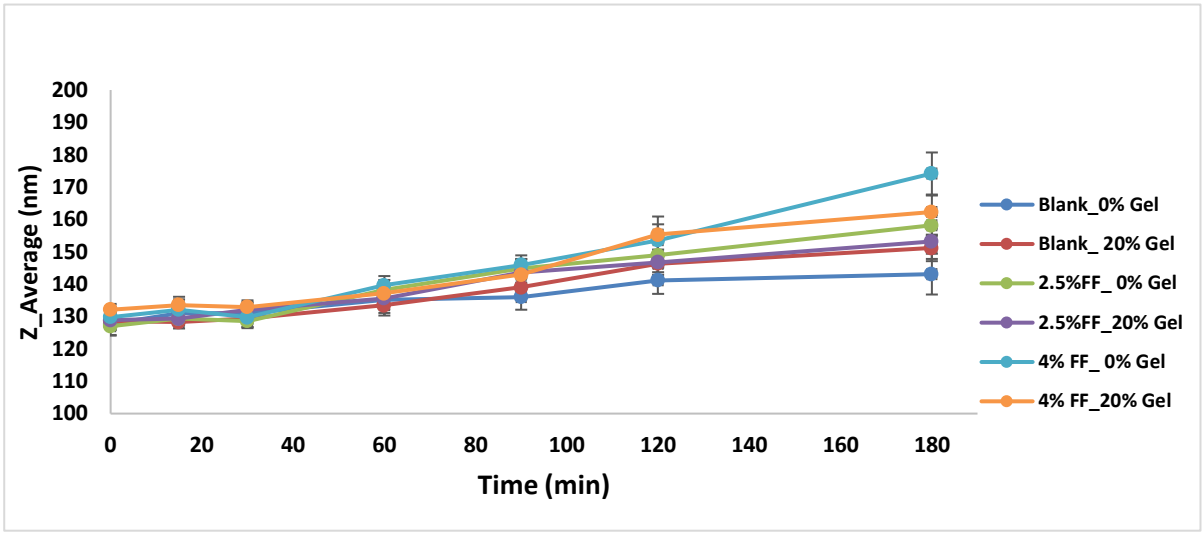
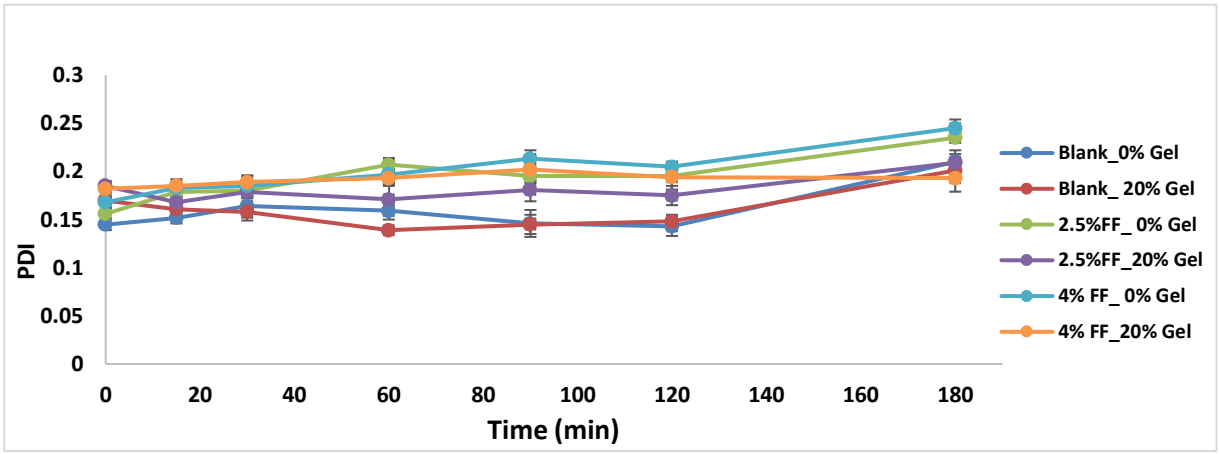


Figure 25. Dissolution profiles of different concentrations of EPC-liposomes in Fasted-State Simulated Gastric Fluid (FaSSGF, pH1.6). (n=3, mean ± SD).

Dissolution results using FaSSGF as the dissolution medium show clear differences in the amount of drug released from the samples compared with the dissolution data using FaSSIF as the dissolution medium. However, the liposomal formulations prepared in the absence and presence of gelatine showed approximately similar dissolution profiles for the release of fenofibrate, as formulations without gelatine released their incorporated drug at a faster rate than those containing gelatine. Nevertheless, it is clear from Figure 25 that the percentage of drug released from the formulations prepared without gelatine was relatively higher, ranging from 50% to 70%. To obtain a better overview of the fate of liposomes, dynamic light scattering was used to evaluate the physical features of particles following their exposure to the acidic FaSSGF medium. The size and PDI of FaSSGF-exposed liposomes are shown in Figure 26.



a)



b)

Figure 26. Size (a) and Polydispersity index (b) of different concentrations of EPC-liposomes in Fasted-State Simulated Gastric Fluid (FaSSIF, pH 6.5), using the USP paddle apparatus. (n=3, mean ± SD).

As Figure 26 illustrates, the particle size in FaSSGF medium clearly increased for all of the DAC-prepared formulations over time, as did the PDI. From the dynamic particle size, as well as by increasing the amount of released drug in the dissolution profile in FaSSGF, it was suggested that the harsh, acidic conditions of FaSSGF medium might lead to the disruption of liposomal features.

By increasing the size of particles, aggregation in the vesicular liposomes was demonstrated, while fenofibrate – as the incorporated drug – is released from the formulations.

The dissolution data for the prepared formulations in different biorelevant media (FaSSIF and FaSSGF) provided some general information about the fate of liposomes. In both media, non-gelatine-containing formulations released fenofibrate faster, with approximately 80% released after the first 30 minutes and more than 95% of drug released after the first hour, whereas gelatine-containing formulations released more than 95% of fenofibrate after 2 hours.

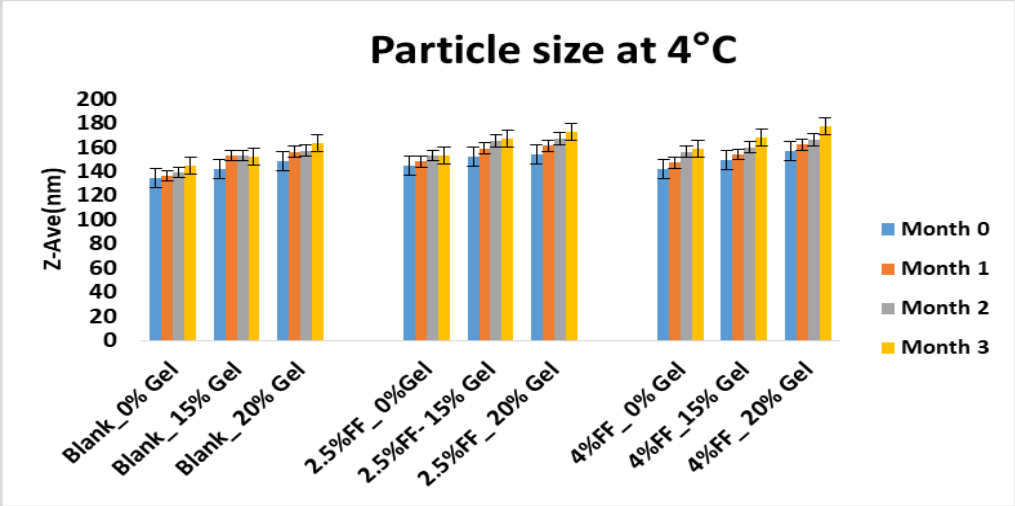
A comparison of the results from the last hour of dissolution testing in biorelevant media for gelatine-containing liposomes showed that formulations were relatively more capable of releasing drug if they had been prepared with a higher fenofibrate loading concentration. Moreover, after comparing the behaviour of gelatine and non-gelatine samples, it could be concluded that gelatine functions as a limiting factor for liposomes to release the incorporated drug at a constant rate, and this could be related to the ability of gelatine to increase the density inside and outside the layers of vesicular liposomes. This finding represents an advantage of gelatine-containing liposomes for use in sustained-release formulations.

The results from the dissolution studies in biorelevant media showed that, for the last 90 minutes of the experiment, all formulations released the same amount of drug, and it is clear from the slope of the curves shown in Figure 23 and Figure 25 that all of the samples did not release a significant quantity of drug from the formulations for this period of time.

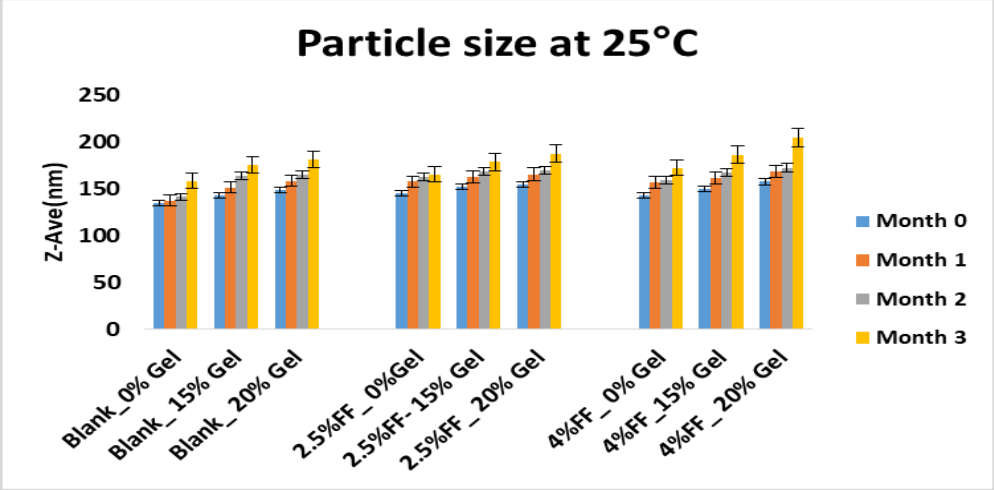
In FaSSGF medium, the particle size increased for all formulations over time, as did the PDI, whereas in FaSSIF medium, both remained relatively constant, suggesting that the liposomes were more stable in an intestinal environment compared with the low pH conditions of gastric fluid.

3.6 Storage Stability

To investigate the physical state of fenofibrate-loaded liposomes over time, a DLS was performed for selected formulations. Figure 27 represents the stability of EPC liposomes prepared by DAC in terms of size and PDI over 3 months.



a)



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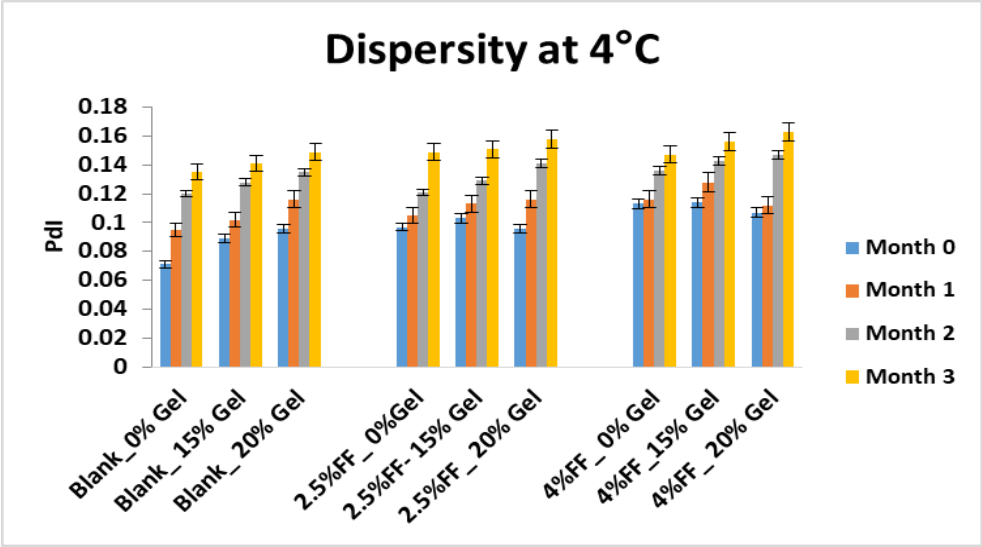

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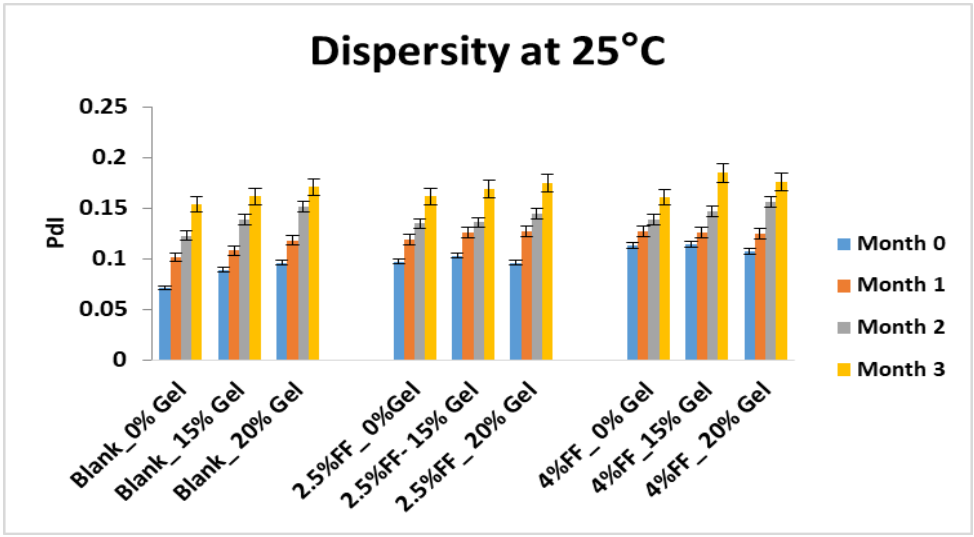


d)

Figure 27. Stability of different samples in size (a and b) and dispersity (c and d) at 4 and 25 °C, respectively.

All preparations showed acceptable stability over 3 months. The size and polydispersity of the liposomes increased slightly in this period, and a constant increase in size of liposomes prepared without gelatine compared with liposomes prepared using gelatine was observed (Figure 27). An

insignificant ($p>0.05$) increase in particle size and distribution was detected between different concentrations, indicating that the content of the formulation did not affect liposome size.

3.7 Dynamic *In Vitro* Lipolysis

To study the digestion and solubilisation of drug formulations in biorelevant media simulating the gastrointestinal environment, an *in vitro* lipolysis model with the continuous addition of calcium was used (0.5 mM final concentration of calcium). In this study the *in vitro* lipolysis model based on the method described by Zangenberg et al., with some modifications (Zangenberg et al. 2001a; Zangenberg et al. 2001b). The lipolysis system consists of two different compartments: the gastric (pH 4) part and the intestinal (pH 6.5) part, which were connected by a transferring pump. An auto-titrator system was incorporated to ensure lipase activity by maintaining the pH. As described in section 2.10.3, the experiment was run for 1 hour and samples were collected at 5, 15, 30 and 60 mins. The collected samples were subjected to ultracentrifugation at high speeds to overcome separation challenges, resulting in T, A and P samples. The T sample represented a total sample, a mixture of aqueous and oily phases containing released and unreleased sample. To evaluate the accuracy of the developed model and the validity of the analysis method, the amount of T sample from the experiment was compared with the expected amount from a theoretical analysis, and the results are shown in Figure 28.

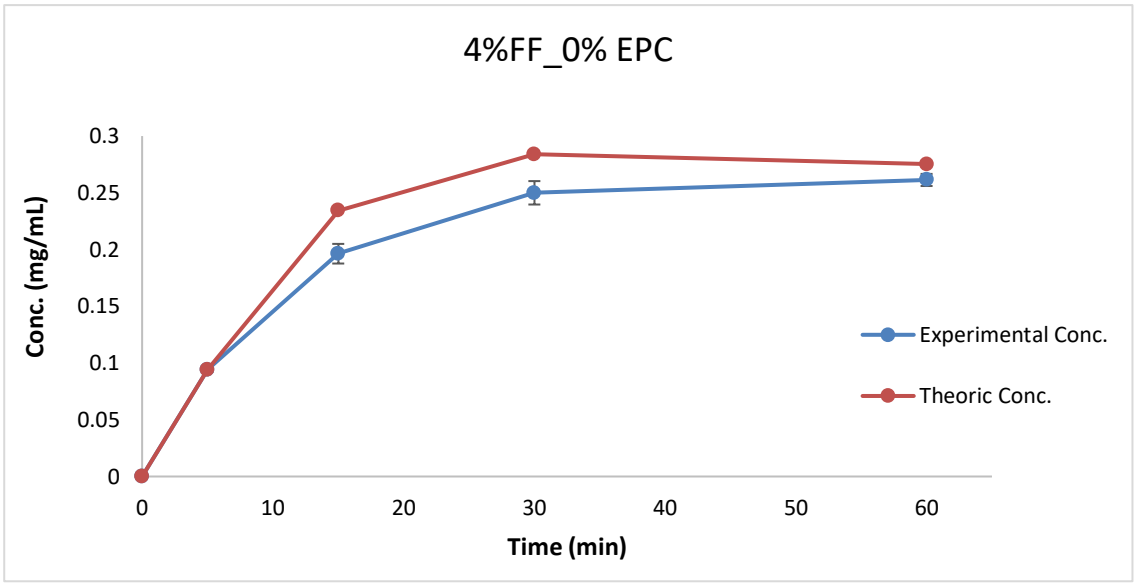
The A (aqueous) sample includes the amount of drug in the aqueous phase, which is the fenofibrate released from the lipid-based formulation and diffused into the aqueous phase of the lipolysis medium. The P (pellet) sample represents the amount of drug detected in the pellet after ultracentrifugation. The pellet contains only the oily phase, and therefore includes undissolved liposomes. The liposomal formulations were prepared with different phosphatidylcholine compositions, namely E-PC, S-PC or Soluthin S 90, both with and without gelatine. The theoretical concentrations were determined according to the initial concentration of drug in the formulation distributed in media, the speed of the transferring pump and the time of sampling. Because a specific amount of drug was removed from the entire volume after each sampling, this amount was also accounted for. Furthermore, the amount of titrating buffer added to the experimental medium led to modifications in the volume, which were also noted. The dynamic particle sizes of prepared formulations are shown in Table 12 and as liposome ranges in terms of size and PDI.

Table 12. Size and distribution of probe sonicated liposomes prepared for lipolysis model (n=3, mean ± SD).

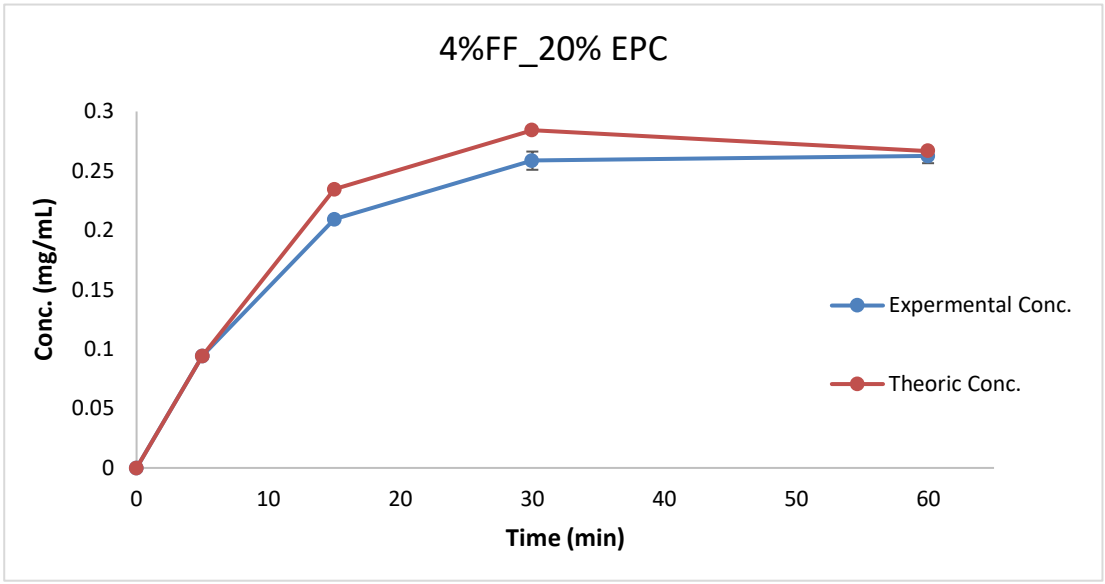
Samples	4%FF_0%_ EPC	4%FF_20%_ EPC	4%FF_0%_ SPC	4%FF_20%_ SPC	4%FF_0%_ S 90	4%FF_20%_ S 90
Size (nm)	162.8± 12.6	173.4±9.2	146.7±12	151.4±9.8	141.9±14.1	144.2±6.6
PDI	0.281±0.016	0.262±0.023	0.282±0.018	0.278±0.023	0.264±0.025	0.251±0.027

3.7.1 Drug recovery

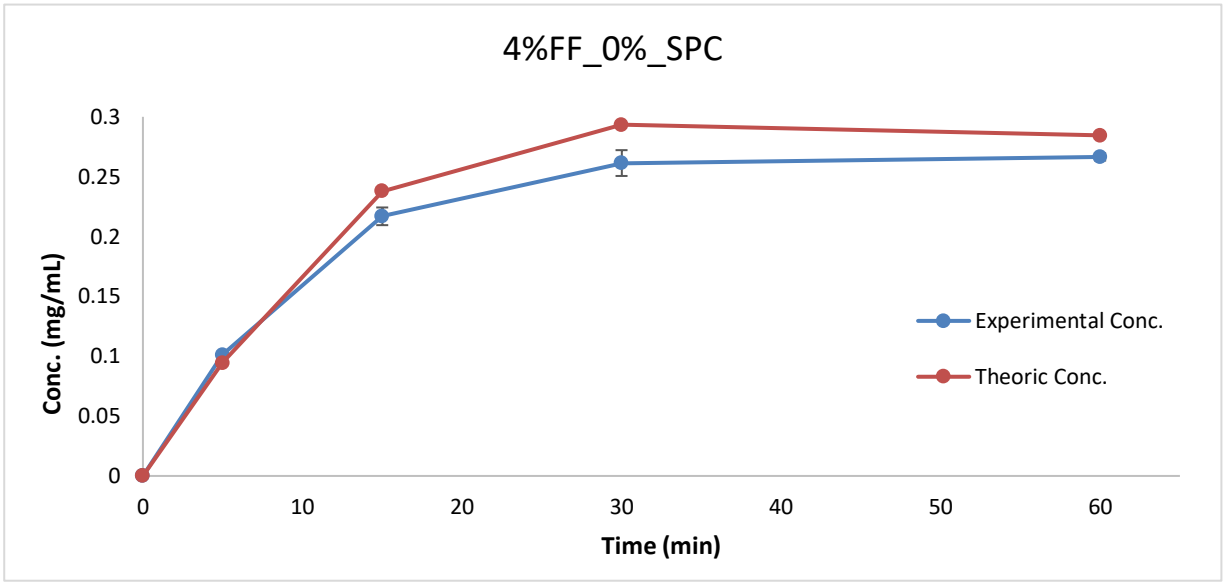
The dissolution experiments were carried out to determine the release of fenofibrate from the respective formulation and shown in Figure 28.



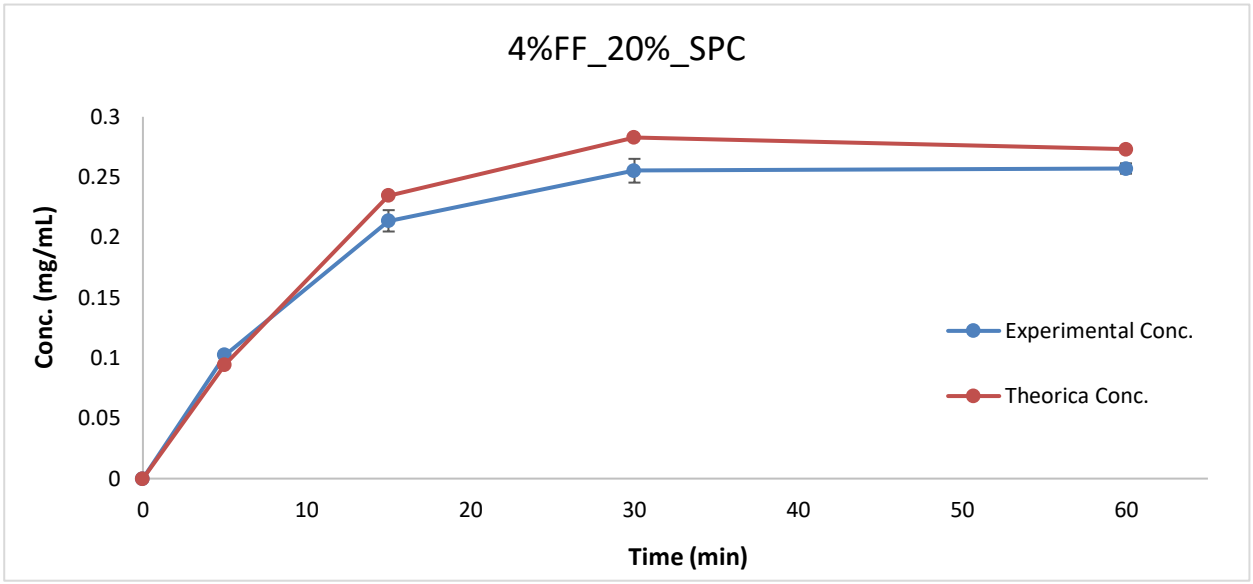
a)


4%FF_20% EPC
0.3
0.25
0.2
0.15
0.1
0.05
0
Conc. (mg/mL)
0
10
20
30
40
50
60
Time (min)
Expermental Conc.
Theoric Conc.

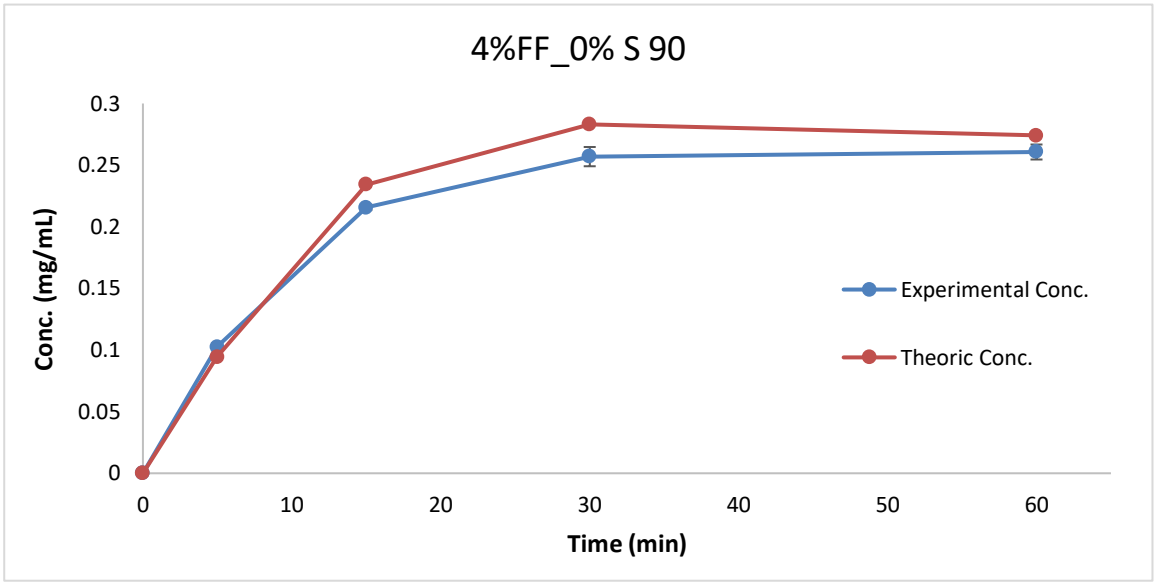
b)


4%FF_0%_SPC
0.3
0.25
0.2
0.15
0.1
0.05
0
Conc. (mg/mL)
0
10
20
30
40
50
60
Time (min)
Experimental Conc.
Theoric Conc.

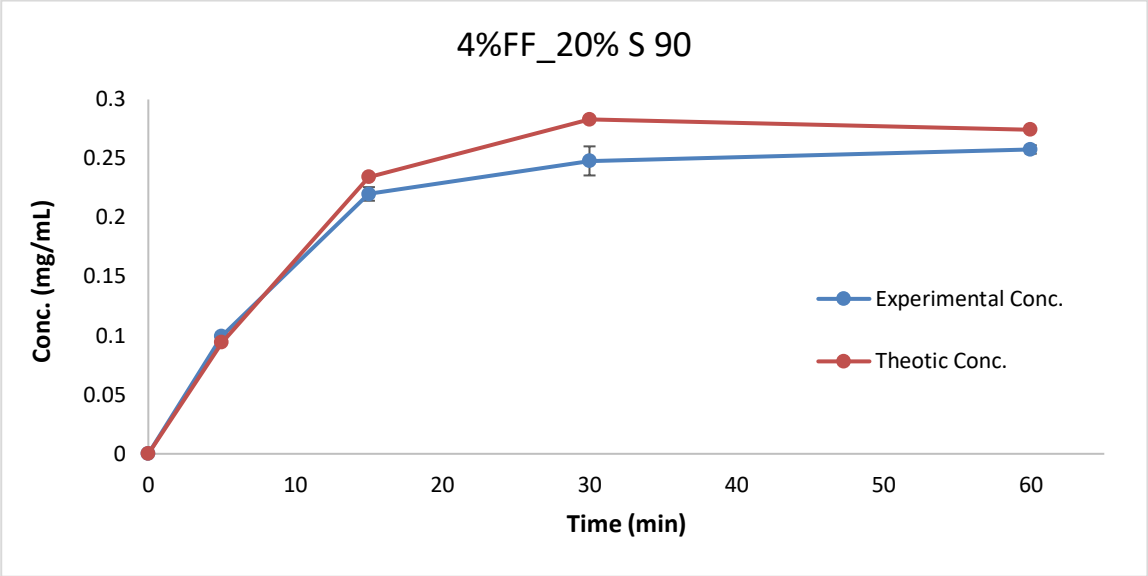
c)


4%FF_20%_SPC
Conc. (mg/mL)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time (min)
Experimental Conc.
Theorica Conc.

d)


4%FF_0% S 90
Conc. (mg/mL)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time (min)
Experimental Conc.
Theoric Conc.

e)



f)

Figure 28. Recovery of total (T) sample in lipolysis model: Comparing the theoretical concentration and total concentration of Fenofibrate in tested samples of liposomes containing different PC compositions: EPC (a and b), SPC (c and d), and S 90 (e and f), with (right side) and without (left side) gelatine.

The results presented in Figure 28 show that there was no significant difference ($p < 0.05$) between the expected theoretical concentration and the experimental concentration from the lipolysis model, except for samples withdrawn after 30 minutes. This may have resulted from experimental errors occurring during transfer between the gastric and intestinal parts, or it might have been caused by uneven distribution of the intestinal suspension by the stirrer. The gastric compartment contained 10 ml of a mixture of media and sample and, after 20 minutes, the entire volume was transferred to the intestinal part using the pump (0.5 mL/min). A comparison of the theoretical concentrations of samples with the experimental data showed the effectiveness of the gastrointestinal compartment as well as verifying that the analytical methods were suitable for use in subsequent lipolysis experiments.

The next step was to assess the T, A and P samples of the *in vitro* lipolysis model. Given that the T sample contained both the oily and aqueous phases, the correlation between the T sample and the

sum of the A and P samples was determined to evaluate the accuracy of the separation methods. Figure 29 illustrates the total recovery of fenofibrate in both the aqueous and non-aqueous phases.

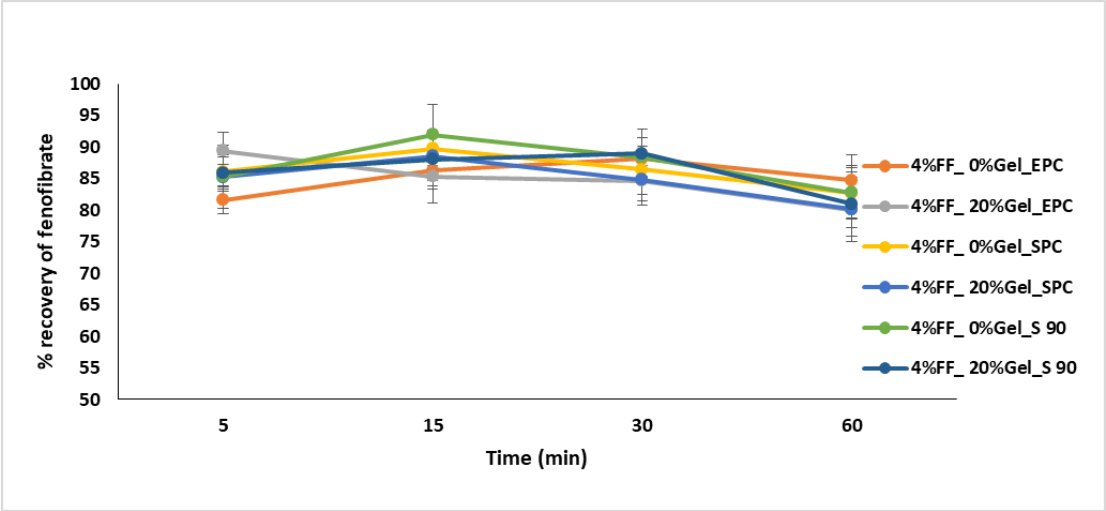
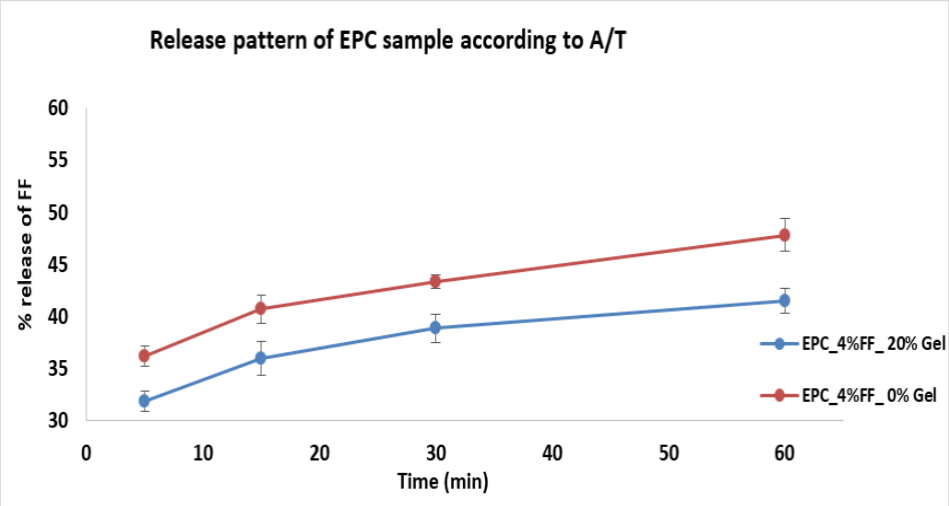


Figure 29. Total recovery of fenofibrate (%) in the pellet and aqueous phase (A+P/T) during one-hour *in vitro* lipolysis (mean ± SD, n=3).

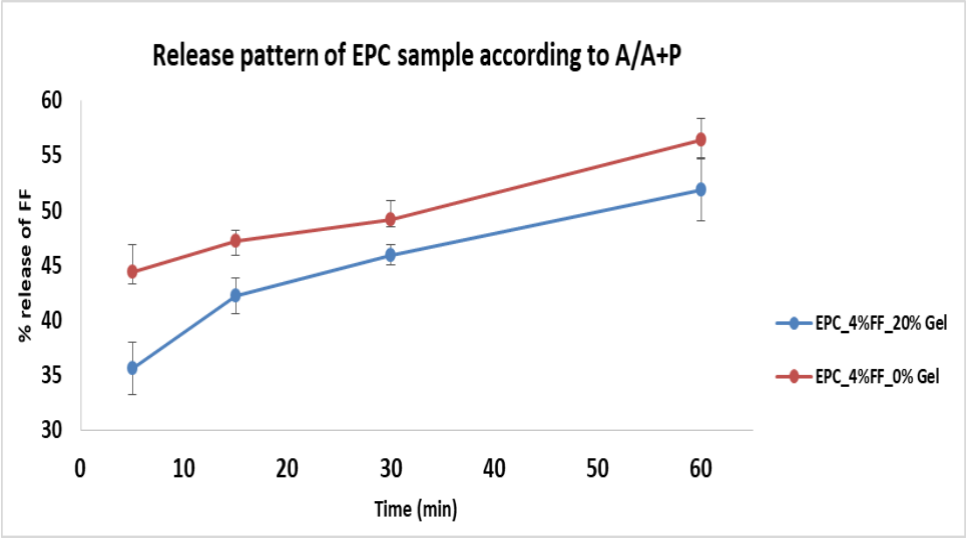
To separate the aqueous and pellet phases, all collected samples were ultra-centrifuged. The pellet phase in the apparatus consists of undigested/partially digested liposomes and calcium soaps of free fatty acids (Larsen et al. 2011). As expected, the amount of fenofibrate in the pellet phase (P sample) followed a drug concentration trend opposite to that in the aqueous phase (A sample). The total fenofibrate recoveries for all prepared samples (both phases combined) upon the lipolysis of liposomes were almost identical and ranged from 80% to 90%. However, theoretically, to the recovery should add up to 100%. Although the amount of drug withdrawn in each sampling was accounted for in the calculations, some portion of the drug was clearly missed at one or more stages of the experiment. This represents a slight decrease in the total content of fenofibrate during the different steps of *in vitro* lipolysis, and may be the result of transferring the pellet phase to the analysis step (ultracentrifugation, re-dispersion and, more importantly, HPLC analysis preparation). The resulting pellet is sticky and compact, with a smooth top layer that makes further transfer somewhat difficult and provides an explanation for the inability of total fenofibrate content to reach 100% recovery. This also explains the rather high variation observed in the recovery of the pellet

phase. Nevertheless, a recovery of 80%–90% was considered acceptable, as the entire experiment comprises different steps for sampling, separation, purification and, finally, analysis. An accurate experimental method is highly recommended for the transfer and determination of the recovery of drug in the pellet phase.

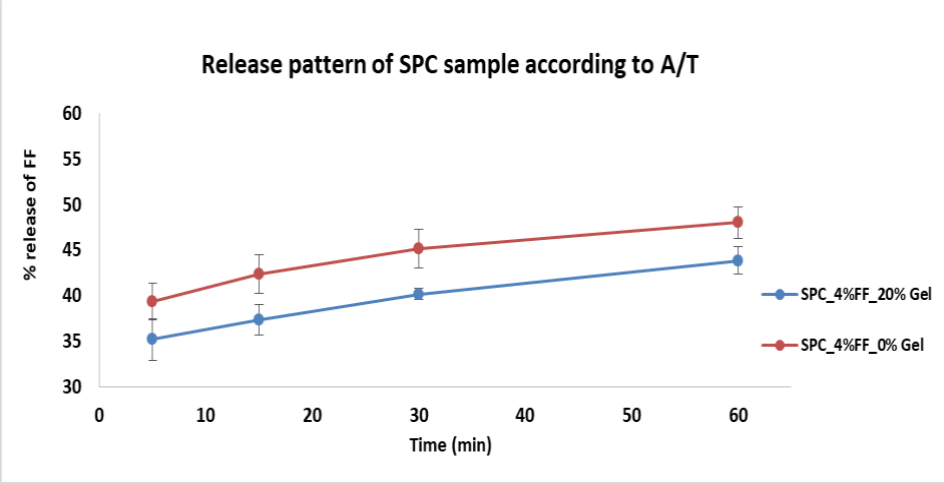
In order to obtain a better understanding of the source of missing sample in these experiments, and the reason why 100% total recovery was not achieved, the concentrations of different phases were evaluated from a different perspective. In this part of the study, the amount of released drug was compared relative to two different elements: the total amount of incorporated drug from the T sample and the sum of the amount of drug in the pellet and aqueous phases. Figure 30 illustrates the release patterns of liposomal samples, where the percentage of drug released from the liposomes for each formulation was calculated as A/T (left side) and A/A+P (right side). The release pattern according to A/T represents the amount of drug in the aqueous phase (A sample) to the total amount of the drug (T sample). The second release pattern was according to A/A+P represents the released fenofibrate in the A sample to the amount of the drug existing in both the supernatant and pellet phases (A+P samples).



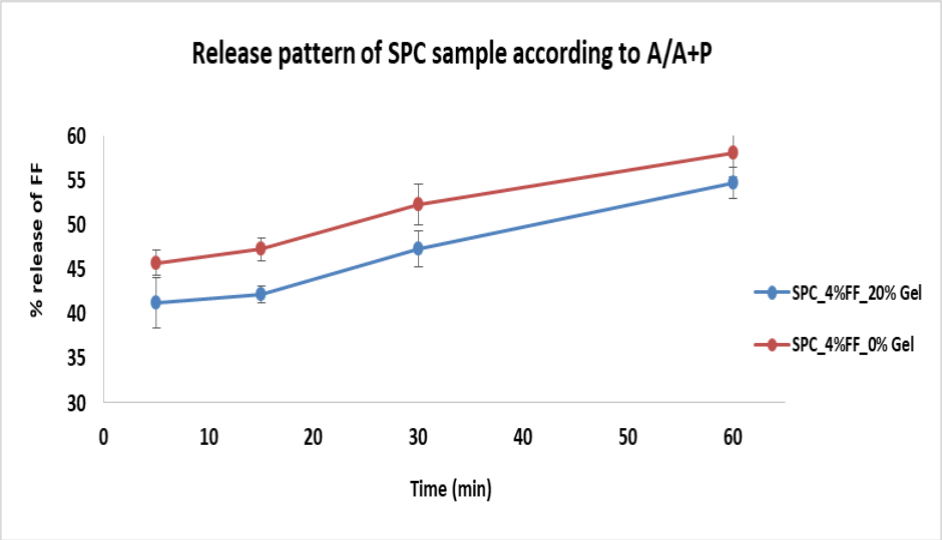
a)


Release pattern of EPC sample according to A/A+P
% release of FF
60
55
50
45
40
35
30
0
10
20
30
40
50
60
Time (min)
EPC_4%FF_20% Gel
EPC_4%FF_0% Gel

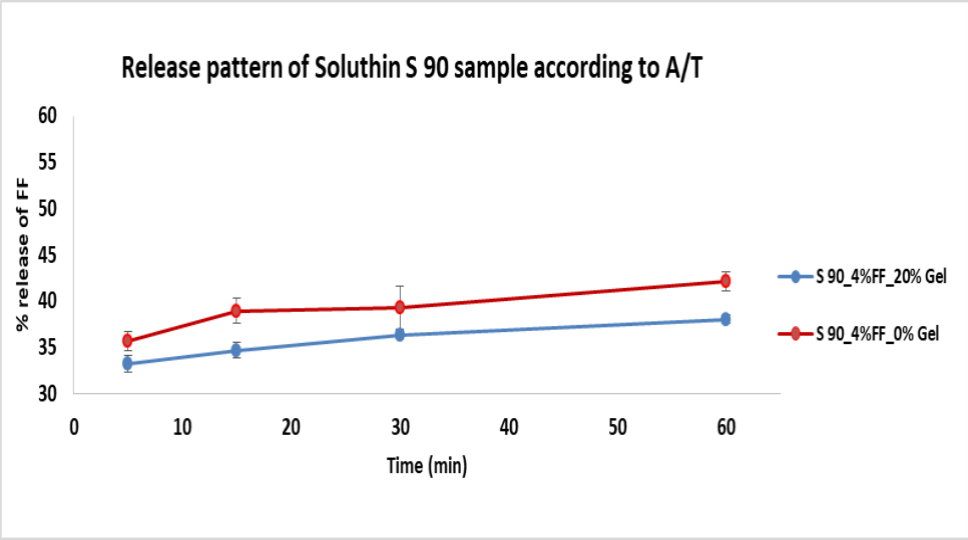
b)


Release pattern of SPC sample according to A/T
% release of FF
60
55
50
45
40
35
30
0
10
20
30
40
50
60
Time (min)
SPC_4%FF_20% Gel
SPC_4%FF_0% Gel

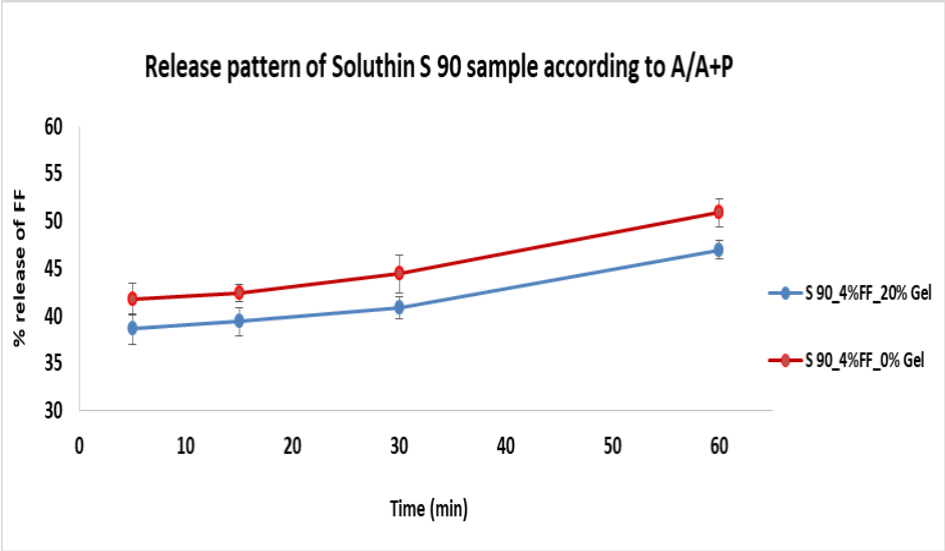
c)


Release pattern of SPC sample according to A/A+P
% release of FF
60
55
50
45
40
35
30
0
10
20
30
40
50
60
Time (min)
SPC_4%FF_20% Gel
SPC_4%FF_0% Gel

d)


Release pattern of Soluthin S 90 sample according to A/T
% release of FF
60
55
50
45
40
35
30
0
10
20
30
40
50
60
Time (min)
S 90_4%FF_20% Gel
S 90_4%FF_0% Gel

e)



f)

Figure 30. Release profile (%) of fenofibrate from liposomes with different compositions according to the A/T (left side; a, c, and e) and A/A+P (right side; b, d, and f), (mean ± SD, n=3).

Although both forms of release profile illustrate an overall increase in the amount of fenofibrate in the aqueous phase as lipid digestion proceeds, the right-side profiles (A/A+P) show more fenofibrate release (%). Since these results were obtained in the same experiment (each right- and left-side figures), this difference represents a slight decrease in the denominator of the second equation (A+P). To obtain further information on the reason for this dissimilarity in concentration, a supplementary experiment was performed. To overcome the issue of pellet recovery, the pellet re-dispersion solvent was first examined. Methanol was used to dissolve the pellet part remaining after ultracentrifugation, especially the oily part consisting of undigested/partially digested liposomes. After comparing the results for re-dispersing the pellet in methanol, acetonitrile, n-hexane or diethyl ether, or a mixture of these solvents (data not shown), methanol was selected as the most suitable solvent for re-dispersing the lipophilic pellets.

The solid phase of the pellet was also evaluated by XRPD, and the resulting data are shown in Figure 33Since fenofibrate is released slowly from liposomes, it is unlikely that precipitation occurs in the pellet phase after dispersal and lipolysis. After carefully repeating and verifying all of the

steps of pellet sample preparation for HPLC analysis, an experimental error was visually detected. After ultracentrifugation, no distinct boundary was observed between the pellet and the aqueous phase, and the upper layer of the pellet phase was partially fixed. Some parts of the pellet phase were therefore missing after separation and removal of the aqueous phase from the solid phase. Due to this unavoidable error, fenofibrate recovery (%) was calculated according to the total samples (A/T) to obtain more accurate results. Figure 31 shows a better representation of the release profile for all formulations.

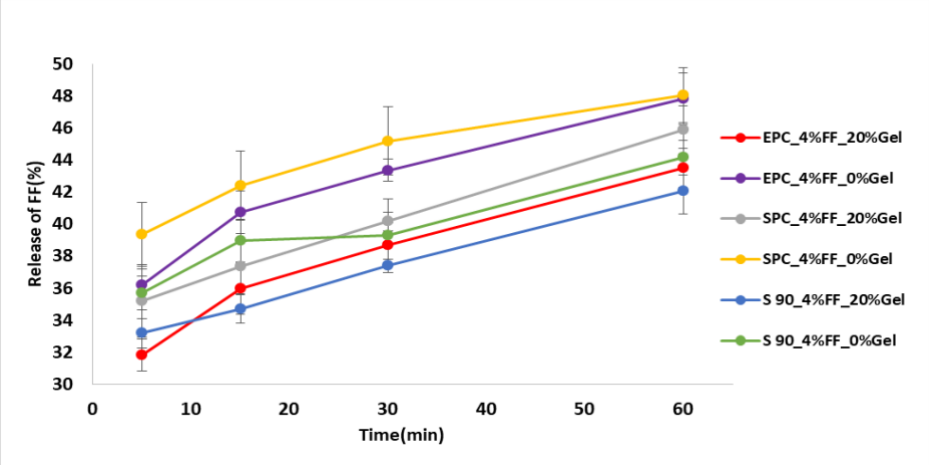


Figure 31. Release profile of fenofibrate (%) of the all of the formulations with three kinds of PCs, with and without gelatine (mean+ SD, n=3).

It was obtained from Figure 31 that, for the different types of liposomes, a general trend (regardless of the type of phosphatidylcholine and amount of gelatine) towards increased release of fenofibrate from the formulations in the aqueous phase was observed as the digestion process continued over time. As shown, there was no significant difference between the different types of prepared formulations in terms of the release profile (%) of fenofibrate from liposomes into the aqueous phase of the lipolysis environment. This may be attributable to the small size of liposomes, which offers a greater surface area for releasing the incorporated drug, or to the pattern of drug distribution in the liposome shell. As described previously, liposomes contain an aqueous and a non-aqueous compartment. Lipophilic drugs such as fenofibrate have an affinity to partition from hydrophilic

core into the lipid surface of liposomes (Müller et al. 2000; Muchow et al. 2008). These specific characteristics correlate with the amount of fenofibrate solubilized in the aqueous phase in the lipolysis study. Since all experimental formulations were prepared with the same size range and similar distribution affinity, they all showed the same drug-release profile. As the digestion process occurs over time, the fatty acids and other lipid digestion products released together with bile salts and phospholipids in the biorelevant lipolysis media facilitate the solubilisation of precipitated drugs by forming micelles (Dahan & Hoffman 2008).

Although fenofibrate was released over time from the liposomal formulation via a lipolysis process, the release profile, which indicates drug solubilisation into the aqueous phase, did not approach 100%. The amount of fenofibrate solubilized into the aqueous phase at 60 min ranged from 42% to 48%. This may have been cause by the very low solubility (5.41 μg/mL ± 0.12 μg/mL) of fenofibrate in the biorelevant medium at 37°C (Borkar 2012). Furthermore, the formation of micelles helps to precipitate fenofibrate, but a burst release due to the previous reasons (small size and shell partitioning) would cause the initial precipitation of fenofibrate. In addition to these two explanations, running the lipolysis model for 60 minutes may have been insufficient to observe a complete digestion process. Performing lipolysis for a longer duration may provide further information about the digestion process and release profile of experimental samples after 1 hour.

3.7.2 NaOH titration

During the *in vitro* lipolysis experiments, lipid digestion occurs and this process produces free fatty acids that decrease the pH of the intestinal compartment. To overcome this challenge and by maintaining the pH of the intestinal compartment within the pH range of 6.5, an auto titrator was used to allow the lipolysis system to eliminate any pH changes occurring due to digestion in the medium by adding NaOH solution (0.5 M). The amount of titrated NaOH correlated reasonably with lipid digestion and, therefore, the drug content in different phases. Figure 32 illustrates the amount of NaOH (mmol) added during the 60 minutes of assessment of *in vitro* lipid digestion in the intestinal compartment. In the early stage of the intestinal evaluation of *in vitro* lipolysis, an

initial lipid concentration from the gastric compartment is observed due to the low pH conditions (pH 4), as well as the availability of gastric enzyme in the mixture which influences lipid digestion and increases the free fatty acids in the experimental media. This leads to a pH reduction, requiring the auto-titrator system to add an adequate volume of NaOH solution. This leads to an additional volume of aqueous suspension in the intestinal compartment and subsequently decreases the final lipid concentration for consideration. A blank lipolysis (without formulation) experiment was carried out to determine the exact amount of NaOH added to the intestinal compartment for titrating the FAs released from the liposomal formulation due to lipid digestion and pH reduction.

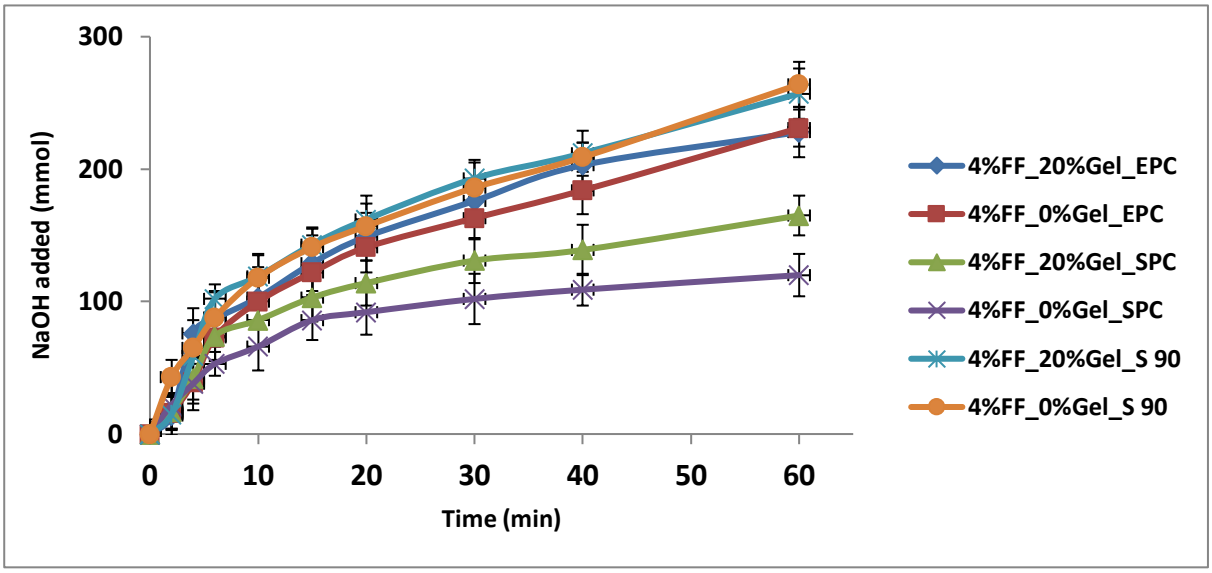


Figure 32. Addition of NaOH (mmol) during hydrolysis of the lipids present in liposomes in the *in vitro* lipolysis model simulating the rat conditions. The data are presented as mean ± SD (n=3).

Figure 32 illustrates the intended amount of NaOH (mmol) added after considering the blank lipolysis data. The amount of FAs released from the liposomes corresponded stoichiometrically with the amount of titrated NaOH at a ratio of 1:1 and offers an indirect indicator of the kinetics of lipid digestion (Dahan & Hoffman 2008). A significant, sharp increase in NaOH titration was observed for all formulations within the first 5 minutes upon rapid lipid digestion, which was due to the exposure of the mixture to pancreatin enzyme and bile salts. However, after 5 minutes, as the

capacity of the intestinal enzyme decreased and precipitation occurred, free fatty acid production and therefore, the acid titration decreased according to the type of phosphatidylcholine formulation. EPC- and S 90-prepared liposomes released more free FAs (especially S 90) compared with SPC-prepared liposomes, and the difference was significant ($p<0.05$). For SPC-prepared formulations, there was a reduced tendency to release FAs after 5 minutes, and it could be concluded that although SPC samples, especially SPC_4%FF_0% Gel (SPC prepared liposomes containing 4% fenofibrate without gelatine), released more drug during lipolysis (Figure 31), most of the drug release (40%) happened in the first 5 minutes of the intestinal step and exclusively at the gastric stage. This shows a reduced potency for SPC samples to tolerate the harsh acidic gastric environment compared with the other types of phosphatidylcholine formulations. After the first 5 minutes, a noticeable decrease in NaOH titration was observed. An obvious explanation for this occurrence is that although the formation of micelles helps precipitated drugs to solubilize into the aqueous phase, due to rapid lipid digestion during the initial exposure to the intestinal environment and the presence of lipolysis enzymes, bile salts and other contributing factors, unavoidable precipitation occurs in the lipolysis process that saturates the activity of the lipase enzyme. This limits the rate of digestion and, subsequently, the production of FAs (Dahan & Hoffman 2008).

Figure 32 representing the digestion and release of FAs, potentially correlates with the release profile of formulations (Figure 31) and supports these data by providing more information on lipid digestion and its source. Figure 31 shows the digestion process from 5 minutes after entering the intestinal section, while the FA titration graph (Figure 32) completes the digestion process, especially the first 5 minutes which is relatively important as it gives more information about the previous step (gastric level). The gastric compartment has a low pH and lipolysis enzymes and the digestion process mostly initiates from this part of GIT, therefore, it is essential and important to have a better understanding about the occurrences in the gastric part. In this study, no statistical data was obtained from the gastric section and therefore, the first 5 minutes of the intestinal part helps to have a better overview about the lipolysis process in gastric and intestinal part. Moreover, in the beginning of intestinal process a rapid lipid digestion occurs as the pancreatin enzyme has a high capacity for digestion, information about the first 5 minutes is very important for evaluating the formulations. Both figures represent the actual lipid digestion process in the intestinal compartment during *in vitro* lipolysis.

3.7.3 Solid phase evaluation

An assessment of the physical state of the precipitates of fenofibrate during *in vitro* lipolysis was carried out. The resulting data provide more details about the lipolysis process; furthermore, XRPD patterns help to explain the *in vitro* and *in vivo* data. It has been shown that amorphous precipitation has a faster dissolution rate compared with the crystalline form – in the case of amorphous precipitation, this implies that drug absorption will not be limited while the crystalline form interferes with dissolution by drug precipitation and consequently has a negative effect on bioavailability (Khan et al. 2016). From previous studies on the fate of poorly water-soluble drugs incorporated into lipid-based formulations, it has been demonstrated that the amorphous precipitated form can be detected while running the *in vitro* lipolysis model (Stillhart et al. 2014; Thomas et al. 2014; Thomas et al. 2013).

The XRPD patterns of the pellet of 4%FF_20% gel_SPC, the pellet, and the blank pellet of 4%FF_20% gel_S 90 after 60 min of lipolysis were compared with the crystalline form of fenofibrate and are shown in Figure 33. The pure fenofibrate drug pattern revealed several characteristic indicators of crystalline fenofibrate, which was in agreement with previous powder diffraction patterns of the crystal structure of fenofibrate (Heinz et al. 2009). Figure 33 shows that the peak positions in XRPD of precipitated fenofibrate available in the pellet clearly did not correlate with the crystalline pattern of spiked fenofibrate. This potentially implies that no crystalline fenofibrate was formed in the pellets during 1 hour of *in vitro* lipolysis. Therefore, the amorphous form or molecular dispersion with a higher dissolution rate (compared with the crystalline form) was available in the pellet and facilities the lipolysis process by increasing the rate of dissolution. The powder diffraction shown in Figure 33 strongly correlates with the dissolution profile of Figure 31 and supports the achieved information about the release profile of the formulations for predicting absorption profiles that were virtually similar for all tested samples.

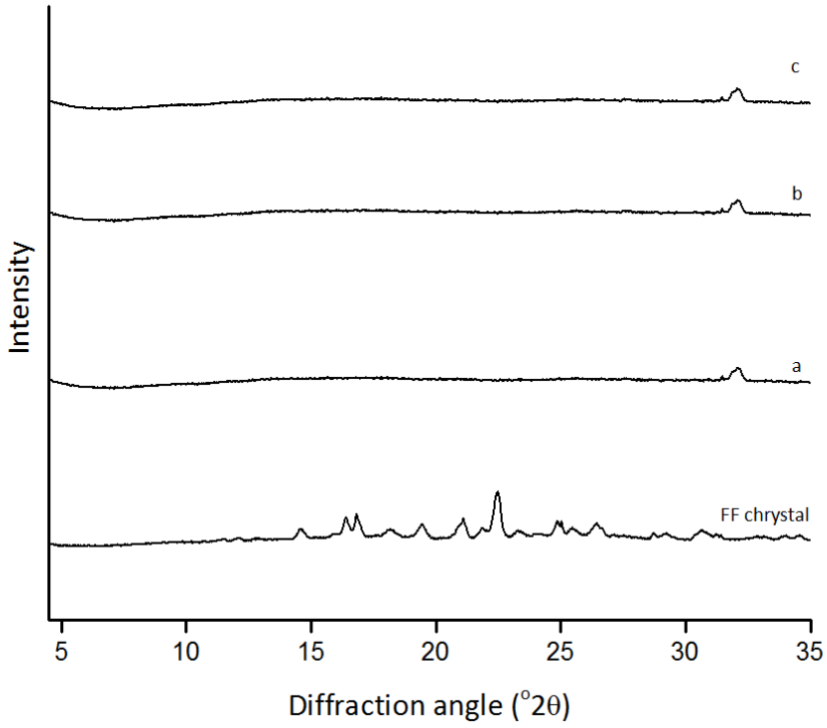


Figure 33. XRPD pattern of pellets obtained after 60 min of lipolysis of the formulations containing a) EPC_4%FF_20%Gel, b) SPC_4%FF_20%Gel, c) S 90_4%FF_20%Gel, compared with crystal.

3.8 *In Vivo* Pharmacokinetics

The aim of the *in vivo* pharmacokinetic study was to evaluate the effect of different types of phosphatidylcholine on the bioavailability of the formulations. Suspensions of different liposomal formulations were orally administered to rats. In order to obtain an overview of the function of the lipid digestion model, the correlation between *in vitro* lipolysis data and *in vivo* bioavailability was investigated. Mean plasma concentration curves following the oral administration of 2.1 mg/kg of fenofibrate formulations in fasted rats are shown in Figure 34.

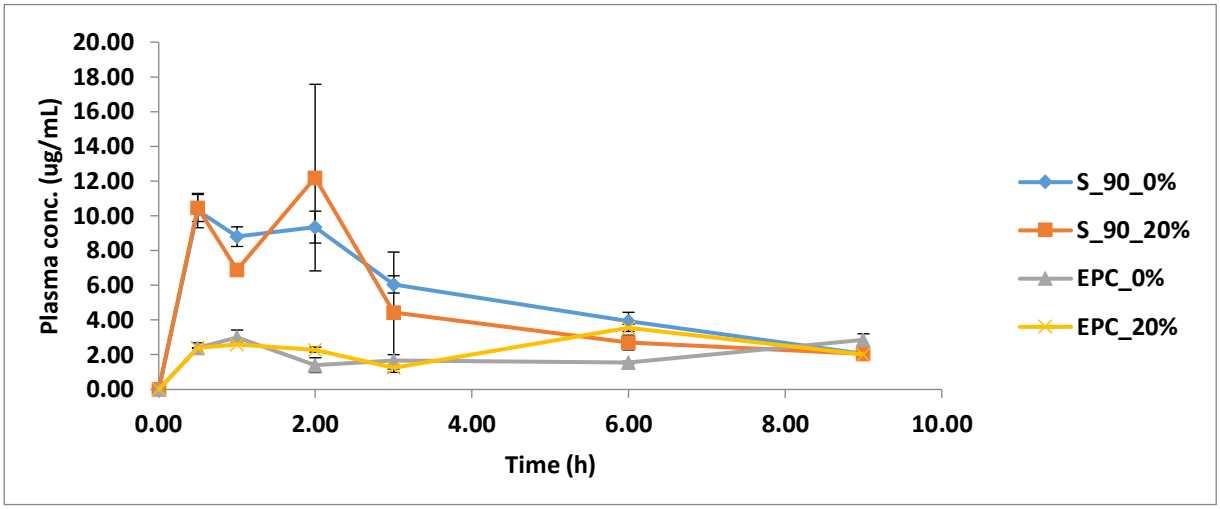


Figure 34. Fenofibric acid concentrations in rat plasma (µg/ml) after the administration of various liposomal formulations of fenofibrate. Data are presented as mean ± SD (n=2).

The formulations prepared using Lipoid Soluthin S90 yielded higher plasma concentrations of fenofibric acid as an active metabolite of fenofibrate compared with the formulations prepared using Lipoid EPC. Thereby, no major differences were observed with respect to the addition of gelatine to the formulations.

Table 13. Pharmacokinetic parameters of fenofibrate following oral administration of four different liposomal formulations (mean ± SD, n=2).

Samples	Size (nm)	PDI	Dose [mg/kg]	AUC [µg/ml∗h]	T_{max} [h]	C_{max} [µg/ml]
S 90_0%Gel.	168±7	0.263±0.018	2.1	48.12±8.32	1.8±0.5	8.53±0.4
S 90_20%Gel.	176±11	0.298±0.019	2.1	42.67±12.54	1.6±0.7	8.12±0.7
EPC_0%Gel.	194±21	0.307±0.031	2.1	17.075±4.97	2.1±1.1	2.64±0.3
EPC_20%Gel.	180±12	0.271±0.023	2.1	21.6±7.26	2.5±1.5	3.29±0.9

The AUC for formulations containing Soluthin S 90 was significantly higher ($p<0.01$) compared with that of the EPC_formulations (Table 13), and was clearly distinct from the plasma concentration-time curves (Figure 34), thus indicating that the formulations containing Soluthin S 90 have greater bioavailability. This can be explained by the type of phosphatidylcholine used for preparing the samples. Soluthin S 90 is a mixed phospholipid consisting of SPC, acid value, lyso-phospholipid and calcium, as described in detail in Table 14. Lyso-phosphatidylcholine (LPC) is a natural surfactant present in the human gastrointestinal tract and is highly recommended (by the FDA) for use in the preparation of drug formulations, especially for liposomal applications (van Hoogevest & Wendel 2014). From previous studies, it has been suggested that natural and low-cost LPCs are effective in the lymphatic transportation of α-tocopherol (a fat-soluble vitamin) in rats (Koo & Noh 2001). The acid value may improve the digestion of the formulation and, more importantly, the existence of calcium in the mixture facilitates the digestion process by forming calcium FA soaps and removing the oil/water interface. Therefore, each composition of the Soluthin S 90 mixture in combination with the different procedures allows the formulation to show effective digestion, absorption and bioavailability.

Table 14. list of the compositions of Soluthin S 90 from lipoid company.

Compounds	Content (%)	Methods
Phosphatidylcholine	87.7 %	LC-PL
Lyso-phosphatidylcholine	2.6 %	LC-PL
Free fatty acids	0.05 %	ADC5
DL-a-Tochopherol	0.1 %	ADC5
Unknown compound (DC)	0.2 %	ADC5
Ethanol	0.3 %	ET
Water	1.4 %	USP<921>Ia
Peroxide	0	POZ
Acid level	4 %	SZ
Calcium	2.7 %	CA

On the other hand, no statistical difference was evident observed for formulations containing EPC and Soluthin S 90 in the absence and present of gelatine. Although the dissolution experiments (Figure 23 and Figure 25) showed a minor difference between formulations containing gelatine and those without gelatine in releasing the incorporated drug from the liposomal VPGs, the *in vitro* lipolysis model showed no significant difference between samples containing 0% and 20% gelatine. In comparison with the USP model, the *in vitro* lipolysis model simulates the gastrointestinal environment in more detail in terms of composition, pH, molarity, enzymes and the digestion speed criteria, as briefly described in section 2.10. Although other research has shown that gelatine can increase the bioavailability of liposomes by increasing the viscosity of the inside and outside of VPGs (Pantze et al. 2014), from the results of the *in vitro* and *in vivo* models, it can be determined that gelatine has no functionally positive effect on the fate of liposomes. Additionally, from the cryo-EM experiment (section 3.2), the microscopy images showed no significant difference in the physical features of both types of fenofibrate-loaded formulations prepared either with or without gelatine. Therefore, the microscopy images could be counted as one the evidence that gelatine has no influence on the stability and, consequently, the bioavailability of liposomal formulations.

The AUC and C_{max} of the S_90_0% sample was significantly higher ($p<0.01$) than that of the other liposomal formulations, although this difference was not statistically significant in the case of S_90_20%. In general, the absence or presence of gelatine had no significant effect on AUC, C_{max} or T_{max}, and this was observed for both EPC- and Soluthin S 90-type liposomal formulations. EPC-type formulations (EPC_0% and EPC_20%) showed a relatively low AUC and C_{max} compared with Soluthin S 90 formulations, with lower bioavailability, and EPC formulations therefore show a lower treatment effect. No significant difference was observed between the T_{max} of the different prepared formulations.

The pharmacokinetic study was based on a study conducted by Anhalt and followed the same administration dose (2.1 mg/kg) for each rat (Anhalt 2012). In that study, oral nanocrystal formulations were used as a drug carrier for fenofibrate to increase its bioavailability in the oral dosage form. After the administration of fenofibrate nanocrystals of different sizes and evaluating the plasma concentration of the API, nanocrystals of 140 nm in size were selected as the most effective formulation. The plasma-time curve showed that fenofibrate-loaded nanocrystals of 140 nm had the best bioavailability, with AUC of 34.532 (µg/ml∗h) and C_{max} of 6.511, and represented

an effective hypolipidaemic formulation to lower the risk of atherosclerosis compared with the pure, crystalline form of fenofibrate with low solubility. Comparing the PK parameters of nanocrystal and liposomal formulations at the same administration dose (2.1 mg/kg), Soluthin S 90-type liposomes showed a remarkably significant increase ($P<0.05$) in AUC and C_{max} (Anhalt 2012), and it could therefore be concluded that the liposome formulation – and specifically Soluthin S 90-type liposomes –have the ability to significantly increase fenofibrate bioavailability and absorption. Although the nanocrystals and liposomes were prepared to almost the same size range, the composition of each formulation was different and this could explain the higher bioavailability of the Soluthin S 90 liposomes. Liposomes are a type of lipid-based formulation that is mostly composed of membrane lipids with hydrophobic head and hydrophilic tails. Therefore, the lipid carrier, by forming chylomicrons, could increase the lymphatic transport of fenofibrate (Hu et al. 2004). For fenofibrate, with a log P value of about 5.24, lymphatic absorption represents an additional method of fenofibrate transport and is a suitable lymphatic model for further studies to investigate the lymphatic transport of fenofibrate (Hu et al. 2011; Chakraborty et al. 2009). Another explanation for the increased AUC and C_{max} of Soluthin S 90 liposomes may be their composition (Table 14), which was briefly described above.

3.8.1 *In vitro* and *in vivo* correlation

Although previous studies on the effect of gelatine on the stability of the oral liposomal formulations demonstrated that gelatine may increase the stability of formulations by increasing the viscosity inside and outside the liposomes, thereby improving bioavailability (Pantze et al. 2014), the *in vitro* lipolysis model in this study demonstrated no significant difference in the formulations in the presence and absence of gelatine, which strongly correlates with the data obtained from the *in vivo* experiments.

Even though the fenofibrate release profile (%) showed no significant difference across all formulations, the *in vivo* results exhibited higher bioavailability for the Soluthin S 90-prepared liposomes compared with the EPC liposomes. Nevertheless, the NaOH titration profile showed that more FAs were released from S 90 formulations in 1 hour of lipolysis, which this FA titration reflects the formulation digestion. NaOH titration showed the results from the beginning of the

intestinal part (time 0), while the fenofibrate release profile (%) showed the amount of drug released after 5 minutes. At the start of pancreatin exposure to lipid-based formulations, digestion is rapid; however, as digestion proceeds and more FAs are released, the capacity of pancreatin for digestion decreases, and drug release is limited by this factor. Thus, the first minutes of sample contact with the intestinal environment are highly critical in term of drug release.

Moreover, the *in vitro* lipolysis model assessed the activity in the intestinal compartment, and not in the gastric part. Of course, lipid digestion mainly occurs in the intestine, although the gastric compartment – due to its low pH and presence of gastric lipolysis enzyme – could efficiently initiate and affect lipid digestion and, therefore, absorption. Another explanation for the lack of correlation between the *in vitro* and *in vivo* models may be the duration of the *in vitro* lipolysis experiments. The *in vitro* model ran for 1 hour while, in the *in vivo* PK studies, rats were under investigation for about 9 hours, and most of the important pharmacokinetic occurrences were observed after 1 hour. This suggests that further studies of more than 1 hour are required to clarify the findings of the *in vitro* studies.

In this study, the presence and absence of gelatine in the *in vitro* rat lipolysis model correlated with the results of the *in vivo* pharmacokinetic studies, and the data on NaOH titration, corresponding with digestion and FA release, almost correlated with the *in vivo* plasma concentration. However, in terms of drug solubilisation, no significant correlation was observed between the *in vitro* and *in vivo* studies, and this was particularly the case with the *in vitro* lipolysis model. The biological environment of the gastrointestinal tract is highly complex with many unknown parameters that can affect the process of digestion and absorption, thus explaining why no suitable model for oral digestion has been established to date. This study strongly supports the construction of a more precise *in vitro* model for LBFs to obtain further correlation between drug solubilisation profiles from *in vitro* lipolysis models and *in vivo* pharmacokinetic profiles. The suggested model should include the gastric simulation model as well as the intestinal part from the beginning of the experiment (time 0), as the first minutes of sample exposure are critical for evaluating formulation digestion, and hence API release.

4. CONCLUSIONS

4.1 Summary and Conclusion

Many new drug candidates show relatively low absorption due to their poor solubility in water. It remains challenging to formulate these poorly water-soluble drugs (PWSDs), although lipid-based drug delivery systems (LbDDS) represent the most successful strategy to date for formulating APIs with low solubility for oral delivery purposes. Among the different types of LbDDS, liposomes are used as drug delivery systems because of their advantageous characteristics such as flexibility, biocompatibility, biodegradability, increased drug therapeutic index and efficacy, increased stability of entrapped drug, reduced side effects, sustained release and drug reservoir properties.

This thesis investigates the biopharmaceutical knowledge of different liposomal formulations obtained using two different phosphatidylcholines (EPC and S 90) in the present and absence of gelatine. The study aimed to characterize the oral lipid-based formulations in terms of physiochemical features, X-ray diffraction, dissolution, *in vitro* lipolysis and *in vivo* pharmacokinetic studies. The results and conclusions are summarized below.

Liposomal formulations were prepared using different methods and were based on particle size and reproducibility of the method. Dual asymmetric centrifugation (DAC) and probe sonication were chosen as experimental methods to prepare fenofibrate-loaded liposomes. The prepared liposomes were used to investigate the physical features of vesicular phospholipid gels (VPGs) and the light-scattering assessed the size and polydispersity of the in range and homogenous liposomes. Cryo_EM was carried out to evaluate the effect of gelatine on the vesicular liposomes, and the obtained images showed no significant difference in the features of the prepared liposomes. Fenofibrate-loaded liposomes had remarkably high entrapment efficiency (95%–99%), which was expected given that fenofibrate has a high lipophilic affinity and was placed within lipid-structured liposomes.

The formation of crystalline fenofibrate was evaluated using X-ray powder diffraction (XRPD) experiments and the obtained diagrams showed no sign of the presence of crystalline fenofibrate in either gelatine-based formulations or those with the high concentrations of fenofibrate. This implies that the dissolution and digestion of liposomal formulations is not restricted by crystalline

fenofibrate. Next, dissolution behaviour of the samples was evaluated in a paddle model. The release of liposome from semi-solid formulations was assessed, and the results showed a negative correlation between the rate of liposome release and gelatine concentration. As gelatine occupies some volume during liposome preparation, the number of released liposomes from the formulation was relatively lower for gelatine-loaded formulations. The USP II model was also used to evaluate the release of fenofibrate from the liposomes using two different biorelevant media: Fasted state-simulated intestinal fluid (FaSSIF), with a pH of 6.5, and fasted state-simulated gastric fluid (FaSSGF), with a pH of 1.6. In both media, non-gelatine-containing formulations released fenofibrate faster, and after the first 30 minutes, approximately 80% and after 1 hour more than 95% of loaded drug was released, whereas gelatine-containing formulations released more than 95% of fenofibrate after 2 hours. The stability of the different formulations was assessed for 3 months at two different temperature conditions (room temperature and refrigerated), and all formulations showed relative stability in terms of size, PDI, and entrapment efficiency.

The prepared liposomes were used in a dynamic *in vitro* lipolysis model to study the digestion and solubilisation of the drug formulations in biorelevant media simulating the gastrointestinal environment. As studies in rats were intended, the lipolysis model was established according to rat physiology and consisted of gastric and intestinal compartments. The release profile of fenofibrate for all liposomal formulations showed a general increasing trend (regardless of the type of phosphatidylcholine and amount of gelatine) towards the release of fenofibrate from the formulations in the aqueous phase, as digestion continued over time. There was no significant difference between the different types of prepared formulations in the release profile (%) of fenofibrate from liposomes into the aqueous phase of the lipolysis environment. Nevertheless, NaOH titration of the lipolysis model showed that the stoichiometric titration of Soluthin S 90-prepared liposomes was higher than that of the other formulations but significantly ($p<0.05$) lower for the SPC-liposomes, indicating that more FAs were released upon digestion.

For the *in vivo* studies, EPC and S 90 liposomes were administrated orally to fasted rats for pharmacokinetic characterization. These *in vivo* studies demonstrated that Soluthin S 90 formulations, regardless of the presence or absence of gelatine, showed significantly higher ($p<0.01$) PK parameters in terms of AUC and C_{max}. All formulations showed a similar T_{max}, and the

presence or absence of gelatine had no significant effect on AUC, C_{max} or T_{max} for both EPC- and Soluthin S 90-type liposomal formulations.

In this study, the absence and presence of gelatine in the *in vitro* rat lipolysis model correlated with the results of the *in vivo* pharmacokinetic studies, and the data from the NaOH titration, corresponding with the digestion and FA release, virtually correlated with the *in vivo* plasma concentration. However, in term of the drug solubilisation profile, no significant correlation was observed between the *in vitro* and *in vivo* studies, particularly in the case of the *in vitro* lipolysis model.

In conclusion, the addition of gelatine, a natural substance, did not have a significant effect on pharmacokinetic parameters and, therefore, the absorption and bioavailability of liposomal formulations. The new phosphatidylcholine mixture, Soluthin S 90, strongly improved the absorption and bioavailability of fenofibrate-loaded liposomes. Further studies should be performed to investigate the effect of fenofibrate-loaded liposomes prepared by Soluthin S 90 on the intestinal permeability of the API and solubilisation capacity of the formulations in an *in vitro* lipolysis model combined with an absorption step. It is also recommended to combine the formulation with different surfactants to choose the optimal formulation. In addition, it is necessary to examine the bioavailability of different PWSDs formulated in Soluthin S 90 liposomes to elucidate the pharmacokinetic effect of such a formulation.

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LIST OF ABBREVIATIONS

API	Active pharmaceutical ingredient
AUC	Area under curve
BBBA	4-bromobenzeneboronic acid
BCS	Biopharmaceutics classification system
C_{max}	Maximal plasma concentration
CMC	Critical micellar concentration
Cryo_EM	Cryogenic electron microscopy
DAC	Dual asymmetric centrifugation
DDS	Drug delivery system
DLS	Dynamic light scattering
FA	Fatty acid
FaSSGF	Fasted state-simulated gastric fluid
FaSSIF	Fasted state-simulated intestinal fluid
FDA	The food and drug administration
FF	Fenofibrate
FFA	Fenofibric acid
Gel	Gelatine
GI	Gastro-intestinal
GIT	Gastro-intestinal tract
HPLC	High performance liquid chromatography
HDL	High-density lipoprotein

IV	Intravenous
IVIVC	*In vitro-in vivo* correlation
kcps	Kilo counts per second
LbDDS	Lipid based drug delivery system
LDL	low-density lipoprotein
MLVs	Multilamellar vesicles
NCE	New chemical entity
NEHI	New England healthcare institute
NLC	Nanostructured lipid carrier
ODD	Oral drug delivery
O/W	Oil in water
Ph.Eur.	European pharmacopia
PBS	Phosphate buffered saline
PDI	Polydispersity index
QC	Quality control
SEM	Standard error mean
SIF	Simulated intestinal fluid
TBU	Tributyrin units
TG	Triglyceride
Tmax	Time required for maximal plasma concentration
USP	United states pharmacopeia
VLDL	Very low-density lipoprotein
VPGs	Vesicular phospholipid gels
XRPD	X-ray powder diffraction