Oral lipid-based drug delivery approaches to improve the intestinal lymphatic transport and systemic bioavailability of a highly lipophilic drug cannabidiol

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LIST OF PUBLICATIONS

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Qin, C., **Feng, W**., Chu, Y., Lee, J., Berton, M., Bettonte, S., Teo, Y., Stocks, M., Fischer, P. and Gershkovich, P., 2020. Development and validation of a cost-effective and sensitive bioanalytical HPLC-UV method for determination of lopinavir in rat and human plasma. *Biomedical Chromatography*, 34 (2020) e4934.

Lee, J., Kim, T., **Feng, W**., Choi, H., Zgair, A., Shin, S., Yoo, S., Gershkovich, P. and Shin, B., 2019. Quantitative Prediction of Oral Bioavailability of a Lipophilic Antineoplastic Drug Bexarotene Administered in Lipidic Formulation Using a Combined In Vitro

Lipolysis/Microsomal Metabolism Approach. *Journal of Pharmaceutical Sciences*, 108(2), pp.1047-1052.

Lee, J., Radhi, M., Cipolla, E., Gandhi, R., Sarmad, S., Zgair, A., Kim, T., **Feng, W**., Qin, C., Adrower, C., Ortori, C., Barrett, D., Kagan, L., Fischer, P., de Moor, C. and Gershkovich, P., 2019. A novel nucleoside rescue metabolic pathway may be responsible for therapeutic effect of orally administered cordycepin. *Scientific Reports*, 9(1).

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LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
2OA	2-Oleoylglycerol with oleic acid
5-HPETE	5-Hydroperoxyeicosatetraenoic acid
ACN	Acetonitrile
ACS	Acyl-CoA synthetase
ADME	Absorption, distribution, metabolism and excretion
ANOVA	Analysis of variance
AUC	Area under the curve
$AUC_{0-\infty}$	Area under the curve from time 0 to infinity
BCS	Biopharmaceutic Classification System
BSU	Bio Support Unit
$CaCl_2$	Calcium chloride
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
CBD	Cannabidiol
CM	Chylomicrons
C_{max}	Maximum concentrations
CNS	Central nervous system
COX	Cyclooxygenase
DDT	4,4-Dichlorodiphenyltrichloroethane

DG	Diacylglycerol
DGAT	Diacylglycerol acyltransferases
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
FA	Final absorbance
FABPpm	Fatty acid binding-protein plasma membrane
FATP	Fatty acid transport protein
FDA	Food and Drug Administration
GALT	Gut-associated lymphoid tissues
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GI	Gastrointestinal
GPCR	G protein-coupled receptor
GT	Glycerol trioleate
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
I-FABP	Intestinal fatty acid binding protein
i.p.	Intraperitoneal
IA	Initial absorbance
IBD	Inflammatory bowel disease
IS	Internal standard
KBr	Potassium bromide
L-FABP	Liver fatty acid binding protein

LA	Linoleic acid
LBP	Lipid-binding proteins
LCFA	Long-chain fatty acids
LCFA	Long-chain fatty acids
LCT	Long-chain triglycerides
LFCS	Lipid formulation categorization system
logD	Distribution coefficient
logP	Partition coefficient
LOX	Lipoxygenase
LT	Leukotriene
LTB4	Leukotriene B4
MCFA	Medium-chain fatty acid
MCT	Medium-chain triglycerides
MG	Monoacylglycerol
MGAT	Monoacylglycerol acyltransferases
MLN	Mesenteric lymph nodes
MS	Multiple sclerosis
MTP	Microsomal triglyceride transfer protein
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAPE	N-acylphosphatidylethanolamine
OA	Oleic acid

OG	Oleic acid with glycerol
PBS	Phosphate-buffered saline
PCTV	Pre-chylomicron transport vesicle
PGI2	Prostacyclin
PLA2	Phospholipase A2
SCT	Short-chain triglycerides
SD	Standard deviation
SEDDS	Self-emulsifying drug delivery systems
SMEDDS	Self-microemulsifying drug delivery systems
$t_{1/2}$	Half-life
TG	Triacylglycerol/Triglycerides
t_{max}	Time to maximum concentration
TNF-α	Tumour necrosis factor-alpha
TPGS	D-α-tocopherol polyethylene glycol 1000 succinate
TRPV1	Transient receptor potential vanilloid type 1
UWL	Unstirred water layer
Δ^9-THC	Delta-9-tetrahydrocannabinol

ABSTRACT

Lipid-based formulations play a significant role in oral delivery of lipophilic drugs. Previous studies have shown that natural sesame oil promotes the intestinal lymphatic transport and oral bioavailability of highly lipophilic drug cannabidiol (CBD). However, both lymphatic transport and systemic bioavailability were also associated with considerable variability. The first aim of this thesis was to test the hypothesis that pre-digested lipid formulations (oleic acid, linoleic acid, oleic acid with 2-oleoylglycerol, oleic acid with 2-oleoylglycerol and oleic acid with glycerol) could reduce variability and increase the extent of the intestinal lymphatic transport and oral bioavailability of CBD. *In vivo* studies in rats showed that pre-digested or purified triglyceride did not improve the lymphatic transport and bioavailability of CBD in comparison to sesame oil. Moreover, the results suggest that both the absorption of lipids and the absorption of co-administered CBD were more efficient following administration of natural sesame oil vehicle compared with pre-digested lipids or purified trioleate. However, this natural oil-based formulation also leads to considerable variability in absorption of CBD [1]. Therefore, the second approach in this thesis was to test the performance of lipid-based formulations with the addition of medium-chain triglyceride (MCT) or surfactants to the sesame oil vehicle *in vitro* and *in vivo* using CBD as a model drug. The *in vitro* lipolysis has shown that addition of the MCT leads to a higher distribution of CBD into the micellar phase. Further addition of surfactants to MCT-containing formulations did not improve distribution of the drug into the micellar phase. *In vivo*, formulations containing MCT led to lower or similar concentrations of CBD

in serum, lymph and mesenteric lymph nodes (MLN), but with reduced variability. MCT improves the emulsification and micellar solubilisation of CBD, but surfactants did not facilitate further the rate and extent of lipolysis. Even though addition of MCT reduces the variability, the *in vivo* performance for the extent of both lymphatic transport and systemic bioavailability remains superior with a pure natural oil vehicle [2].

These results lead to the hypothesis that differences in composition of vegetable oils lead to differences in promotion of intestinal lymphatic transport of lipophilic drugs. Therefore, the differences in composition of sesame, sunflower, peanut, soybean, olive and coconut oils and their corresponding role as vehicles in promoting CBD lymphatic targeting and bioavailability were investigated in this thesis. The comparative analysis suggested that the fatty acids profile of vegetable oils is overall similar to the fatty acids profile in the corresponding chylomicrons in rat lymph. However, arachidonic acid (C20:4), was introduced to chylomicrons from endogenous nondietary sources in all cases. Overall, fatty acid composition of natural vegetable oils vehicles affected the intestinal lymphatic transport and bioavailability of CBD following oral administration in this work. Olive oil led to the highest concentration of CBD in the lymphatic system and systemic circulation and low variability in comparison to other natural vegetable oils following oral administration in rats.

The natural rapeseed oil bodies also used as lipid-based vehicles to facilitate CBD oral bioavailability and lymphatic transport in this thesis. The oral bioavailability of CBD was

1.7-fold higher in oil bodies-based formulation than rapeseed oil-based formulation in rats. This finding indicates that oil bodies could potentially to improve lipophilic drug systemic exposure and lymphatic targeting in comparison to simple oils, and their other pharmaceutical properties as a drug delivery carrier needs to be further investigated.

Overall in this thesis, olive oil and oil bodies are preferred lipid vehicles for improving intestinal lymphatic transport and bioavailability of co-administered CBD following oral administration.

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1. GENERAL INTRODUCTION

1.1. Challenges associated with absorption of lipophilic drugs

1.1.1. Drug lipophilicity

Lipophilicity is a crucial parameter in drug development for evaluating the pharmacological and therapeutic effect of compounds and is characterised by a balance of hydrophobic and polar interactions. It is a particularly critical physicochemical characteristic that influences the pharmacokinetics properties of novel drug candidates, including absorption, distribution, metabolism and excretion (ADME). Partition coefficient (logP) or distribution coefficient (logD) between aqueous phase and octanol are referred to define the lipophilicity of compounds, when compounds are in a single electrical form [3,4]. The shake-flask method and potentiometric titration are two experimental approaches for measuring lipophilicity in isotropic solvent and aqueous systems [5,6]. The dosage form of compounds is also partially determined by the lipophilicity of drug molecules, because they have to cross the lipid bilayer of cellular membranes during absorption [7].

1.1.2. Absorption of lipophilic drugs

The absorption of drugs is partially influenced by lipophilicity, which is regulated by two factors: solubility and permeability. The major transport route for most drugs is the passive diffusion through the cell membranes, especially for highly lipophilic drugs [8]. Oral drug delivery is a preferred administration route that is acceptable by patients and the pharmaceutical industry, due to convenience and patient compliance.

However, lipophilic drugs have reduced oral bioavailability due to their poor aqueous solubility. Despite the fact that lipophilic drugs can be absorbed from the small intestine, the major physiological challenge for these drugs during the absorption is solubility in the aqueous environment in the intestinal lumen, then to be absorbed through the intestinal epithelial membrane [9,10]. In addition, there is a mucus layer on the villi of the intestinal membrane, also known as the unstirred water layer (UWL), which is thought to be a considerable impediment to the passive diffusion of lipophilic compounds [11–13]. Therefore, the primary challenge in enhancing the oral absorption of these lipophilic drugs is to improve their water solubility. Furthermore, the dosage form of drugs significantly impacts on their solubility and permeability, and further influences bioavailability.

There is also a drug categorization by Biopharmaceutics Classification System (BCS): high permeability, high solubility as the Class 1; high permeability, low solubility as the Class 2; low permeability, high solubility as the Class 3; and low permeability, low solubility as

the Class 4 (Figure 1-1) [14]. The oral dosing of Class 3 and 4 compounds is frequently linked with low bioavailability [15]. Improving the bioavailability of lipophilic drugs can be achieved by applying different strategies in oral drug delivery technologies, such as lipid-based formulations.

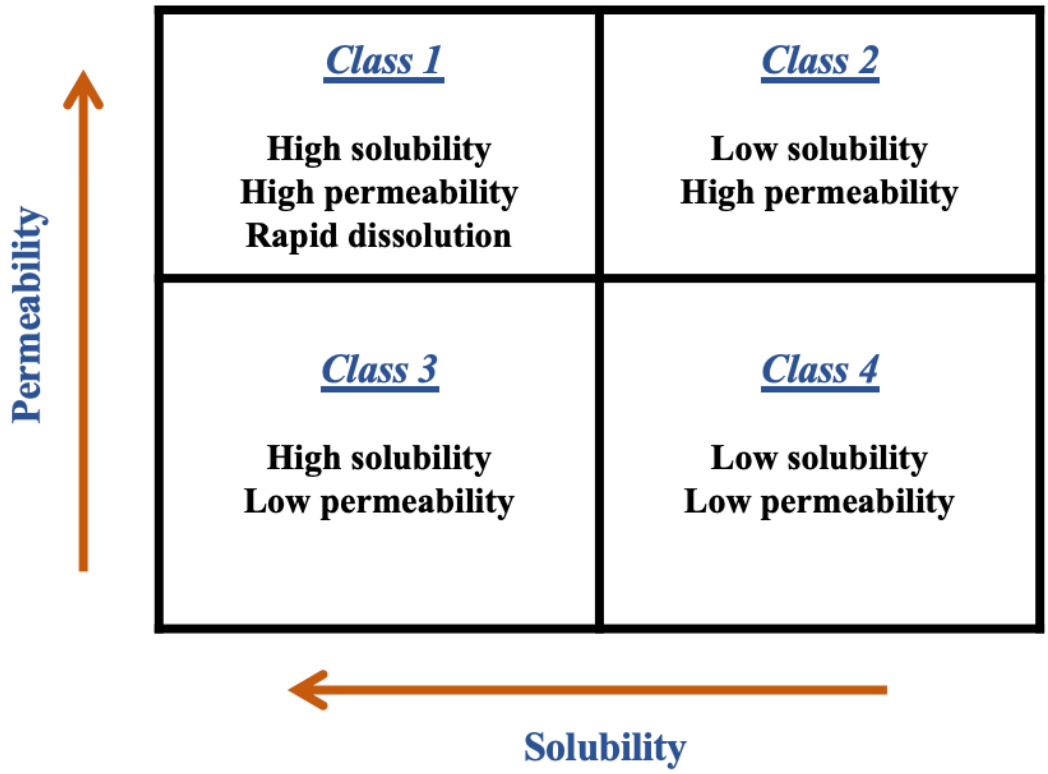


Figure 1- 1 : Schematic description of Biopharmaceutics Classification System (BCS). The BCS is proposed by Amidon et al. to provide the perspective of the intestine absorption following oral administration of compounds based on their solubility and permeability characteristics. Adapted from [14].

1.2. Lipid-based formulations

1.2.1. Categories of lipid-based formulations

In recent decades, there has been an increase in interest in lipid-based formulations to deliver poorly water soluble or poor permeability compounds. In general, lipid-based formulations for oral delivery of lipophilic drugs contain a single excipient, such as triglycerides, mixed mono- and di-glycerides, or two or more excipients, including triglycerides, surfactants or co-surfactants [16,17]. The major purpose of lipid-based formulations is to enhance the dissolution process and aqueous solubility of lipophilic drugs, therefore improving their oral bioavailability [18,19]. Colin Pouton proposed a classification system for lipid-based formulations, which includes four categories in the lipid formulation categorization system (LFCS) [17]. The choice of lipid-based formulations can be influenced by the physicochemical properties of compounds, thus LFCS is used as a guideline to develop the suitable formulation for these drugs.

The LFCS for four categories is summarised in Table 1-1. When there is only one type of excipients, such as oils or mixed triglycerides, mono- and di-glycerides in the lipid-based formulations, it is referred to as LFCS type I lipid system [18–20]. In general, excipients that are used for LFCS type I lipid system are usually food gradient, therefore they are safe for oral administration [21]. However, the LFCS type I lipid system cannot disperse on its own, it must be digested by the pancreatic lipase and mixed with bile salts, and phospholipids in the gastrointestinal (GI) tract [17]. Furthermore, there is a limited capacity

for dispersion in the aqueous medium for LFCS type I lipid systems because there are no surfactants in the formulations [21].

Table 1-1. Summary of lipid formulation classification system (LFCS) [16,17,21].

	Type I	**Type II**	**Type IIIA**	**Type IIIB**	**Type IV**
Oils (Tri-, di- and mono-glycerides)	100	40-80	40-80	< 20	-
Water-insoluble surfactants	-	20-60	-	-	0-20
Water-soluble surfactants	-	-	20-60	20-50	30-80
Hydrophilic co-solvent	-	-	0-40	20-50	0-50
Particle size of dispersion (nm)	Coarse	100-250	100-250	50-100	< 50
Characteristics	Non-dispersing	SEDDS	SEDDS/ SMEDDS	SEDDS/ SMEDDS	Micellar solution

SEDDS: self-emulsifying drug delivery systems; SMEDDS: self-microemulsifying drug delivery systems.

LFCS type II and III lipid systems also contain certain levels of oils. The difference between two systems is that the LFCS type III lipid system is better suited for more hydrophilic drugs due to the presence of hydrophilic co-solvents and water-soluble surfactants in the system [16,21]. Both LFCS type II and III formulations are spontaneously dispersed in aqueous conditions. Depending on the particle size of the dispersion, the type II system is referred to as self-emulsifying drug delivery systems (SEDDS) and self-microemulsifying drug delivery systems (SMEDDS) for the type III formulation [22–24].

Moreover, natural vegetable oils are the most commonly utilised excipients in lipid-based formulations. The natural plant-derived oil normally comprises combinations of triglycerides with different chain lengths, saturated and unsaturated fatty acids [25]. Triglycerides are classified into different types based on the chain length of their fatty acids, which are short-chain triglycerides (SCT, when carbons number is lower than 5), medium-chain triglycerides (MCT, when carbons number is 6-12) and long-chain triglycerides (LCT, when carbons number is higher than 12) [26–29]. Coconut, palm and palm kernel oils are well known to be rich in MCT, with the majority of MCT being saturated C8-triglycerides [30–33]. Moreover, other natural vegetable oils, such as sesame, peanut, soybean, sunflower and olive oils mostly contain LCT, which are commonly employed in the development of lipid-based formulations [25,34,35].

1.2.2. Digestion of lipid-based formulations

As described in Section 1.2.1, some lipid-based formulations are more likely to be in an oily form, and they spontaneously disperse in the aqueous environment in the gastrointestinal tract following oral administration. The digestion of lipid-based formulations initiates in the stomach, and the stomach agitation assists formulations to become crude oil-in-water emulsions [36,37]. The triglycerides in lipid-based formulations will be partially hydrolysed by gastric lipase into free fatty acids and diglycerides in the gastric condition [38,39]. The remaining triglycerides and diglycerides subsequently flow into the small intestine with stomach juices, where they are digested by pancreatic lipase [40,41].

The pancreatic lipase acts specifically on sn1- and sn3- ester groups of triglycerides to produce free fatty acids and 2-monoglycerides [42,43]. The oral lipids and hydrolysed lipid products stimulate the release of bile salts and phospholipids from the liver to the duodenum, where they come together to form mixed micelles [44–47]. The lipophilic compounds in lipid-based formulations will be encapsulated and associated with the lipophilic core in these mixed micelles during the digestion process, therefore the aqueous solubility of these drugs is enhanced by co-administration with lipids. These mixed micelles subsequently diffuse across the UWL together with lipophilic drugs and are broken down at the membrane, then monomers permeate the membrane of epithelial cells

in the small intestine [38,39,48,49]. The digestion process of lipid-based formulation is summarised and presented in Figure 1-2.

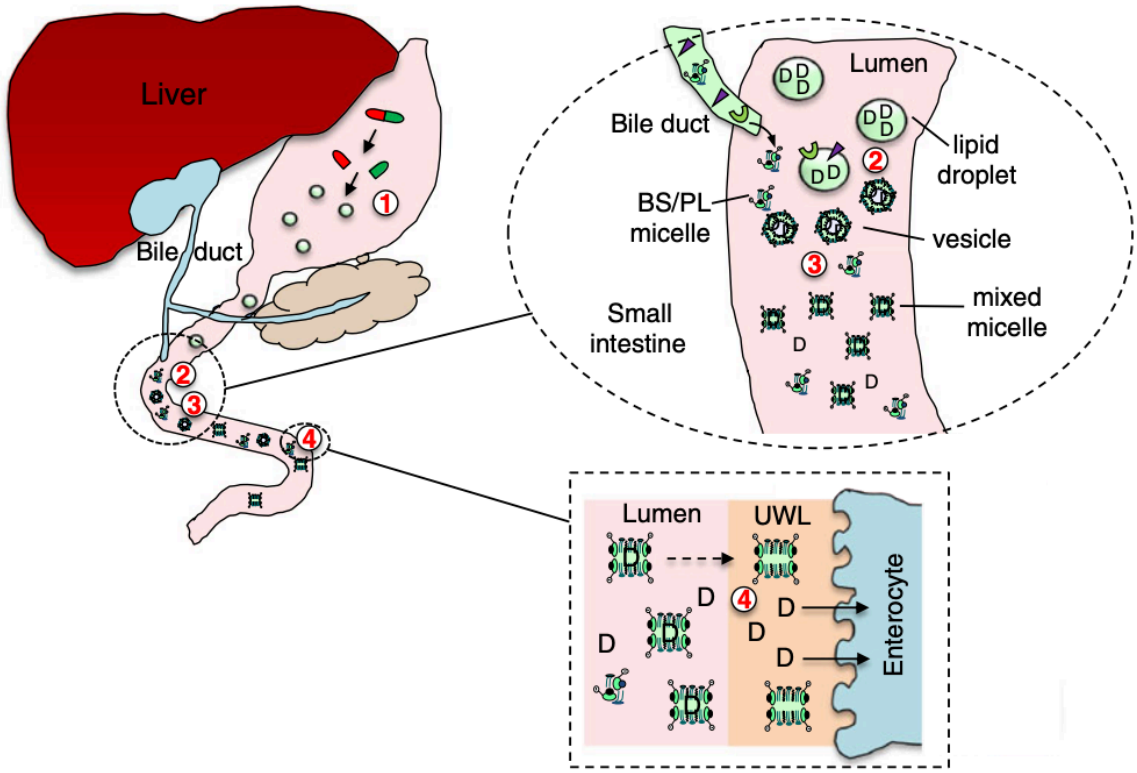


Figure 1- 2 . Schematic description of the digestion process of lipid-based formulation following oral administration. (1) Oil in water emulsion in the stomach. (2) Oil in water emulsion in the intestinal lumen. (2) Mixed micelles in the small intestine. (4) Mixed micelles are broken down near the membrane, and the monomers permeate the membrane into enterocyte. D: drug; UWL: unstirred water layer. Adapted from [40].

1.2.3. *In vitro* lipolysis system

With the increased interest in lipid-based formulations for improving the oral bioavailability of poorly water-soluble drugs, *in vitro* lipolysis has emerged as an effective technique for assessing the performance of lipid-based formulations prior to the *in vivo* stage (Figure 1-3). The primary function of *in vitro* lipolysis system is to mimic the lipid digestion process in the upper intestinal tract to predict lipid digestion and potential drug absorption [50,51]. The pancreatic lipase is a major enzyme involved in the digestion of dietary fat that selected in the *in vitro* lipolysis model [52,53]. The pH range for pancreatic lipase is from 6.5 to 8.0 and its activity is pH dependent [54,55]. Especially when hydrolysis of dietary fat occurs mostly in the duodenum, the pH for lipolysis is close to 6.8 [56,57]. The digestion process also requires the involvement of bile acids and phospholipids. Due to the fact that pancreatic lipase functions predominantly on the surface of the oil-in-water droplets, bile salts, phospholipids and co-lipases could improve the pancreatic lipase catalytic activity by reducing the surface area of oil droplets [50,58]. Moreover, calcium has been proposed to play an important role during the lipid digestion process because calcium can form the calcium soaps by the elimination of free fatty acids [59]. The free fatty acids also restrict the pancreatic lipase activity due to adherence to the interface of the oil droplets or emulsions during lipid digestion [60,61].

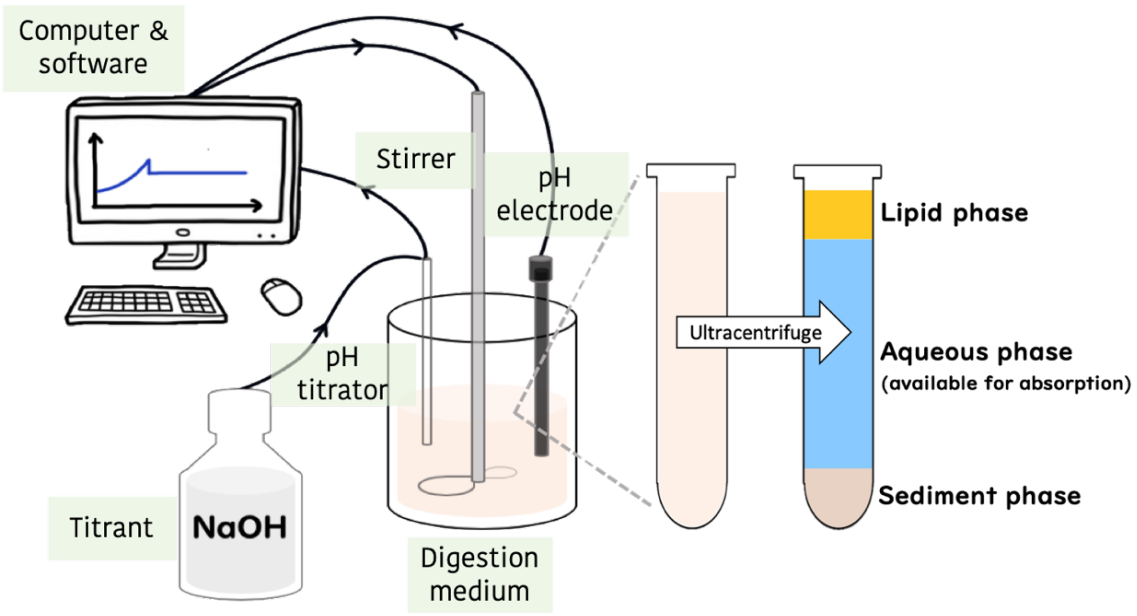


Figure 1- 3 . The diagram of *in vitro* lipolysis. Adapted from [62].

Therefore, the *in vitro* lipolysis system is designed to comprise the bile acids, phospholipids and calcium in order to imitate intestinal fluids. The concentration of bile salts and phospholipids is according to the state of food consumption. For human intestinal fluids, the bile salts concentration is between 2 and 5 mM in the fasting condition and 8 to 15 mM in the fed state [63–65]. According to previous studies, the concentration of bile salts in the *in vitro* lipolysis is usually in the range of 5 mM to 30 mM for different dietary states, and the bile salts versus phospholipid ratio is 4:1 [66–69]. The most common source of bile salts and pancreatic lipase for *in vitro* lipolysis is from porcine origin. However, the differences between human and porcine bile salts that pig bile salts comprise additional hyocholic and hyodeoxycholic bile salts compared to humans [70]. The *in vitro* lipolysis model consists of a computer, pH titrator and digestion media (Figure 1-3). Samples obtained from *in vitro* lipolysis can be separated into different phases after ultracentrifuge. The aqueous micellar phase in the middle contains mixed micelles. Therefore, the drug

level in the aqueous phase following *in vitro* lipolysis is the amount that is considered most likely to be absorbed in the small intestine during digestion.

1.3. Intestinal lymphatic system

1.3.1. Anatomy and physiology of intestinal lymphatic system

The lymphatic system and lymph fluid were identified and described centuries ago. In addition to the more well-known blood circulation, the lymphatic system comprises a large number of lymphatic vessels with a primary function of removing extravasated fluids from the periphery back to the systemic circulation [71]. In contrast to blood circulation, which forms a closed loop, the lymphatic network can return fluid to the blood vessels unidirectionally. In the case of the intestinal lymphatic system, the drainage begins in the GI tract, then lymph flows into the thoracic duct at the end of posterior and subclavian veins to enter the cardiovascular system (Figure 1-4) [72,73]. The largest lymph ducts in the lymphatic system are the thoracic duct and the right lymphatic duct (mesenteric lymph duct) [74].

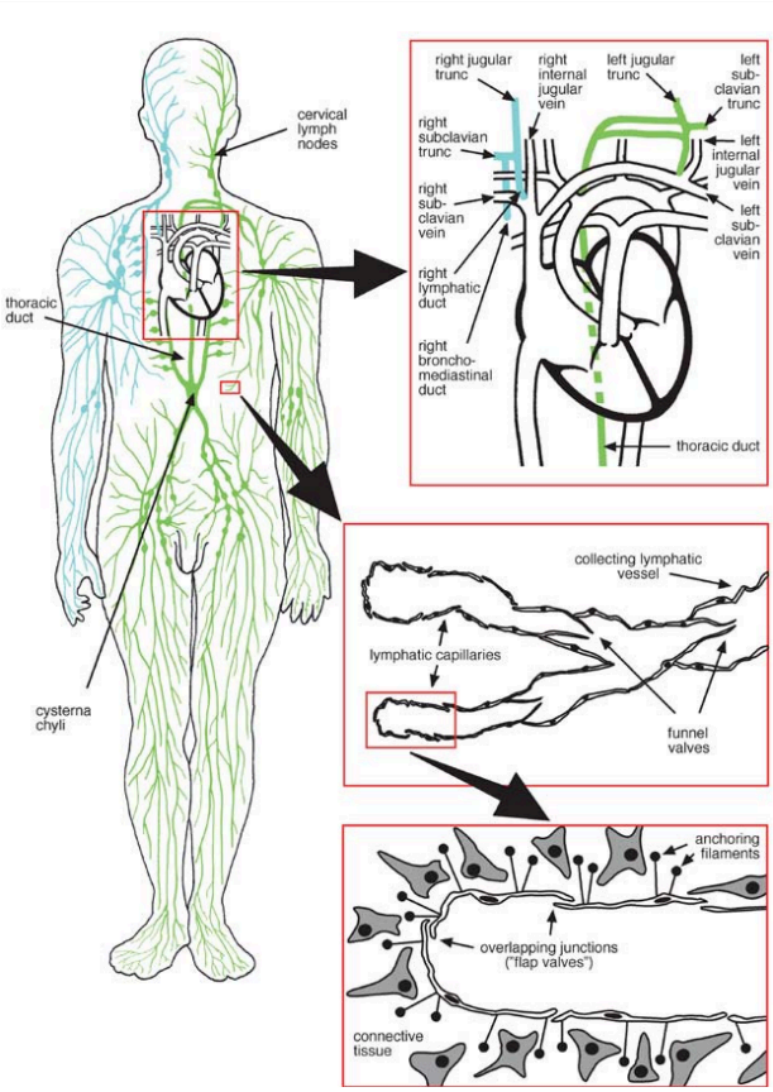


Figure 1- 4 . Schematic description of the lymphatic system. Adapted from [74].

The lymphatic system is composed of lymphatic veins, lymph fluids, immune cells and organs, all of which play a critical role in the immune response. Immune cells including T and B cells, as well as antibodies, are regulated and proliferating in the lymph nodes before being disseminated into systemic circulation [74]. Lymphatic capillaries in the intestinal lymphatic system, also known as lacteals, are found only in intestinal villi and congregate in the mesentery, which is located near the small intestine [75]. Lacteals are located underneath enterocytes and surrounded by blood capillaries of artery and vein, and their

length can achieve up to 70 % of villus (Figure 1-5) [72,76]. Furthermore, the apical membrane of mucosal enterocytes and the intestinal lymphatic system are important organs for the absorption of dietary fats, such as LCT and fat-soluble vitamins [72].

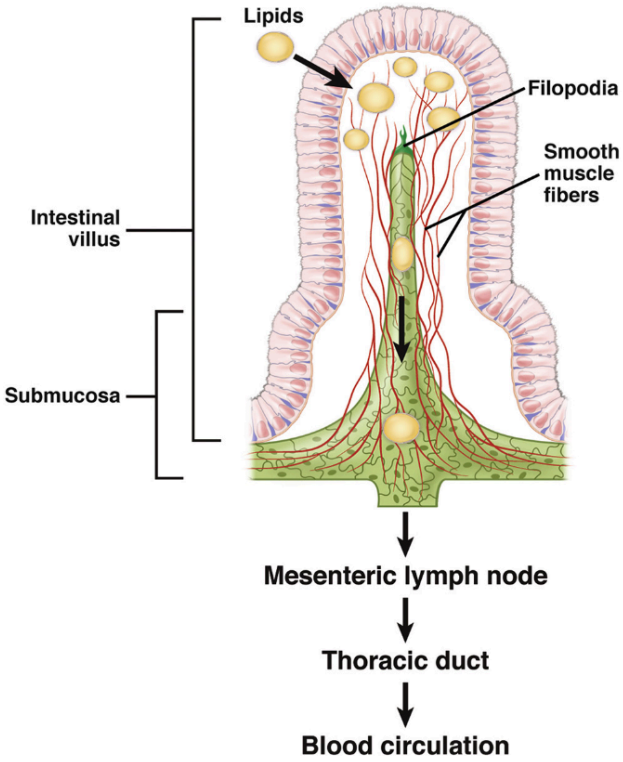


Figure 1- 5 . Schematic description of a single lacteal under enterocytes in the intestinal villus. Adapted from [72].

1.3.2. Chylomicrons formation

It has been well demonstrated that dietary LCT can be digested and absorbed from the small intestinal lumen and that they are the exogenous source of chylomicron formation [77,78]. As described in Section 1.2.2 above, free long-chain fatty acids (LCFA) and 2-monoglycerides are hydrolysed from dietary LCT by lipases, and lipid digestion products

are subsequently transported into enterocytes by the association with mixed micelles in the GI tract. Even though passive diffusion is responsible for the transfer of LCFA and 2-monoglycerides into enterocytes, numerous lipid-binding proteins (LBP) have been shown to be involved in this process, and these LBP are found to be expressed in the small intestine [79]. These LBP, such as fatty acid transport protein (FATP) and fatty acid binding-protein plasma membrane (FABPpm), deliver LCFA to the endoplasmic reticulum (ER) within the enterocyte for LCT synthesis and chylomicron formation [79–81].

In the enterocytes, the re-synthesis of triglycerides takes places in the ER membrane [82,83]. Enzymes involved in the triglyceride re-synthesis are acyl-CoA synthetase (ACS), monoacylglycerol acyltransferases (MGAT) and diacylglycerol acyltransferases (DGAT), and their activities can be found on cytoplasmic surface of the ER [84,85]. ACS are produced endogenously and function to LCF, that transform LFA to fatty acyl-CoA to mediate the fatty acid influx [86]. The primary function of MGAT is to convert monoacylglycerol (MG) with fatty acyl-CoA into diacylglycerol (DG), and DG with fatty acyl-CoA are subsequently catalysed by DGAT to form triglycerides [87].

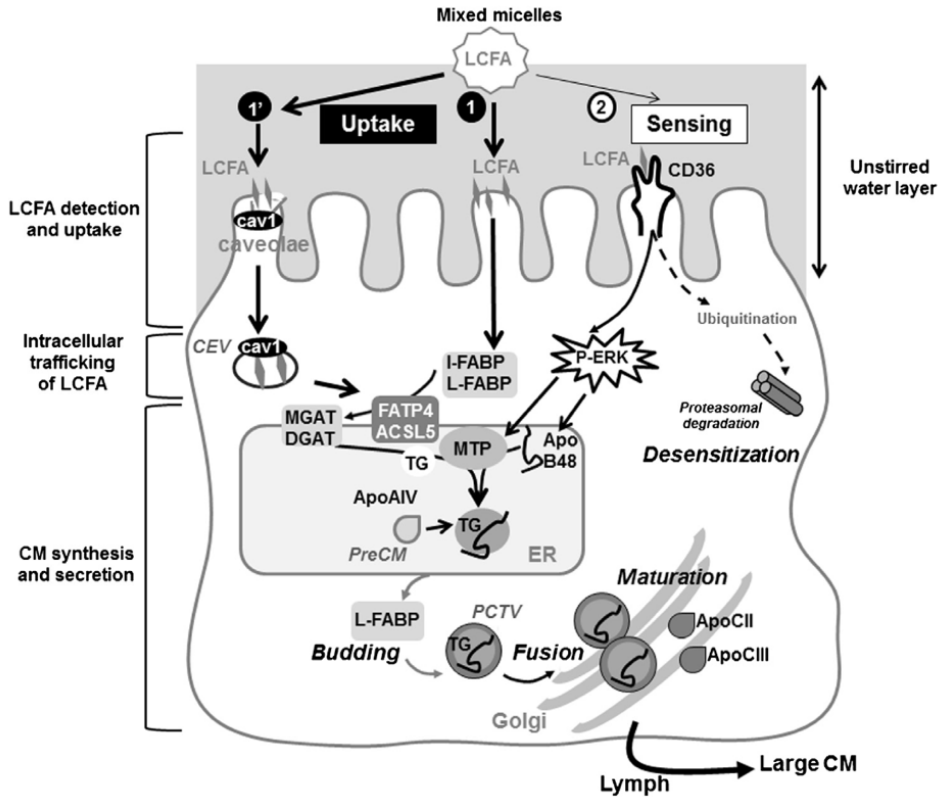


Figure 1- 6 . Schematic description of chylomicrons formation. LCFA: long-chain fatty acid; MGAT: monoacylglycerol acyltransferases; DGAT: diacylglycerol acyltransferases; I-FABP: intestinal fatty acid binding protein; L-FABP: liver fatty acid binding protein; MTP: microsomal triglyceride transfer protein; Apo B48: apolipoprotein B48; PCTV: pre-chylomicron transport vesicle; TG: triacylglycerol. Adapted from [79].

These re-synthesised triglycerides subsequently reach the cisternae of the endoplasmic reticulum, and it has been proposed that triglycerides can saturate the phospholipid membrane because of their limited solubility in phospholipids (about 3 mol%) [88]. As a result, when triglycerides surpass the solubility of phospholipid bilayers, the bilayer can separate and form small lipid droplets [83]. Therefore, pre-chylomicrons are produced by integrating apolipoprotein B48 (apo B48) onto the phospholipid membrane of these lipid droplets in the cisternae of the endoplasmic reticulum, then diffusion to the Golgi apparatus

[83,89]. Mature chylomicrons are formed in the Golgi and eventually secreted and delivered to the lymphatic system (Figure 1-6) [89].

1.3.3. Gut-associated lymphoid tissues

Gut-associated lymphoid tissues (GALT) are composed of mesenteric lymph nodes (MLN), intraepithelial lymphocytes, intestinal lamina propria and Peyer's patches in the mucus membrane of the small intestine [90]. GALT are also the biggest group of lymphoid tissues that contribute to immune response modulation by the intestinal humoral immune system in the GI tract. The major immune cells in GALT are B- and T-lymphocytes, which play a significant role in regulating the balance of resistance and immunity to both endogenous and exogenous antigens [91,92]. Therefore, in diseases related to immune response, such as human immunodeficiency virus (HIV) infection and auto-immune diseases, targeting drugs to the GALT may result in optimal therapeutic effects on these diseases. Furthermore, as described in Sections 1.2.2 and 1.3.2, chylomicrons can be considered as possible lipid carriers to deliver lipophilic drugs through the intestinal lymphatic system, and this process is also known as intestinal lymphatic transport [93]. LCT, particularly when presented in oral lipid-based formulations, can promote the formation of chylomicrons and therefore enhance the intestinal lymphatic transport of co-administered lipophilic drugs.

1.4. Cannabidiol

1.4.1. Natural cannabinoids

Phytocannabinoids are the natural compounds derived from cannabis that have been investigated for centuries for their potential medicinal applications. The primary natural cannabinoids are delta-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD), which were identified and isolated in the 1930s by Mechoulam and Gaoni [94,95]. Unlike Δ^9-THC, CBD has a non-psychoactive effect and lower affinity to the cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB_2) [96,97]. CB_1 and CB_2 receptors are both G protein-coupled receptor (GPCR) members, with CB_1 receptors being located primarily in the central nervous system (CNS) and certain peripheral tissues, whilst CB_2 receptors are prominent in certain peripheral tissues [98]. These receptors regulate the pharmacological actions of endocannabinoids (2-arachidonoylglycerol (2-AG) and anandamide as well as exogenous cannabinoids (such as CBD and Δ^9-THC) [98,99]. Even though CBD has been reported to shown low affinity for CB_1 and CB_2 receptors, studies have demonstrated that CBD has a negative allosteric modulation to CB_1 receptor by inhibiting the 2-AG and Δ^9-THC efficiency and potency to CB_1 receptors [100]. In addition, *in vitro* studies have shown that CBD has an inverse agonism impact on human CB_2 receptors, which may alleviate inflammatory symptoms [101]. Furthermore, studies in rats have shown that CBD can regulate transient receptor potential vanilloid type 1 (TRPV1) to attenuate the hyperalgesia in a rat acute inflammation model [102,103].

1.4.2. The application of cannabidiol (CBD)

In recent years, there has been a growing interest in the pharmacological effect of CBD, due to the fact that CBD has a broad therapeutic dose window and less adverse effects than THC. Many *in vitro* and *in vivo* studies have demonstrated that CBD has activity on tumour necrosis factor-alpha (TNF-α) and depresses the proliferation of cytokines, therefore, CBD has been found to have anti-inflammatory activity [104–106]. It has been reported that CBD significantly reduces the inflammatory and hyperalgesic effects of an acute inflammation rat model following oral administration of CBD in cremophor, ethanol, and saline (v/v/v, 1:1:18) emulsion at daily dose 5-40 mg/kg for three days [107].

Furthermore, CBD has been shown to alleviate the symptoms developed by auto-immune diseases, such as multiple sclerosis (MS), inflammatory bowel disease (IBD) and rheumatoid arthritis [108–111]. In the collagen-induced arthritis mice model, CBD was reported to protect joints from severe damage by oral administration (25 mg/kg per day in olive oil) and by intraperitoneal (i.p., 5 mg/kg per day) injection [112]. CBD was also found to limit T cell infiltration into CNS and reduce cytokines release when used to imitate MS symptoms in experimental auto-immune encephalomyelitis (EAE) mice, and the dose of CBD was 20 mg/kg per day by i.p. injection [113]. In addition to auto-immune diseases, CBD has been investigated to have anti-invasive and antiproliferative effects on a variety of tumours, therefore it can be potentially used for anti-cancer treatment [114–116].

1.4.3. Current formulations of CBD

Despite the fact that CBD has promising therapeutic effects on many diseases, the administration route of CBD has been limited due to its physicochemical properties. CBD is a highly lipophilic compound with low water solubility (12.6 mg/L) but can be well solubilized in lipids, and CBD has also been proven to have high membrane permeability [117–119]. The oral bioavailability of CBD in lipid-free formulation ranges between 6 and 8% for humans and rats [120,121]. Therefore, many studies are being conducted to investigate different types of formulations to improve the oral bioavailability of CBD. One of the current CBD-based medicines on the market is EPIDIOLEX®, which was approved by the Food and Drug Administration (FDA) in 2018 for the treatment of epilepsy in children [122]. EPIDIOLEX® is an oil solution that contains 100 mg/mL CBD in dehydrated alcohol and sesame oil, with an oral daily recommended dose of 5 mg/kg [122–124].

Lipid-based formulations have been shown to improve the oral bioavailability of CBD in the preclinical laboratory stage. For example, compared to CBD oil, an oral nano-emulsion of CBD composed of vitamin E acetate, Tween® 20, ethanol and water can increase the total CBD absorption by 65% in rats [125]. In the pure sesame oil, CBD also found to enhance the oral bioavailability compared to the lipid-free formulation [120,126]. Furthermore, oral SNEDDS formulation employing LCT, Tween® 20, Span® 80, Cremophor® RH40, lecithin and ethanol as the vehicle, can produce comparable CBD

plasma concentrations with less variabilities in drug absorption than sesame oil vehicle in both humans and rats [127,128].

1.5. Project hypothesis and aims

As explained in Section 1.4, the development of lipid-based formulations for CBD can improve oral bioavailability. It has also been described in Sections 1.2.2 and 1.3.2 that using LCT as the lipid vehicle can promote the intestinal lymphatic transport and chylomicron productions. CBD has been shown to have a high affinity to chylomicrons, with a chylomicron association of nearly 70% for humans and 73% for rats [120]. Therefore, administering CBD in LCT-based lipid vehicle not only enhances the oral bioavailability but also promotes the lymphatic transport. In addition, because of the immunomodulatory properties of CBD at high concentrations, direct targeting of CBD into the lymphatic system could potentially result in better treatment outcomes of conditions associated with the immune system, such as autoimmune diseases. Even though previous studies have already shown that CBD in sesame oil leads to high CBD concentrations in the intestinal lymphatic system [120,129], sesame oil-based formulations are associated with considerable variability in drug absorption when compared to other formulations. Therefore, the focus of this thesis was on optimizing oral lipid-based vehicles for delivering CBD through the intestinal lymphatic system and reducing the variability in drug absorption associated with pure sesame oil vehicle.

Chapter 3:

Using pre-digested lipid such as oleic acid and linoleic acid to avoid lipid digestion step, with a hope to reduce the variability and increase the extent of CBD delivery to the intestinal lymphatic system and systemic circulation as compared to sesame oil or purified LCT.

Chapter 4:

Addition of MCT and surfactants to sesame oil as an attempt to improve the emulsification and micellar solubilization of CBD in GI tract, therefore enhancing the intestinal lymphatic transport and bioavailability of CBD with potentially reduced variability.

Chapter 5:

Natural vegetable oils have different fatty acids compositions and lipid contents, which may result in different performance as a vehicle for delivering CBD to the lymphatic system and systemic circulation when administered orally. Therefore, this chapter has compared the lipid profiles of several vegetable oils, as well as the intestinal lymphatic transport and bioavailability enhancement properties of CBD in co-administered vegetable oils.

Chapter 6:

Loading CBD into plant derived oil bodies to make oil bodies-based formulation, containing natural oil bodies proteins. Oil bodies are extracted from the rapeseeds. The intestinal lymphatic transport and systemic absorption of CBD following the administration of CBD in rapeseed oil bodies and the corresponding rapeseed oil was compared.

2. MATERIALS AND METHODS

2.1. Materials

Cannabidiol (CBD, ≥ 98%) was obtained from THC Pharm (Frankfurt, Germany). Sesame oil, sunflower oil, peanut oil, soybean oil, olive oil, coconut oil, rapeseed oil, methyl pentadecanoate, glyceryl trioctanoate, polyoxyethylene sorbitan trioleate (Tween® 85), polyethylene glycol sorbitan monooleate (Tween® 80), sorbitan monolaurate (Span® 20), d-α-Tocopherol polyethylene glycol 1000 (TPGS), oleic acid (~ 97%), linoleic acid (≥ 99%), glycerol (≥ 98%), glycerol trioleate (≥ 97%), potassium bromide (KBr), sodium hydroxide solution (NaOH, 1M), L-α-phosphatidylcholine (~ 60%, from egg yolk), Trizma® maleate, sodium taurocholate hydrate, pancreatin from porcine pancreas (8 × USP specifications), probucol, 4,4-dichlorodiphenyltrichloroethane (DDT) and serum triglyceride determination kit were purchased from Sigma-Aldrich (Dorset, UK). Trimethylsulfonium hydroxide (0.25 M solution in ethanol), sodium chloride (NaCl) and calcium chloride ($CaCl_2$) anhydrous were purchased from Fisher Scientific (Leicester, UK). The 2-oleoylglycerol (≥ 94%) was custom-synthesised by BiBerChem Research Limited (Newcastle upon Tyne, UK). The rapeseed oil body was kindly provided by the division of food science, University of Nottingham (Sutton Bonington Campus, Nottingham, UK). Rat plasma was purchased from Sera Laboratories International (West Sussex, UK). All other solvents and reagents were analytical or high-performance liquid chromatography (HPLC) grade purchased from Fisher Scientific (Leicester, UK).

2.2. Lipid-based formulations

2.2.1. Preparation of cannabidiol (CBD) in pre-digested and digestible lipid vehicles

This methodology was used to generate experimental that results are described in Chapter 3.

The five tested formulations can be classified into two groups: digestible or pre-digested lipid-based vehicles. The digestible lipid-based vehicles included sesame oil and glycerol trioleate (GT). The pre-digested lipid-based vehicles included oleic acid (C18:1, OA), linoleic acid (C18:2, LA), oleic acid with 2-oleoylglycerol (2:1, molar ratio, 2OA) and oleic acid with glycerol (3:1, molar ratio, OG). All six lipid-based formulations contained fully solubilised CBD at a concentration of 12 mg/mL.

2.2.2. Preparation of CBD in the medium-chain and long-chain triglyceride-based vehicles

This methodology was used to generate experimental that results are described in Chapter 4.

All lipid vehicles were prepared in volumetric flasks. Sesame oil and triglyceride-C8 (Tri-C8) mixture were firstly prepared, then surfactants were blended in lipid mixture using a magnetic stirrer (1000 rpm) under 37 °C in water bath until TPGS was fully dissolved. Lipid-based formulations were prepared by solubilizing CBD in the pre-mixed lipid vehicle under the same conditions as described above, then transferred into glass scintillation vials. Sesame oil was used as a vehicle for control group, and as the long-chain triglyceride (LCT) component in other formulations. In addition to sesame oil control group, five lipid-based formulations were prepared: Formulation 1 (F1, sesame oil:Tri-C8:Tween® 80, 5:3:2, v/v/v with 10 mg/mL TPGS), Formulation 2 (F2, sesame oil:Tri-C8:Tween® 85, 5:3:2, v/v/v with 10 mg/mL TPGS), Formulation 3 (F3, sesame oil:Tri-C8, 1:1, v/v), Formulation 4 (F4, sesame oil:Tri-C8:Tween® 85, 2:2:1, v/v/v with 10 mg/mL TPGS) and Formulation 5 (F5, sesame oil:Tri-C8:Tween® 85:Span® 20, 5:3:1:1, v/v/v/v with 10 mg/mL TPGS) (Table 2-1). Concentration of CBD was 50 mg/mL in all formulations, which were freshly prepared on the day of experiment for all *in vitro* and *in vivo* tests.

Table 2-1. The composition of the developed lipid-based formulations.

Formulation No.		Control	F1	F2	F3	F4	F5	HLB
Lipid	Sesame oil (v/v, %)	100	50	50	50	40	50	7
	Tri-C8 (v/v, %)	-	30	30	50	40	30	7
Surfactant	Tween® 85 (v/v, %)	-	-	20	-	20	10	11
	Tween® 80 (v/v, %)	-	20	-	-	-	-	15
	Span® 20 (v/v, %)	-	-	-	-	-	10	8.6
	TPGS (mg/mL)	-	10	10	-	10	10	13.2

Tri-C8, triglyceride-C8; TPGS, d-α-Tocopherol polyethylene glycol 1000; HLB, hydrophilic–lipophilic balance.

2.2.3. Preparation CBD in the lipid-free vehicle

This methodology was used to generate experimental that results are described in Chapter 5.

A mixture of 80:10:10 propylene glycol:ethanol:water containing 12 mg/mL of solubilised CBD was used as the lipid-free formulation. CBD solution was firstly prepared by dissolving CBD in ethanol, then glycol was added in CBD solution. The lipid-free formulation was accomplished by adding water to the previously prepared CBD in solution glycol and ethanol at a constant rate to avoid precipitation. The formulation was prepared using a 1000 rpm magnetic stirrer at room temperature.

2.2.4. Preparation CBD in natural vegetable oils

This methodology was used to generate experimental that results are described in Chapter 5.

CBD (12 mg/mL) was solubilised in six different natural vegetable oil vehicles: sesame sunflower, peanut, soybean, olive, coconut and rapeseed oils. All formulations were prepared in the volumetric flask at room temperature and mixed with the magnetic stirrer spinning at 1000 rpm.

2.2.5. Preparation of oil bodies-based formulation

2.2.5.1. Assessment of CBD association with oil bodies

This methodology was used to generate experimental that results are described in Chapter 6.

The CBD stock solution was prepared by dissolving CBD 0.6 mg/mL in ethanol in the volumetric flask at room temperature. The crude oil bodies cream was diluted in 0.9% physiological saline (1 g/mL) to increase oil body mobility.

The association of CBD with diluted oil bodies cream was determined based on a previously reported assay with minor modification [130]. CBD in ethanol stock solutions (20 μL) and 1 mL diluted oil bodies cream were prepared in the glass test tubes, and the association was initiated by magnetic stirring at 400 rpm in a 37 °C water bath for one hour.

2.2.5.2. Purification of oil bodies-based formulation

This methodology was used to generate experimental that results are described in Chapter 6.

Following the association experiment described in section 2.2.5.1, the CBD-loaded oil bodies were separated from the reaction mixture by a density gradient ultracentrifugation method to obtain the oil bodies-based formulation. This method has been modified based on a previous report, 0.145 g KBr was mixed with association reaction mixture to yield density of 1.1 g/mL, then transferred into the polyallomer ultracentrifuge tubes [120,130]. The density gradient solutions were prepared by adding KBr to sterile isotonic saline to achieve final densities of 1.0046, 1.019 and 1.063 g/mL. These gradient solutions were layered on top of the reaction mixture samples in polyallomer ultracentrifuge tubes, following the order of 4 mL 1.063 g/mL, 3 mL 1.019 g/mL and 2 mL 1.0046 g/mL solution from bottom to top to build density gradient layers. Then all samples were centrifuged at 268,350 *g* and 15 °C for 30 min by ultracentrifuge (SORVALL® TH-641 Rotor, Thermo

Fisher Scientific, UK). Following ultracentrifugation, the CBD-loaded oil bodies pellet on the top layer was collected using the spatula and transferred in Eppendorf tubes. A small quantity of distilled water was added to dissolve the CBD-loaded oil bodies pellet until the pellet became a fluid cream. Then the reconstitute CBD-loaded oil bodies cream was stored at 4 °C for further analysis.

2.3. *In vitro* lipolysis assay

2.3.1. Simulated human small intestinal fluid buffer preparation for *in vitro* lipolysis

This methodology was used to generate experimental that results are described in Chapters 4-6.

The lipid digestion process in the GI tract was mimicked by the *in vitro* lipolysis system. The composition of simulated human intestinal fluid digestion buffer at the fasted condition has been reported before [2,120,131]. Briefly, the complete buffer consisted of 50 mM Trizma® maleate, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM sodium taurocholate hydrate and 1.25 mM lecithin in water to mimic fasted state intestinal fluids. The pancreatic lipase extract was prepared in the incomplete digestion buffer, composed of 50 mM Trizma® maleate, 5 mL 150 mM NaCl and 5 mM $CaCl_2$. One gram of pancreatin was blended into the incomplete digestion buffer and vortexed for 15 min at room temperature. The

supernatant of lipase extract was collected after centrifugation at 5 °C, 1160 *g* for 15 min, and then stored on ice.

2.3.2. Assessing lipid-based formulations digestion in *in vitro* lipolysis system

This methodology was used to generate experimental that results are described in Chapters 4-6.

Per oral medicines are assumed to be taken together with approximately 250 mL of water. Thus, the total volume used for *in vitro* lipolysis was scaled down 10 times compared with human parameters. Briefly, 22.2 mL complete digestion buffer containing lipid formulations was pre-mixed at 37 °C water bath for 15 min. To initiate the lipolysis reaction, 2.2 mL lipase extract was then added and a pH adjustment within a range of 6.8 ± 0.05 was obtained using 1 M NaOH solution by means of pH-stat titrator (LabX light v3.1, T50 Graphix, Mettler Toledo Inc., Leicester, UK). The reaction was assumed as complete when the addition rate of 1 M NaOH was slower than 3 μL/min or terminated following 1 h reaction.

The final lipolysis medium was collected and ultra-centrifuged at 268,350 *g*, 37 °C for 1.5 h. As a result, three distinct layers were obtained: the top layer (the undigested lipid phase), the middle layer (aqueous micellar phase) and the bottom layer (sediment phase). The top

layer was collected using glass Pipettes and stored in scintillation vials, while the aqueous micelle phase was directly transferred into the Falcon tubes. The bottom precipitation was reconstituted with 2 mL of water and transferred into new Falcon tubes. All three lipolysis fractions have been stored at –80 °C for further analysis.

2.4. Animal experiments

2.4.1. Animals

This methodology was used to generate experimental that results are described in Chapters 3-6.

Animal welfare and all experimental procedures were reviewed and authorised by the United Kingdom Home Office and University of Nottingham Ethical Review Committee in accordance with the Animals [Scientific Procedures] Act 1986. Male Sprague Dawley rats (340 to 380 g, Charles River Laboratories, UK) were used for *in vivo* pharmacokinetic and biodistribution studies. All animals were housed in the University of Nottingham Bio Support Unit under regulated temperature and humidity, 12 h light-dark cycle, with unrestricted access to water and food.

2.4.2. Jugular vein cannulation for *in vivo* pharmacokinetics study

This methodology was used to generate experimental that results are described in Chapters 3-6.

Prior to the experiments, all rats were weighed and placed in an anaesthetised chamber. When no righting reflex was observed, the rat was placed on a warm platform with the continuously supplied isoflurane. Warmed saline (4 mL) and 1 kg/mL meloxicam were then injected subcutaneously to replenish body fluids and reduce the pain and inflammation post-surgery, and eye gel was applied to keep eyes moist. The upper body of rats was shaved to expose the right ventral and dorsal necks, and the skin surface was cleaned using 70% isopropyl ethanol wipes. Rat was wrapped and ready for the surgery, isoflurane anaesthesia was maintained throughout the operation.

A 1 to 1.5 cm incision was made with scissors in the ventral neck where the pulsation one the right neck can be observed. Then the jugular vein was exposed by blunt dissection with gentle tissue scissors, and the surrounding tissues were cleaned using forceps and cotton tips. Two lengths of 4-0 silk surgical were thread placed under the jugular vein, the longer one closer to the head and the shorter one further away. The wound was moistened with warm saline before turning the rat to the opposite side. A 0.5 cm incision was made on the

dorsal neck, and the cannula was pulled subcutaneously from the dorsal neck to the front wound using a guide, before turning back the rat.

The longer thread closer to the head side was ligated and stabilised on the table, while the shorter thread was tied in a semi-closed loop. A tiny incision was made on the surface of the jugular vein with the spring scissors and forceps between two threads, and the cannula flashed within heparinised saline was inserted into the jugular vein from the incision. The prepared semi-closed loop was promptly ligated after ensuring that the cannula was properly functioning by injecting and withdrawing heparinised saline. The longer thread was then used to secure the cannula along the jugular vein, and the incisions were sewn up on both sides of the vein using 4-0 silk suture. The section of the cannula exposed outside the body from the dorsal neck wound allows blood collection, and then a metal pin was plugged into the end of the cannula to prevent blood leakage. Rats for pharmacokinetic studies underwent jugular vein cannulation surgery and were allowed to recuperate for two nights before the *in vivo* pharmacokinetics experiment.

2.4.3. *In vivo* pharmacokinetics assay

This methodology was used to generate experimental that results are described in Chapters 3-6.

Table 2-2. Formulation summary for *in vivo* pharmacokinetics studies.

Chapter	Vehicles	CBD concentration (mg/mL)	Dose (mg/kg)	PK time points (h)	n
3	Sesame oil, pre-digested lipids and purified lipids	12	12	1, 2, 3, 4, 5, 6, 8, 10, 12	6
4	Sesame oil and F1 to F5 (Table 2-1)	50	25	0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12	6
5	Lipid-free and natural vegetable oils	12	12	1, 2, 3, 4, 5, 6, 8, 10, 12	6
6	Rapeseed oil	12	12	1, 2, 3, 4, 5, 6, 8, 10, 12	6
	Oil body	6			

CBD, cannabidiol; PK, pharmacokinetics

All formulations were prepared freshly on the day of the experiments and administered by oral gavage. Post-surgery animals have fasted one night up to 16 hours before oral administration of the tested formulations. Formulations were administered based on the body weights. For pharmacokinetics study, 0.2 mL blood was withdrawn from cannula at pre-selected time points following drug administration and collected into 20 mM K_3EDTA-containing Eppendorf tubes. CBD concentrations in different vehicles, administration doses and time points for blood collection for individual chapters are summarised in **Table 2-2**. Plasma samples were obtained from the blood samples by centrifugation at 3000 *g* for 10 min and kept at –80 °C until analysis. Phoenix WinNonlin Version 6.3 software

(Pharsight, Mountain View, USA) was used to calculate the pharmacokinetic parameters by a non-compartmental approach.

2.4.4. *In vivo* biodistribution study

This methodology was used to generate experimental that results are described in Chapters 3-6.

Biodistribution studies were carried out based on the plasma t_{max} determined in pharmacokinetic studies. All rats were housed with free access to water and fasted overnight up to 16 hours before the biodistribution experiment. All formulations were prepared on the day of the experiment and administered the same manner as for the pharmacokinetic studies. Animals were sacrificed humanely at 1.5 or 2.5 hours post-dosing for the experiment in Chapter 4 and at plasma t_{max} or one hour prior to t_{max} (t_{max} - 1 h) following formulations feeding for studies in Chapters 3, 5 and 6. Approximately 50 µL of lymph fluid was withdrawn from the superior mesenteric lymph duct using a 1 mL syringe. Mesenteric lymph nodes (MLN) were harvested and carefully isolated from the surrounding tissue. Blood samples from the posterior vena cava were then collected, and serum samples were isolated by centrifugation (3000 g, 10 min). All obtained samples were stored at –80 °C until analysis. In addition, after the completion of pharmacokinetic study at 12 hours, animals were sacrificed, and MLN were collected for further analysis.

2.5. Bioanalytical procedures

2.5.1. *In vitro* lipolysis sample preparation for HPLC-UV

This methodology was used to generate experimental that results are described in Chapters 4-6.

The sample preparation procedure was performed using a combination of protein precipitation and liquid-liquid extraction before injection into HPLC system as previously described [2,120]. Briefly, fifty microliter of lipid phase, 100 μL of micelle phase and 100 μL sediment phase were used for sample preparation in 16 × 100 mm glass test tube. Ten microliters of 2 mg/mL probucol in acetonitrile stock solution as the internal standard (IS) was spiked into lipid phase, and 1 mg/mL into micelle and sediment phases. Then 0.3 mL tetrahydrofuran and 3 mL *n*-hexane were added into test tube to extract CBD. After 3 min vortex, the test tubes were centrifuged at 1160 *g* for 10 min at room temperature. The upper organic layer was transferred to a clean test tube and evaporated under a gentle stream of nitrogen gas (Techne Dri-Block Sample Concentrator, Cambridge, UK) at 35 °C to dryness. The dry residue was then reconstituted in 1 mL acetonitrile for all phases, and 20 μL sample was injected into HPLC system.

2.5.2. Determination CBD concentration in oil body-based formulation by HPLC

This methodology was used to generate experimental that results are described in Chapter 6.

Purified oil body-based formulation (described in section 2.2.5.2) was diluted 1000 times in water, and 100 µL of diluted oil body formulation sample was used for the liquid-liquid extraction method to measure CBD concentration. The preparation procedure for determining CBD in oil body-based formulation using HPLC was the same as the method described in section 2.5.1.

2.5.3. Animal tissue sample preparation

This methodology was used to generate experimental that results are described in Chapters 3-6.

The sample preparation procedure for plasma (or serum) and tissue samples was performed using a combination of protein precipitation and liquid-liquid extraction before injection into HPLC system as previously described [1,2,129,132]. Briefly, for the determination of CBD in serum or plasma, 10 µL of DDT stock solution (IS, 50 µg/mL) in acetonitrile was spiked into 100 µL of the serum or plasma samples. Cold acetonitrile (450 µL) was added

for protein precipitation and the sample was vortex-mixed, followed by addition of 450 µL water. The drug and IS were extracted by vortex-mixing with 3 mL of *n*-hexane for 5 min. Following the centrifugation at 1160 *g* for 10 min, the upper organic phase was transferred to a clean test tube and evaporated to dryness under nitrogen at 37 °C. The residue was reconstituted in 100 µL acetonitrile and 40 µL sample was injected into the HPLC system.

Due to very high concentrations of CBD in lymph fluid, a 10 µL lymph fluid sample was diluted into 90 µL of blank rat plasma. The isolated MLN were homogenized (POLYTRON® PT 10–35 GT, Kinematica AG, Luzern, Switzerland) with HPLC-grade water in the ratio of 1:2 (w/v). Then 100 µL of diluted lymph fluid or MLN homogenate samples were used for HPLC sample preparation in the same manner as serum or plasma samples above.

2.5.4. Sample preparation for fatty acid analysis

2.5.4.1. Separation of chylomicrons from lymph fluid sample

This methodology was used to generate experimental that results are described in Chapter 5.

The chylomicrons were separated from lymph fluid samples by density gradient ultracentrifugation, using a methodology modified from a previously published paper [130].

The lymph fluid sample was obtained from the biodistribution study, as described in section 2.4.4. Due to a limited and variable volume of lymph fluid that can be collected from each rat, the sample was firstly diluted using various volumes of phosphate-buffered saline (PBS) up to 1 mL. Then 1 mL diluted lymph fluid samples were mixed with 0.1425 g KBr to achieve 1.1 g/mL final density. A density gradient was then built by dissolving appropriate amounts of KBr in PBS buffers with densities of 1.006, 1.019 and 1.063 g/mL. The chylomicrons were separated by density gradient ultracentrifugation (SORVALL® TH-641 Rotor, Thermo Fisher Scientific, 268,350 *g*, 35 minutes, 15°C), and the top layer of approximately 1 mL was collected. The chylomicron samples were stored at 4 °C for further analysis.

2.5.4.2. Triglyceride extraction from chylomicrons

This methodology was used to generate experimental that results are described in Chapter 5.

The triglyceride was extracted from the separated chylomicrons samples (section 2.5.4.1) using the lipid extraction method. In brief, separated chylomicron samples (1 mL) were vortex-mixed for 1 min with 2.4 mL chloroform/methanol mixture (2:1, v/v) and 0.6 mL 0.9% (w/v) NaCl solution. Following centrifugation at 1160 *g* and 4 °C for 10 min, three distinct layers were obtained: upper layer (clear liquid waste), solid debris (protein) and lower layer (lipids in solvent). The lowest layer, which contained triglyceride, was

transferred to a clean glass test tube. The remaining mixture was extracted again by adding 2.4 mL 2:1, v/v chloroform/methanol mixture (without any additional saline) and transferring and combining lower phase from all extractions, followed by centrifugation. Then the second remaining mixture was extracted a third time using the same procedure. All lower phases were collected and then evaporated to dryness under nitrogen at 37 °C. The dried residue was reconstituted in 500 µL chloroform for further analysis.

2.5.4.3. Sample preparation for the determination of fatty acids composition

This methodology was used to generate experimental that results are described in Chapters 5-6.

Fatty acid compositions were determined for vegetable oils and chylomicrons samples. Approximately 0.01 g vegetable oil was dissolved in 2 mL chloroform. Then 1 mL of the vegetable oil and chloroform mixture or 500 µL chylomicrons extract in chloroform (as described in Section 2.5.4.2) were added into 200 µL trimethylsulfonium hydroxide solution (0.25 M in methanol) for the hydrolysis of triglyceride and subsequent methylation of fatty acids. The reconstituted chylomicrons in chloroforms samples (300 µL), as described in section 2.5.4.2, were also prepared with 200 µL trimethylsulfonium hydroxide (TMSH) solution. After 10 min reaction, the lipid extracts were filtered through a 0.45 µm filter and analysed using Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS) [133].

2.5.5. Triglyceride level determination

This methodology was used to generate experimental that results are described in Chapters 3-6.

The triglyceride level in *in vitro* lipolysis fractions (section 2.3.2), serum and lymph fluid samples (section 2.4.4) was measured, and performed using a commercially available kit (TR0100, Sigma, Gillingham. UK) following the manufacturer's instructions. The Free Glycerol Reagent, Triglyceride Reagent and Glycerol Standard were provided in the commercial kit. Water was used as the reference in this study. Free Glycerol Reagent (200 μL) was firstly added into the 96-well plate (flat well shape), and 2.5 μL of reference (water), Glycerol Standard, and samples were pipetted in the plate, respectively. The plate was incubated at 37 °C for 5 mins followed by a gentle mixture. The absorbance of reference, standard and samples were measured at 540 nm wavelength by UV multilabel reader (PerkinElmer® EnVision® 2104 Multilabel Reader) for the initial absorbance (IA), and the glycerol concentration was calculated as:

$$\frac{(\text{IAsample} - \text{IAblank})}{(\text{IAstandard} - \text{IAblank})} \times \text{concentration of standard}$$

Then 50 μL of reconstituted Triglyceride Reagent was added to each reference, standard and samples, and mixed properly to continue incubating at 37 °C for 5 mins. The UV for

all samples were measured again at 540 nm for final absorbance (FA), and the total triglyceride concentration was calculated as:

$$\frac{(\text{FAsample} - \text{FAblank})}{(\text{FAstandard} - \text{FAblank})} \times \text{concentration of standard}$$

The final true triglyceride concentration was based on glycerol concentration and total triglyceride concentration, and calculated as (when F = 0.81/1.01 = 0.8):

$$\frac{(\text{FAsample} - (\text{IAsample} \times \text{F}))}{(\text{FAstandard} - (\text{IAblank} \times \text{F}))} \times \text{concentration of standard}$$

2.6. Analytical conditions

2.6.1. HPLC conditions for *in vitro* and formulation samples

This methodology was used to generate experimental that results are described in Chapters 4-6.

The analysis of CBD in *in vitro* lipolysis fractions (section 2.5.1) and oil body formulation samples (section 2.5.2) were performed by means of a validated HPLC method [2,120]. The system consisted of Waters 600 Pump, Waters 717 Autosampler and Waters 2996 Photodiode Array Detector. The CBD and IS (probucol) were detected at 220 nm wavelength. For lipolysis fractions and oil body formulation samples, the separation was

achieved using an ACE Excel Super C18 100 × 4.6 mm, 5 μm particle size column, protected by an ACE C18-PFP 3 μm guard cartridge. The mobile phase was a mixture of acetonitrile and water in a ratio of 92:8 (v/v) at isocratic conditions. The flow rate was set at 0.6 mL/min and 43 °C for column temperature. The lower limit of quantification of CBD was 20 ng/mL all samples. Retention times of CBD and IS (probucol) were 6.5 and 12.5 min, respectively.

2.6.2. HPLC conditions for animal samples

This methodology was used to generate experimental that results are described in Chapters 3-6.

The analysis of CBD in rat plasma, serum, lymph fluid and lymph nodes were performed by HPLC system (Waters Alliance 2695 separations module coupled with a Waters 996 photodiode array detector. For rat plasma, serum and tissue samples, separation was achieved using ACE C18-PFP 150 × 4.6 mm, 3 μm column, protected by an ACE C18-PFP 3 μm guard cartridge, as previously reported) [1,2,129,132]. The mobile phase consisted of acetonitrile: water (62:38, v/v) at isocratic conditions. Flow rate was set at 1 mL/min, column temperature was maintained at 55 °C, and injection volume was 40 μL. The CBD and IS (DDT) were detected at 220 nm wavelength. Retention times of CBD and IS (DDT) were 8.7 and 22 min, respectively. The lower limit of quantification of CBD was 10 ng/mL for plasma, serum and lymph fluid samples, and 20 ng/mL for MLN.

2.6.3. GC-MS/MS conditions for the determination of fatty acids composition

This methodology was used to generate experimental that results are described in Chapters 5-6.

The composition of the fatty acids was assessed by means of GC-MS/MS (Trace GC Ultra; Thermo Scientific) equipped with an auto-injection system (AS3000) and coupled to a quadrupole mass spectrometer (DSQ II Quadrupole GC-MS/MS; Thermo Scientific). Fatty acid determination conditions in GC-MS/MS have been slightly modified from previously published works [133,134]. The capillary column (Phenomenex Zebron ZB-FFAP, 30 m x 0.22 mm internal diameter, 0.25 μm film thickness) was used, 10 μL of each sample was injected. The oven temperature was initially held at 80 °C for 1 minute and then increased to 250 °C at a rate of 5 °C/min for 2 minutes. The spit flow of the carrier gas (helium) was 25 mL /min. Identification was obtained by comparing the mass spectra with a standard library through the Thermo Scientific Xcalibur software program (NIST/EPA/NIH Mass Spectral Library, Version 2.3, NIST 17, Gaithersburg, MD, USA).

2.7. Data analysis

This methodology was used to generate experimental that results are described in Chapters 3-6.

All results ($n \geq 3$) were presented as mean ± standard deviation (SD). All graphs and statistical analysis were generated using GraphPad Prism v9.2d (GraphPad software, San Diego, CA, USA). Phoenix WinNonlin 6.3 Professional was applied for non-compartmental pharmacokinetic analysis of the obtained plasma concentrations. One-way analysis of variance (ANOVA) was performed for three or more experimental groups, followed by Dunnett's post-hoc comparison or Tukey's multiple comparisons tests to evaluate the statistical significance of differences. In addition, the two-tailed unpaired Student *t-test* was applied for statistical analysis to determine differences between two experimental groups. The results were considered to be statistically significant when the *p*-values were less than 0.05.

3. NATURAL SESAME OIL IS SUPERIOR TO PRE-DIGESTED LIPID FORMULATIONS AND PURIFIED TRIGLYCERIDES IN PROMOTING THE INTESTINAL LYMPHATIC TRANSPORT AND SYSTEMIC BIOAVAILABILITY OF CANNABIDIOL [1]

The results described in this Chapter have been published by the *European Journal of Pharmaceutics and Biopharmaceutics:*

W. Feng, C. Qin, Y.J. Chu, M. Berton, J.B. Lee, A. Zgair, S. Bettonte, M.J. Stocks, C.S. Constantinescu, D.A. Barrett, P.M. Fischer, P. Gershkovich, Natural sesame oil is superior to pre-digested lipid formulations and purified triglycerides in promoting the intestinal lymphatic transport and systemic bioavailability of cannabidiol, Eur. J. Pharm. Biopharm. 162 (2021) 43–49.

3.1. Introduction

The oral route is the preferred method of administration of drugs due to convenience and patient compliance. Many natural compounds and synthetic drugs or drug candidates can possess high lipophilicity and poor water solubility. Despite pharmacological activity *in vitro*, oral bioavailability of such lipophilic molecules is usually low, which limits further

development of these compounds. Lipid-based formulations represent a common approach to increase bioavailability of lipophilic drugs [135–140].

Due to the poor water-solubility of lipophilic compounds, bile surfactants can promote the micellar solubilisation in the intestinal lumen and absorption of lipophilic drugs [38]. The main organic solutes secreted in bile are bile acids, phospholipids, and cholesterol. These molecules improve the solubility of lipophilic drugs by forming mixed micelles that diffuse to the membrane of the enterocyte. However, the capacity of this mechanism is limited without the presence of dietary lipids or pharmaceutical lipid excipients [141,142].

Most dietary lipids and pharmaceutical lipid excipients are not absorbed intact following oral administration. Instead, they undergo a complicated digestion and intraluminal processing in the upper gastrointestinal (GI) tract. The most common dietary lipids and pharmaceutical lipid excipients are triglycerides of different chain lengths. Following oral administration, triglycerides are partially digested in the stomach by gastric lipases, which preferentially hydrolyse the ester bonds of triacylglycerol, producing free fatty acids and diglycerides [38,39]. When remaining triglycerides and diglycerides are released into the duodenum, pancreatic secretions in the small intestine continue the triglyceride digestion process. The sn1- and sn3- ester bonds of triglycerides are hydrolysed by pancreatic lipase to yield fatty acids and 2-monoacylglycerol [42].

The amphiphilic products of the lipid digestion process, together with bile salts and phospholipids, form mixed micelles. Lipophilic drugs co-administered with dietary lipids or with lipid excipients frequently associate with the lipophilic core of mixed micelles. These micelles diffuse through the unstirred aqueous layer to the membrane of the enterocytes. It is believed that due to the lower pH near the membrane the mixed micelles disassemble and monomers cross the membrane of the cell [38,39,48,49]. The long-chain fatty acids and monoglycerides are re-esterified to triglycerides that form a hydrophobic core of large lipoproteins (chylomicrons), with which lipophilic drugs can associate and therefore can be transported to the intestinal lymphatic system rather than the portal vein [143,144].

Previous experiments have shown that oral administration of cannabidiol (CBD) together with sesame oil improves the bioavailability and lymphatic transport of CBD [120,129]. CBD is one of the main components of cannabis and is metabolized extensively in animals and humans, and has no psychiatric effects, as opposed to other phytocannabinoids [145]. CBD potentially has high medicinal value and has been reported to be of therapeutic benefit in many types of disease, such as cancer, anxiety, schizophrenia, and immune system disorders [106,146–149]. However, the oral bioavailability of CBD is limited by its poor water solubility and substantial hepatic first pass metabolism, whereby it is metabolised by oxidation predominantly by CYP3A4 and CYP2C19 [150,151].

Lipid-based drug delivery is a promising approach to improve the oral bioavailability of CBD. Zgair *et al.* have previously reported that when CBD is co-administered orally with sesame oil, the bioavailability of the drug is around 3-fold higher compared to administration without lipids [120]. A subsequent study demonstrated that the levels of CBD in lymph fluid were 250-fold higher than in plasma and substantially above the immunomodulation activity threshold [129]. Furthermore, both *in vitro* and *in vivo* studies have shown that CBD can alleviate the symptoms of inflammation caused by auto-immune diseases through its effects on T lymphocytes and its ability to decrease the release of cytokines in the lymphatic system [129,152]. It has been shown that CBD can reduce the levels of pro-inflammatory cytokines TNF-α and IL-1b of cells from immune cells at high concentrations of 20 μg/mL and microglial cells at a concentration above 1 μM [106,129]. Thus, targeting CBD to the immune cells within the lymphatic system can have a great significance for the treatment of autoimmune diseases.

Even though the co-administration of CBD with digestible vegetable (sesame) oil leads to high concentrations within the intestinal lymphatic system and higher bioavailability compared to lipid-free formulation, this is associated with substantial variability [120,129]. Due to the complexity of the lipid digestion process in the intestinal lumen, we have hypothesized that the intraluminal digestion could be the rate-limiting step in the processing of the formulation and the absorption of CBD through the lymphatic system, and thus, a source of variability. Therefore, to reduce the variability and increase the efficiency of the lymphatic transport of CBD we have designed a library of pre-digested

formulations of CBD consisting of excipients which are a product of intestinal digestion of triglyceride. These formulations do not have to go through digestion process in the intestine to facilitate the absorption and lymphatic transport of CBD, and if hypothesis is correct, should lead to lower variability and potentially to enhanced lymphatic transport and bioavailability of CBD.

Sesame oil mostly contains linoleic and oleic acids, and these two fatty acids are the primary fats obtained from the natural human diet [153,154]. Many studies have reported that these long-chain fatty acids can facilitate the delivery of the lipophilic drug through the intestinal lymphatic system [130,142,155–157]. However, there is a limited number of reports that directly compare the natural vegetable oil and the corresponding free fatty acids and monoglycerides, or purified triglyceride for the enhancement of the intestinal lymphatic transport and oral bioavailability of lipophilic drugs [158].

Therefore, the aim of this study was to test the hypothesis that pre-digested lipid formulations could reduce variability and increase the extent of the intestinal lymphatic transport and oral bioavailability of CBD compared to digestible natural vegetable oil or purified triglyceride (Figure 3-1).

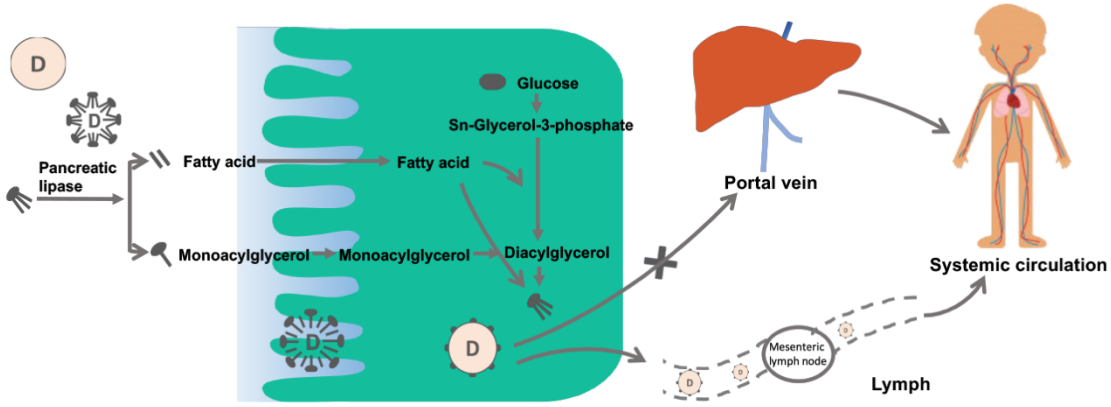


Figure 3- 1 . The schematic description of lymphatic transport of lipid-based formulations. D: drug.

3.2. Experimental design

CBD was formulated in pre-digested and digestible lipid-based formulations and the preparation of formulations is described in Section 2.2.1. All formulations were assessed by *in vivo* pharmacokinetics and biodistribution studies in rats as described in Section 2.4. Biological samples from *in vivo* experiments were prepared for HPLC analysis. The sample prepare procedure is described in Section 2.5.2, and HPLC conditions in Section 2.6.2. The triglyceride levels were determined in *in vivo* samples as described in Section 2.5.5. The experimental design is summarised in the general scheme flowchart and presented in Figure 3-2.

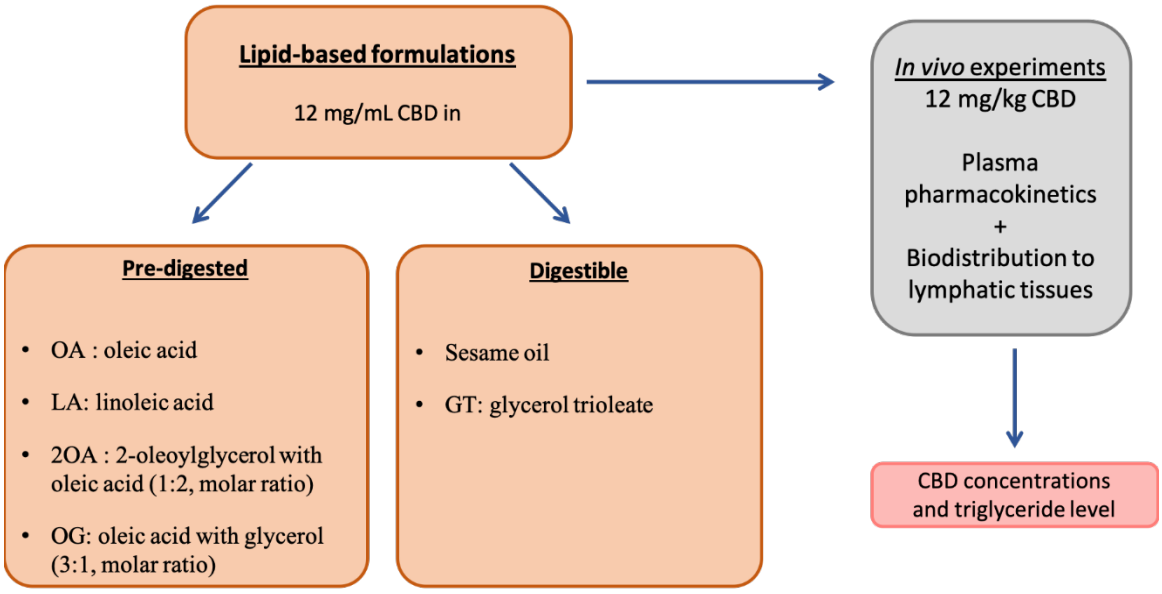


Figure 3- 2 . Flowchart of experimental design of comparing the bioavailability and lymphatic targeting of CBD in co-administered pre-digested and digestible lipid-based formulations. CBD, cannabidiol.

3.3. Results

3.3.1. *In vivo* pharmacokinetics of CBD administered in different lipid-based formulations

Plasma concentration-time profiles of CBD following oral administration of six tested lipid-based formulations are shown in Figure 3-2. The pharmacokinetic parameters derived from plasma concentration-time profiles are summarised in Table 3-1. There was no statistically significant difference in the area under the plasma concentration-time curve (AUC) for pre-digested lipid-based formulations or purified triglyceride in comparison to the sesame oil group. However, the C_{max} was significantly lower for linoleic acid (LA) and

2-oleoylglycerol with oleic acid (2OA) vehicles, and t_{max} was substantially prolonged and variable in 2OA group.

3.3.2. Bio-distribution of CBD into mesenteric lymph, MLNs and serum following oral administration in different lipid-based formulations

The bio-distribution of CBD into serum, mesenteric lymph and MLNs was assessed following oral administration of sesame oil, oleic acid, linoleic acid, oleic acid with glycerol and glycerol trioleate formulations. Oleic acid with 2-oleoylglycerol (2OA) vehicle did not proceed to the biodistribution assessment stage due to the extremely unfavourable plasma pharmacokinetic profile of CBD administered in this formulation, especially prolonged and variable t_{max} (Figure 3-3 and Table 3-1). The animals were sacrificed, and all samples were collected at plasma t_{max} and one hour prior to t_{max} (t_{max} – 1h) following oral administration. The triglyceride levels and CBD concentrations were measured in the rat serum and lymph fluid and shown in Figure 3-4 and 3-5, respectively. Administration of sesame oil formulation resulted in higher triglyceride serum concentrations compared to the oleic acid group, the glycerol with oleic acid group and the glycerol trioleate group at t_{max} – 1 hour (Figure 3-4(a)). The triglyceride concentrations in lymph were also the highest following the administration of sesame oil compared to all other groups at t_{max} (Figure 3-5(b)). There were no statistically significant differences in

CBD concentrations in serum or lymph fluid samples at both time points for the sesame oil group in comparison to the other formulation groups.

Table 3-1. Pharmacokinetic parameters of cannabidiol (CBD) calculated from plasma concentrations-time profiles following oral administration.

Formulations	$t_{1/2}$ [a,b] (h)	t_{max} [c] (h)	C_{max} [a,d] (ng/mL)	$AUC_{0-\infty}$ [a,e] (h*ng/mL)	n
Sesame oil	1.9±0.4	4	225±112	821±296	6
Oleic acid (OA)	2.6±0.9	3	134±70	604±140	6
Linoleic acid (LA)	2.1±0.9	4	108±63*	602±248	6
2-Oleoylglycerol with oleic acid (2OA)	3.1±1.7	6	71±18**	512±163	6
Oleic acid with glycerol (OG)	2.9±0.8	3	125±44	584±160	5
Glycerol trioleate (GT)	1.9±0.6	3	154±74	560±229	7

[a] Mean ± SD (*n*=5-7).

[b] Half-life.

[c] Time to maximum concentration in plasma.

[d] The maximum concentration in plasma.

[e] AUC from 0 to infinite time.

*, ** are statistically significantly different from sesame oil (*, p <0.05, **, p <0.01).

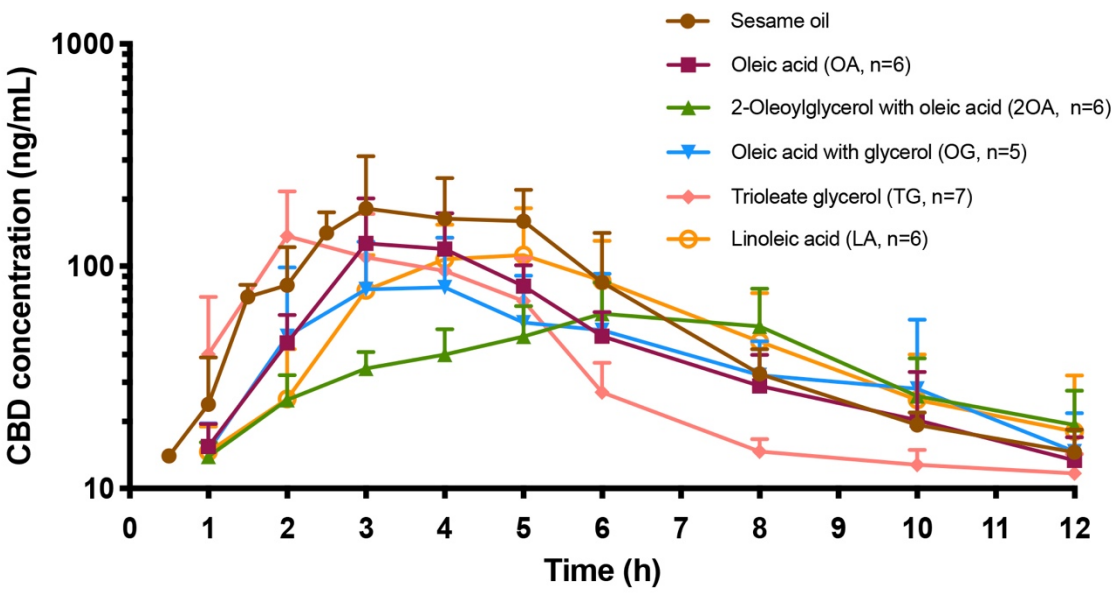


Figure 3-３. Plasma concentration-time profile of CBD (mean ± SD) following oral gavage administration of 12 mg/mL CBD solution in sesame oil, glycerol trioleate (GT), oleic acid (OA), linoleic acid (LA), oleic acid with 2-oleoylglycerol (2:1, molar ratio, 2OA), and oleic acid with glycerol (3:1, molar ratio, OG). The CBD dose was 12 mg/kg for all formulations.

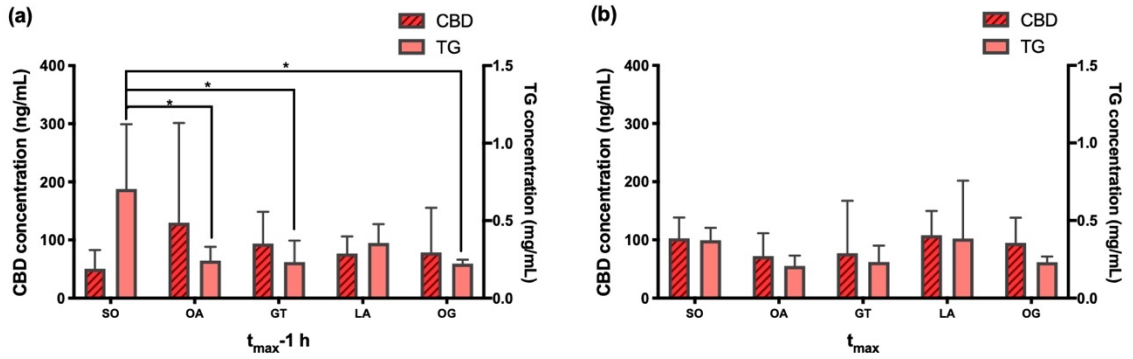


Figure 3-４. Triglyceride (TG) and CBD concentrations in serum. SO, sesame oil; OA, oleic acid; GT, glycerol trioleate; LA, linoleic acid; OG, oleic acid with glycerol (3:1, molar ratio). CBD was orally administered in lipid-based formulations at a dose of 12 mg/kg to rats. (a) The concentration of CBD and triglyceride level in rat serum at one-hour prior to t_{max} (t_{max} – 1 h). (b) The concentration of triglyceride and CBD in rat serum at t_{max}. All data are presented as mean ± SD, *n=4*. Statistical analysis was

performed using one-way ANOVA with Dunnett's multiple comparisons test. All experimental groups were compared to the sesame oil group. * p <0.05.

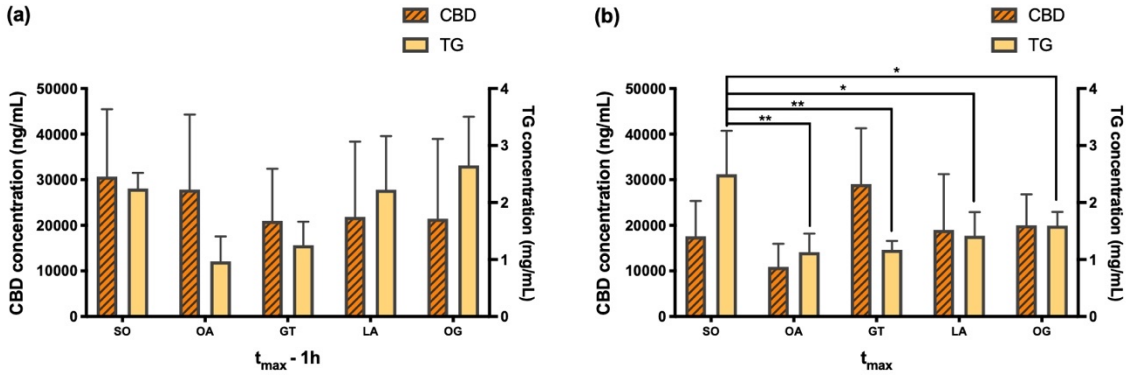


Figure 3-5. Triglyceride (TG) and CBD concentrations in lymph fluid. SO, sesame oil; OA, oleic acid; GT, glycerol trioleate; LA, linoleic acid; OG, oleic acid with glycerol (3:1, molar ratio). CBD was orally administered in lipid-based formulations at a dose of 12 mg/kg to rats. (a) The concentration of triglyceride and CBD in lymph fluid at one-hour prior to t_{max} (t_{max} – 1 h). (b) The concentration of triglyceride and CBD in lymph fluid at t_{max}. All data are presented as mean ± SD, *n=4*. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. All experimental groups were compared to the sesame oil group. * p < 0.05, ** p < 0.01.

The levels of CBD in MLN are shown in Figure 3-6. There were no significant differences in CBD concentrations in MLN following administration of all pre-digested lipid-based formulations or purified lipid-based formulation compared to sesame oil vehicle at both time points.

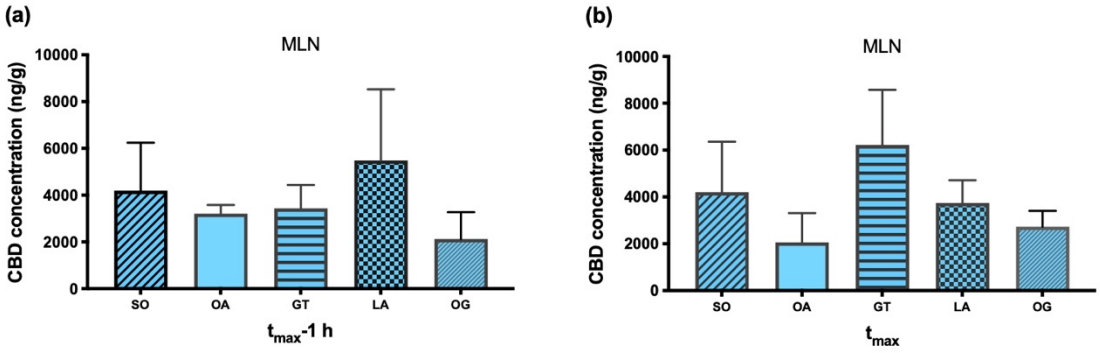


Figure 3- 6 . Distribution of cannabidiol (CBD) into mesenteric lymph nodes (MLN). SO, sesame oil; OA, oleic acid; GT, glycerol trioleate; LA, linoleic acid; OG, oleic acid with glycerol (3:1, molar ratio). CBD was orally administered in lipid-based formulations at a dose of 12 mg/kg to rats. (a) Concentrations of CBD in MLN at one-hour prior to t_{max} (t_{max} – 1 h). (b) Concentrations of CBD in MLN at t_{max}. All data are resented as mean ± SD, *n=4*. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. All experimental groups were compared to the sesame oil group.

3.4. Discussion

It has been reported previously that lipid-based formulations can increase the intraluminal solubility of lipophilic drugs and enhance the drug absorption. Moreover, oral administration of highly lipophilic drugs in lipid-based formulations containing long-chain triglycerides or long-chain fatty acids results in intestinal lymphatic transport and therefore avoids hepatic first-pass metabolism [142,159]. CBD is a highly lipophilic drug which has high affinity to both rat and human chylomicrons and extensive intestinal lymphatic transport when administered orally with a long-chain triglyceride vehicle (sesame oil) [120,129]. This leads to extremely high concentrations of the drug within the mesenteric

lymph fluid and mesenteric lymph nodes and enhanced immunomodulation [129]. However, despite the efficiency of the sesame oil vehicle in promoting the intestinal lymphatic transport of CBD, it is also associated with considerable variability in intestinal lymphatic transport and bioavailability [129].

The digestion of triglycerides in the intestinal lumen is a complicated multi-step process. Therefore, we have hypothesized that the digestion of lipids in the intestinal lumen is a primary source of variability observed in the oral bioavailability and intestinal lymphatic transport of the highly lipophilic drug CBD [120,129]. Therefore, in this work, pre-digested lipid-based formulations were investigated and compared to the natural sesame oil-based formulation, as well as purified triglyceride vehicle.

It was found that the C_{max} of CBD following the administration of the digestible sesame oil-based formulation was higher than for pre-digested lipid-based formulations (Table 3-1). However, there is no statistically significant difference in AUC between pre-digested lipid-based formulations or purified triglyceride versus sesame oil-based formulation (Table 3-1). Moreover, variability similar to that of the sesame oil vehicle has been found in the linoleic acid and glycerol trioleate groups. Thus, pre-digested lipids or purified triglyceride have not reduced the variability and have not improved the oral bioavailability of CBD compared to natural sesame oil. In fact, quite consistently, a tendency for higher AUC (although not statistically significant) was observed in the natural sesame oil group compared to all other pre-digested or purified vehicles. A lack of statistical significance in

this observed tendency could be largely attributed to high variability, especially in the sesame oil, linoleic acid and purified triglyceride groups.

Unlike other pre-digested lipid-based formulation groups, in case of 2-oleoylglycerol with oleic acid-based formulation the CBD absorption has been substantially delayed. It is unclear at this stage why the 2-oleoylglycerol excipient led to delayed and variable absorption of CBD. However, due to the unfavourable effect of this specific vehicle on plasma pharmacokinetics of CBD, this formulation did not proceed for further biodistribution studies.

The biodistribution studies were carried out at plasma t_{max} and t_{max} -1 hour time points. It has been previously reported that the maximum concentration in lymph for drugs with substantial intestinal lymphatic transport appears at earlier time point compared to the peak concentrations in plasma [129]. In addition to determination of CBD, triglyceride concentration has been also measured in lymph fluid and serum samples to assess the correlation between the absorption of CBD and the co-administered lipids.

In the current study, administration of natural sesame oil-based formulation resulted in the highest CBD concentrations and highest levels of TG in serum at one hour prior to t_{max} (Figure 3-4(a)) and lymph fluid at t_{max} (Figure 3-5(b)) compared to all other formulations. There are no significant differences in the CBD levels in the mesenteric lymph node tissues for purified triglyceride or pre-digested formulations compared to the sesame oil-based

formulation (Figure 3-6). However, there is still a substantial variability that could be seen for all lipid-based formulations, and neither pre-digested lipid-based formulations nor trioleate vehicle improve the CBD levels in intestinal lymphatic system compared to sesame oil-based formulation.

In theory, the pre-digested lipids do not have the need for hydrolysis by intestinal lipases and instead can directly form mixed micelles together with endogenous bile salts and phospholipids. This could lead to faster and more efficient absorption of co-administered lipophilic drug with lower variability. However, the t_{max} for the linoleic acid group was the same as the sesame oil group, and t_{max} for oleic acid group and glycerol with oleic acid group was the same as for trioleate glycerol (Table 3-1). Therefore, the intraluminal digestion step does not seem to affect substantially the rate of absorption of lipids and of co-administered CBD.

In this study, pre-digested lipids or purified triglycerides have not improved the CBD bioavailability and lymphatic transport compared to the natural vegetable (sesame) oil. Moreover, taken together, the results suggest that both the absorption of lipids and the absorption of co-administered CBD was more efficient following administration of natural sesame oil vehicle than of pre-digested lipids or purified trioleate.

Although the exact reasons for better performance of natural sesame oil vehicle compared to pre-digested lipids or purified trioleate are unclear, there could be several potential

explanations. Sesame oil is a natural oil containing various lipids, mainly triglycerides and phospholipids. Triglycerides present in sesame oil have different chain lengths and degrees of saturation [154,160–162]. It is possible that the various triglyceride composition in sesame oil might provide a more favourable composition for mixed micelles in the intestinal lumen compared to a single type of triglyceride. Even though the levels of phospholipids in sesame oil are relatively low, they might facilitate emulsification of the triglycerides in the intestinal lumen and formation of mixed micelles.

Moreover, there are several minor constituents in the natural plants and seed oils that may have bioactive properties that can affect the absorption of lipids and co-administered drugs. These minor, mostly small molecule constituents can have protective antioxidant effect on the triglyceride, but also can potentially serve as co-factors in multiple key stages of absorption of lipids, such as lipid digestion or chylomicron assembly. Among such well-known minor components of natural lipids are tocopherols. Notably, α-tocopherol and γ-tocopherol are the most abundant tocopherols present in natural oil [163,164]. The levels of tocopherols in sesame oil varies from 0.21 to 0.8 g/kg oil [165]. The absorption of α-tocopherol and γ-tocopherol is similar to that of lipids, in association with the mixed micelles in the intestinal lumen [166]. The tocopherols are regarded as fat soluble antioxidants preventing the unsaturated fatty acids oxidation [164].

Another potential minor constituent identified in the plant and vegetable oils are phytoestrogens. The lignan family are the main phytoestrogens and can reach substantial

amounts in natural vegetable oils [167]. The metabolism of lignans in the GI tract is associated with gut bacteria leading to formation of enterolignans [168]. The content of lignans in sesame oil varies from 6.5 to 17.3 g/kg oil [163]. The main lignans present in sesame oil are sesamin, sesamolin and sesaminol. Sesamin is present in the sesame oil at highest levels (0.07% to 0.61%) compared to other lignans [169,170]. It has been reported that the plant lignans have antioxidant properties, which provide the thermal and storage stability to the lipids [171,172]. Some lignans in sesame oil can prevent lipid peroxidation in cells [173,174]. Therefore, it might be hypothesised that lignans in sesame oil prevent the triglyceride oxidation or peroxidation before or after digestion in the GI tract.

3.5. Conclusion

In this study, we have compared pre-digested lipids and purified trioleate to the sesame oil as the lipid vehicle for oral delivery of CBD *via* the intestinal lymphatic system. The *in vivo* studies have shown that the pre-digested or purified lipids do not improve the extent or decrease the variability of CBD bioavailability and lymphatic transport in comparison to the natural sesame oil. Therefore, the intraluminal digestion step does not seem to affect substantially the rate and extent of absorption of lipids and of co-administered CBD. Moreover, taken together, the results suggest that both the absorption of lipids and the absorption of co-administered CBD was more efficient following administration of natural sesame oil vehicle than of pre-digested lipids or purified trioleate.

The various small molecule constituents or the diverse forms of fatty acids in the natural oil could contribute, in a synergistic effect, to the absorption of lipids and co-administered lipophilic drugs. The mechanisms, and constituents responsible for more efficient absorption of lipids and co-administered highly lipophilic drug (CBD) following administration of natural sesame oil compared to pre-digested or purified lipids should be further investigated. Moreover, future studies should assess if these beneficial effects on absorption of lipids and co-administered lipophilic drug are limited to sesame oil or could be also achieved using other natural vegetable oils.

4. INCLUSION OF MEDIUM-CHAIN TRIGLYCERIDE IN LIPID-BASED FORMULATION OF CANNABIDIOL FACILITATES MICELLAR SOLUBILIZATION *IN VITRO*, BUT *IN VIVO* PERFORMANCE REMAINS SUPERIOR WITH PURE SESAME OIL VEHICLE [2]

The results described in this Chapter have been published by the Journal of *Pharmaceutics:* W. Feng, C. Qin, E. Cipolla, J.B. Lee, A. Zgair, Y. Chu, C.A. Ortori, M.J. Stocks, C.S. Constantinescu, D.A. Barrett, P.M. Fischer, P. Gershkovich, Inclusion of Medium-Chain Triglyceride in Lipid-Based Formulation of Cannabidiol Facilitates Micellar Solubilization In Vitro , but In Vivo Performance Remains Superior with Pure Sesame Oil Vehicle, Pharmaceutics. 13 (2021) 1–15.

4.1. Introduction

Approximately 30 to 60 % of new active drugs have poor aqueous solubility issues, and conventional formulations usually do not facilitate the absorption of these compounds from the gastrointestinal (GI) tract [25]. Lipid-based formulations have been proposed to facilitate the intraluminal solubility and systemic bioavailability of these poorly water-

soluble compounds [25,34,175,176]. The most common lipidic core excipient used in various lipid-based formulations is different vegetable fats, such as sesame oil [1,120,129].

The main component in most natural vegetable oils is long-chain triglycerides (LCTs), which are digested in the intestinal lumen by lipases to generate free fatty acids and monoglycerides. Since the lipases act at the surface of the oil droplets, the emulsification step of the lipidic formulation in the intestinal tract is extremely important for the digestion of lipids and the absorption of the co-administered lipophilic drug. The triglyceride lipolysis products together with bile salts and phospholipids then form mixed micelles in the intestinal lumen. Mixed micelles facilitate the transport of the lipophilic compounds further across the unstirred aqueous layer and into the enterocytes. Triglycerides are then re-synthesized intracellularly from long-chain fatty acids and monoglycerides, and large lipoproteins (chylomicrons) are then assembled in the enterocytes. The chylomicrons are taken up by lymph lacteals rather than blood capillaries due to their large size [177,178]. The association of lipophilic compounds with chylomicrons in the enterocytes facilitate the intestinal lymphatic transport of drugs and avoids hepatic first pass metabolic loss.

Cannabidiol (CBD), a BCS class II drug, is a highly lipophilic ($clogD_{7.4}$ 6.53) non-psychoactive phytocannabinoid, which has substantial first-pass metabolism contributing to low oral bioavailability [149,179]. For highly lipophilic drugs with $logD_{7.4}$ above 5, the intestinal lymphatic transport is frequently a useful drug delivery route to avoid hepatic

first-pass metabolic loss and increase bioavailability, for example halofantrine, Vitamin D_3 and tetrahydrocannabinol (THC) [120,129,141,176,180,181].

CBD has therapeutic potential in the treatment of auto-immune and inflammatory diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA) and allergic asthma [107,182–184]. The immunosuppressive effects of CBD have been extensively studied, and it was found that CBD could suppress the tumour necrosis factor (TNF) and interleukin (IL) cytokines production from both rats and human mononuclear cells [129,185–187]. It has been also shown that sesame oil vehicle facilitates the transport of CBD through the intestinal lymphatic system. When CBD was administered orally in natural sesame oil vehicle to rats, the systemic bioavailability of CBD has increased 2.8-fold compared to the lipid-free vehicle, and the drug concentration in lymph fluid was 250-fold higher compared to plasma [120,129]. The clinical study has also shown sesame oil has 8-fold increases in CBD oral absorption than CBD powder in healthy male volunteers [126]. However, both the intestinal lymphatic transport and systemic bioavailability of CBD administered in sesame oil vehicle were associated with substantial variability [1,120,129].

In the present study we hypothesize that enhancement of the emulsification and micellar solubilisation of CBD in GI tract could contribute to reduction in the variability of the absorption of the drug. The rationale for this hypothesis comes from a previously conducted *in vitro* lipolysis study suggesting that less than 15% of triglyceride-C18 (Tri-C18) was digested in simulated human intestinal fluids [131]. On the other hand, the same lipolysis

experiment showed that more than 90% of medium-chain triglyceride (MCT), such as triglyceride-C8 (Tri-C8), was hydrolysed under the same condition [131,188]. Thus, addition of MCT to the sesame oil could improve the CBD micellar solubilization following lipid digestion in GI tract, and consequently enhance the drug absorption and the intestinal lymphatic transport.

To enhance further the emulsification and micellar solubilisation, nonionic surfactants can be used. In this study, selection of surfactants was based on their hydrophilic–lipophilic balance (HLB) value. Surfactants with HLB value ranging from 8 to 18 are suitable for the oil in water emulsification. Therefore, Tween 85, Tween 80 and Span 20 have been selected for this study [189,190]. In addition, a derivative of Vitamin E, d-α-tocopherol polyethylene glycol 1000 succinate (TPGS), which has been previously shown as a powerful solubilizer for lipophilic compounds, has also been selected [191–193]. The free α-tocopherol released from TPGS could also prevent fatty acid oxidation process [194–196]. It has also been reported that TPGS can enhance lymphatic transport of lipophilic compounds by stimulating the chylomicron secretion in Caco-2 cells [197].

Therefore, the aim of this study is to test the hypothesis that addition of MCT and surfactants to long-chain triglyceride vehicle (sesame oil) improves the emulsification and micellar solubilisation of CBD *in vitro*, and eventually leading to increased intestinal lymphatic transport and drug bioavailability with reduced variability *in vivo*.

4.2. Experimental design

CBD was formulated in 6 different lipid-based vehicles, sesame oil was used as a vehicle for the control group, and preparation of lipid-based formulations is described in Section 2.2.2. Six lipid-based formulations were assessed by *in vitro* lipolysis and *in vivo* pharmacokinetic and biodistribution studies as described in Sections 2.3 and 2.4, respectively. Sample from *in vitro* lipolysis and *in vivo* experiments were prepared for HPLC analysis as described in Sections 2.5.1 to 2.5.3. The HPLC conditions are described in Sections 2.6.1. and 2.6.2. The triglyceride levels were determined in *in vitro* and *in vivo* samples as described in Section 2.5.5. The experimental design is summarised in the general scheme flowchart and presented in Figure 4-1.

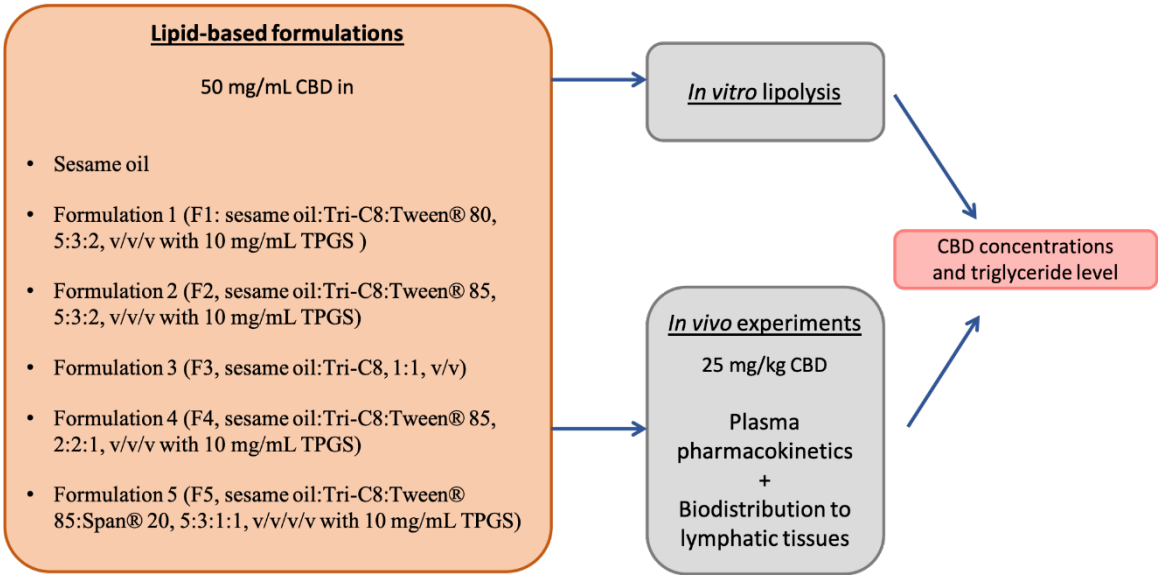


Figure 4- 1 . Flowchart of experimental design of comparing the bioavailability and lymphatic targeting of CBD in co-administered 6 lipid-based formulations. CBD, cannabidiol; TPGS: d-α-tocopherol polyethylene glycol 1000 succinate.

4.3. Results

4.3.1. *In vitro* lipolysis of lipid-based formulations

The processing of lipid-based formulations in the intestinal lumen has been assessed using *in vitro* lipolysis system (Figure 4-2). The CBD distributed into micelle phase represents the fraction readily available for absorption from the small intestine [50]. Figure 4-2 clearly shows that addition of the MCT resulted in trend for higher CBD distribution into micellar phase. Moreover, when the ratio of tri-C8 and sesame oil in Formulation 3 and Formulation 4 reached 1:1, the micellar phase had statistically significantly higher amount of CBD than the sesame oil group.

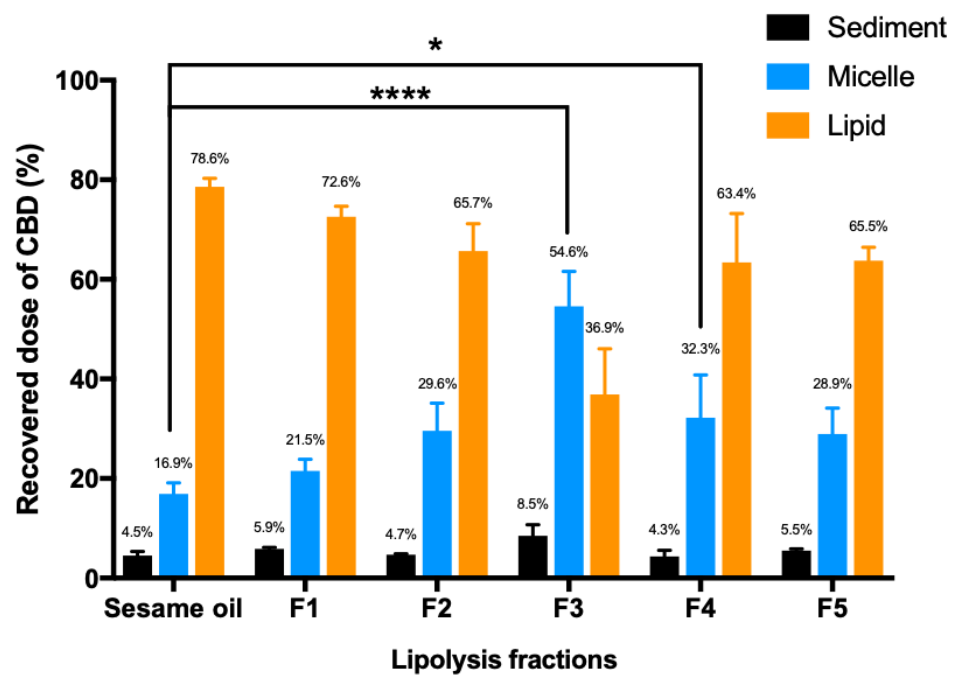


Figure 4- 2 . Distribution of CBD in sediment, micelle and lipid phases (n=3, mean ± SD) following the *in vitro* lipolysis of 6 lipid-based formulations of CBD. One-way

ANOVA was used for statistical analysis, followed by Dunnett's post-hoc test with sesame oil serving as a control group. *, p <0.05, ****, p <0.0001. The experiment was terminated when addition rate of NaOH was slower than 3μL/min.

The recovery of triglyceride, diglyceride and monoglyceride in lipolysis phases have been calculated and shown in Figure 4-3. The lipid fractions in the micellar phase are mainly diglycerides or monoglycerides, and a significantly higher levels of these lipids have been found in the sesame oil group compared to other five formulations in this study. The lipid phase represents the proportion of lipidic formulation that has not been hydrolysed completely, and sesame oil group has significantly higher amount of triglycerides in lipid phase compared to Formulation 3, Formulation 4 and Formulation 5.

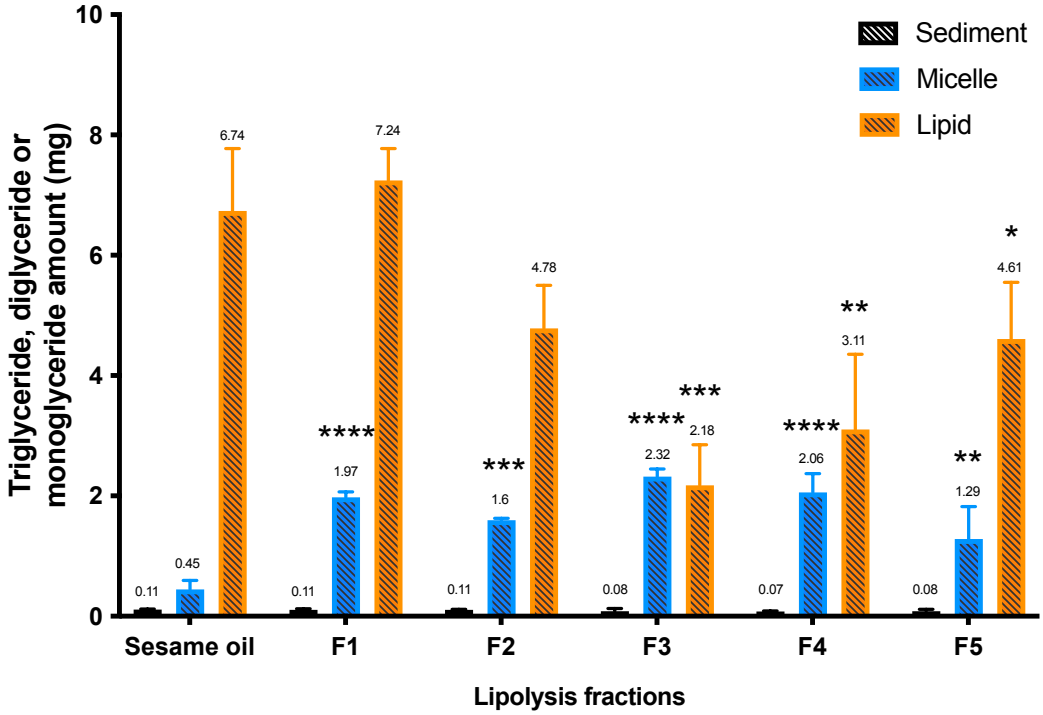


Figure 4- 3 . The amount of triglyceride, diglyceride and monoglyceride in lipolysis fractions following *in vitro* lipolysis of six lipid-based formulations (n = 3, mean ±

SD). One-way ANOVA was used for statistical analysis, followed by Dunnett's post-hoc test with sesame oil serving as a control group. * p <0.05, **, p <0.01, ***, p <0.001, ****, p <0.0001.

4.3.2. *In vivo* pharmacokinetics

Formulations 3 and 4 were selected to proceed to *in vivo* pharmacokinetic and biodistribution studies due to higher distribution of the drug to the micellar phase in *in vitro* lipolysis experiments. Pure sesame oil vehicle was also selected for *in vivo* studies to serve as a control group. The plasma concentration-time profiles of CBD following oral administration of these formulations are presented in Figure 4-4. The pharmacokinetic parameters including half-life, t_{max}, C_{max} and the plasma concentration-time curve (AUC) are outlined in Table 4-1. The AUC of CBD concentrations for sesame oil formulation is significantly higher than the Formulation 3. However, there are no statistically significant differences for other parameters when Formulations 3 and 4 are compared to the sesame oil group.

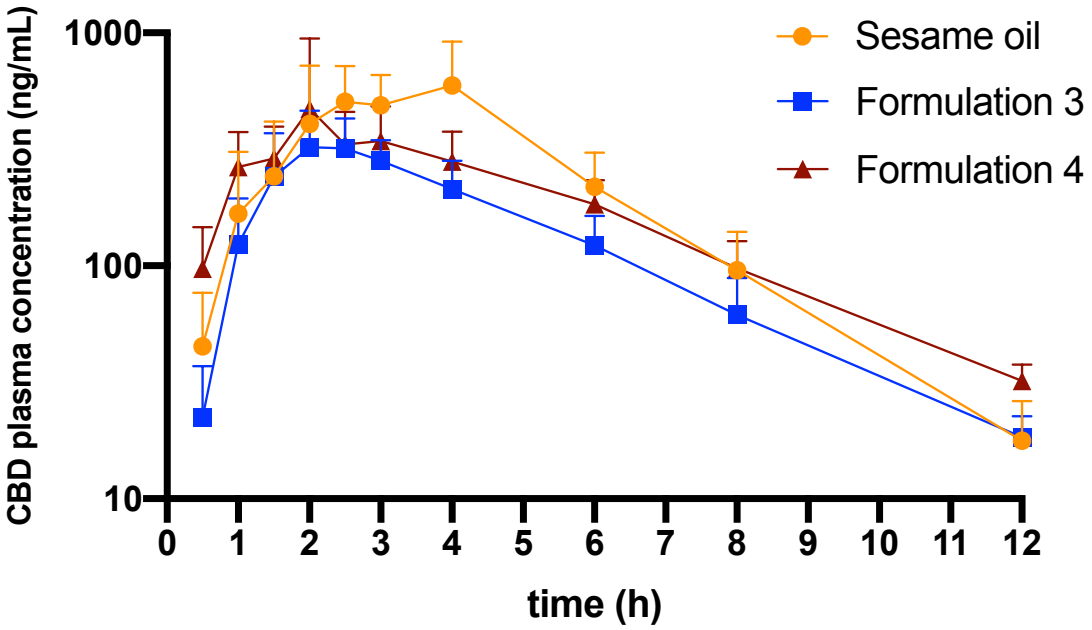


Figure 4- 4 . Plasma concentration-time profiles of CBD following oral administration of sesame oil-based formulation, Formulation 3 (sesame oil:tri-C8, 5:5, v/v) and Formulation 4 (sesame oil:Tri-C8:Tween® 85, 2:2:1, v/v/v with 10 mg/mL TPGS) (n=5-6, mean ± SD). The concentration of CBD in all formulations was 50 mg/mL, the administered dose of CBD was 25 mg/kg in all treatment groups.

Table 4-1. Pharmacokinetic parameters of cannabidiol (CBD) derived from plasma concentration-time profiles following oral administration in different lipid-based formulations (mean ± SD (n=5-6)).

Administration/ formulation	$t_{1/2}$[a] (h)	t_{max}[b] (h)	C_{max}[c] (ng/mL)	$AUC_{0-\infty}$[d] (h*ng/mL)	n
Sesame oil	1.6 ± 0.1	2.5-4	724 ± 318	2702 ± 909	5
Formulation 3	2.3 ± 0.3	2-2.5	371 ± 85	1512 ± 224**	6
Formulation 4	2.5 ± 0.5	2-3	562 ± 390	2131 ± 373	6

[a] Half-life

[b] time to maximum concentration in plasma

[c] The maximum concentration in plasma

[d] AUC from 0 to infinity

, statistically significantly different from sesame oil (, p <0.01)

4.3.3. Biodistribution

The blood, lymph fluid and MLNs were collected in the biodistribution experiments at pre-determined time points. The time points chosen are based on the *in vivo* plasma pharmacokinetics results and cover the early and late periods in the absorption phase of CBD. The triglyceride levels have also been measured in serum and lymph fluid samples, and are presented together with CBD concentrations in Figure 4-5 and Figure 4-6. There are no statistically significant differences in triglyceride levels at both 1.5 and 2.5 hours

time points, when Formulation 3 and Formulation 4 are compared to the sesame oil group (for both serum (Figure 4-5) and lymph (Figure 4-6) samples). It has been found that CBD concentration in serum at 2.5 hours following oral administration of pure sesame oil-based formulation is significantly higher than after administration of Formulation 4 (Figure 4-5b). For CBD concentrations in the lymph fluid, there are no significant differences between Formulations 3 or 4 when they are compared to sesame oil at 1.5 and 2.5 hours (Figure 4-6). The administration of CBD in sesame oil leads to a higher concentration of CBD in MLNs at 2.5 hours compared to Formulation 4 (Figure 4-7b), whereas no differences were found at 1.5 hours (Figure 4-7a). The MLNs have been also collected at 12 hours time point at the end of *in vivo* pharmacokinetic study, and the levels of CBD following administration of sesame oil-based formulation were significantly higher than for Formulation 4 (Figure 4-7c).

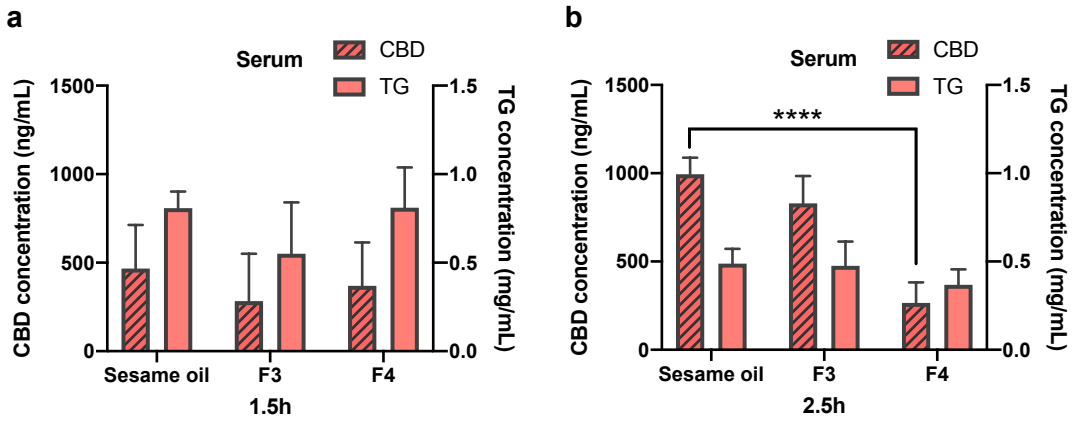


Figure 4- 5 . Concentrations of CBD and triglyceride in rat serum. CBD was orally administered in sesame oil, Formulation 3 (F3, sesame oil:tri-C8, 5:5, v/v) and Formulation 4 (F4, sesame oil:tri-C8:Tween 85, 4:4:2, v/v/v with 10 mg/mL TPGS) at a dose of 25 mg/kg in rats (50 mg/ml CBD content in each formulation). (a) The

concentration of CBD and triglyceride level in rat serum at 1.5 hours post-administration. (b) The concentration of CBD and triglyceride level in rat serum at 2.5 hours post-administration. All data are shown as mean ± standard deviation (SD) (n=4). Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post-hoc test with sesame oil serving as a control group. ****, p <0.0001.

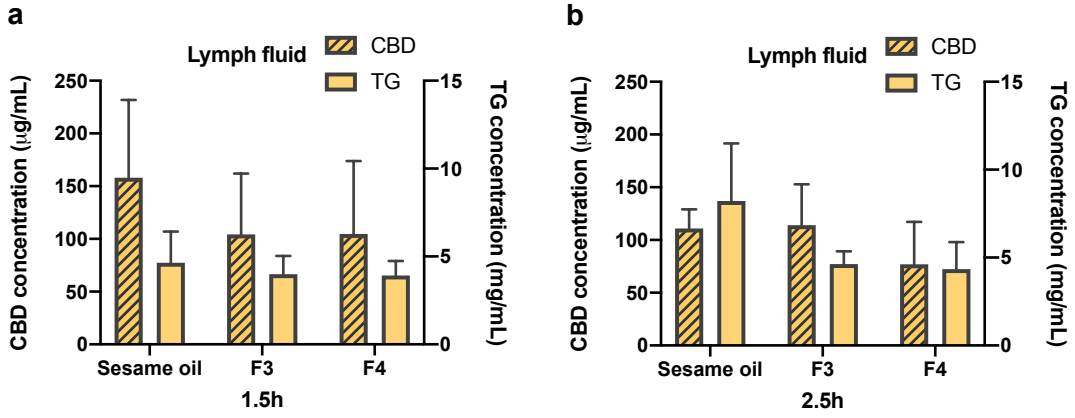


Figure 4- 6 . Concentrations of CBD and triglyceride in lymph fluid. CBD was orally administered in sesame oil, Formulation 3 (F3, sesame oil:tri-C8, 5:5, v/v) and Formulation 4 (F4, sesame oil:tri-C8:Tween 85, 4:4:2, v/v/v with 10 mg/mL TPGS) at a dose of 25 mg/kg in rats (50 mg/ml CBD content in each formulation). (a) The concentration of CBD and triglyceride in lymph fluid at 1.5 hour post-administration. (b) The concentration of CBD and triglyceride in lymph fluid at 2.5 hours post-administration. All data are shown as mean ± SD, n=4). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test with sesame oil serving as a control group.

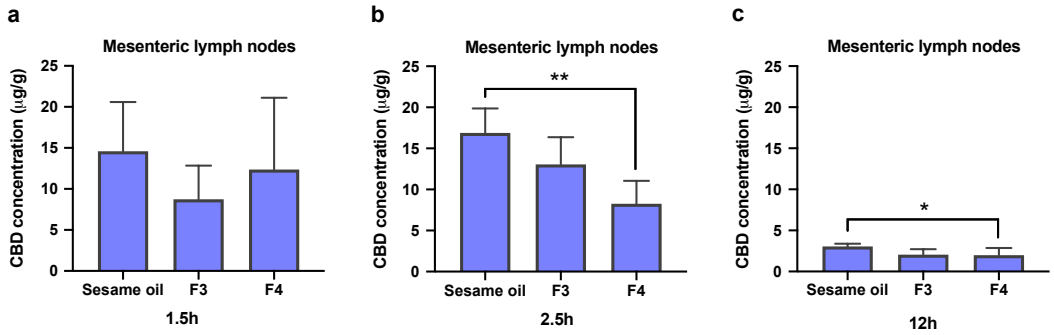


Figure 4- 7 . Concentrations of CBD in mesenteric lymph nodes (MLNs). CBD was orally administered in sesame oil, Formulation 3 (F3, sesame oil:tri-C8, 5:5, v/v) and Formulation 4 (F4, sesame oil:tri-C8:Tween 85, 4:4:2, v/v/v with 10 mg/mL TPGS) at a dose of 25 mg/kg in rats (50 mg/ml CBD content in each formulation). (a) The concentration of CBD in MLNs at 1.5 hours post-administration. (b) The concentration of CBD level in MLNs at 2.5 hours post-administration. (c) The concentration of CBD in MLNs at 12 hours post-administration. All data are shown as mean ± SD, n=4). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test with sesame oil serving as a control group. *, *p* <0.05, **, *p* <0.01.

4.4. Discussion

Even though sesame oil-based formulation facilitated the oral bioavailability and intestinal lymphatic transport of CBD in previous studies, the substantial inter-subject variability is a major issue which needs to be addressed [120,129]. The pre-digested lipid-based formulations have been previously suggested to minimise such variability by completely avoiding LCT digestion step in the intestinal lumen. However, it was shown in our previous work that the pre-digested lipids did not reduce the variability or enhance the intestinal

lymphatic transport or systemic bioavailability of CBD in comparison to sesame oil vehicle [1].

Multiple previous works have suggested that addition of the MCTs into lipid-based formulations can enhance the emulsification and micellar solubilisation, and eventually the bioavailability of lipophilic drugs [176,198–202]. Previously reported *in vitro* lipolysis data also suggest that the MCTs are digested more readily in the simulated human intestinal fluids compared to the LCTs [131,188,203,204]. Moreover, addition of surfactants into lipid-based formulation can enhance the micellar emulsification in the GI tract. Therefore, addition of the MCT and surfactants to the natural sesame oil has been attempted in this study for improvement of micellar solubilisation of CBD and reduction of the *in vivo* variability in bioavailability and lymphatic transport observed with sesame oil vehicle.

4.4.1. The design of lipid-based formulations

When lipid-based formulations are designed for a highly lipophilic compound such as CBD, with proven substantial intestinal lymphatic transport, a certain proportion of LCT must remain in the formulation to maintain the intestinal lymphatic transport element of the absorption [1,120,129]. Therefore, the proportion of sesame oil was maintained at 40 to 50 % of the total lipid volume in Formulations 1-5. The dose and concentration of CBD in the formulations were dominated by two major factors: 1) the clinical oral therapeutic dose

range of CBD in humans, 2) the size range of oral soft gelatine capsules suitable for oral administration.

CBD and its therapeutic potential have been studied for decades. CBD has been suggested as a potential therapeutic agent for diverse medical conditions, including autoimmune disease, inflammations, cancer or schizophrenia. The reported dose range in humans for different conditions is also very wide from 16 mg to 3000 mg per day [205–208]. CBD has an inverted U-shaped dose-response curve in animal models and human volunteers, with the most effective single oral dose in humans being around 300 mg [208–210].

The largest soft gelatine capsules that can be found on the market are 25 oblong capsules, which contain approximately 1.5 mL (for example 1500 mg Evening primrose oil and Starflower oil produced by Holland & Barrett (Hinckley, United Kingdom)). Therefore, the concentration of CBD in formulations in this study (50 mg/mL) is based on mimicking realistic human use condition of 300 mg CBD dose in 3 mL (two 25 oblong capsules) lipid vehicle.

To note, the original concentration of CBD considered was 100 mg/mL (to mimic one 25 oblong capsule administration). However, our previous work showed that CBD concentration above 80 mg/mL in lipid-based formulations could affect the digestion process during *in vitro* lipolysis. [211].

The rat model was utilized to assess the lipid-based formulations in this study. The dose of CBD was calculated in rats using allometric scaling from the human dose. Assuming 300 mg CBD dose in 70 kg adult human, the allometrically scaled dose in rats is 26 mg/kg [212]. Therefore, a dose of 25 mg/kg CBD was administered to rats in this study.

4.4.2. *In vitro* lipolysis

Based on the *in vitro* lipolysis results in this study, mixing MCT with sesame oil, as predicted, indeed leads to a trend of higher CBD distribution into aqueous micellar phase compared to sesame oil vehicle (Figure 4-2). The statistical significance has been found for Formulation 3 and 4, when compared to the sesame oil-based formulation (control). Unlike Formulations 1, 2 or sesame oil vehicle, the proportion of Tri-C8 is 50% of total lipids for Formulation 3 and Formulation 4 (Table 2-1). Such high ratio of Tri-C8 in these formulations results in overall more efficient digestion during the *in vitro* lipolysis process.

Lower levels of triglycerides remain in lipid phase for Formulation 3, Formulation 4 and Formulation 5 compared to the sesame oil group following *in vitro* lipolysis (Figure 4-3). This suggest that the higher proportion of Tri-C8 indeed leads to more efficient lipid digestion and a higher CBD distribution into the micellar phase for Formulation 3 and Formulation 4 compared to the sesame oil (Figures 4-2 and 4-3). In addition, the measured "triglyceride" (free glycerol and glycerol released from triglyceride, diglyceride and monoglyceride) levels in micellar phase also suggest that addition of Tric-C8 to the

formulation enhances the lipid digestion and micellar solubilisation of CBD in comparison to sesame oil vehicle.

The lipolysis results indicate that addition of surfactants (HLB value from 8.6 to 15) affects lipid digestion *in vitro*. During the digestion of lipids, the surfactants in lipid-based formulations are readily displaced from the lipid droplets surface by bile acids [213,214]. This allows lipase to approach the interface of the oil-in-water emulsion and initiate lipolysis [215]. The hydrolysed fatty acids and 2-monoglycerides compete with the surfactants at the lipid droplets surface. The excess of fatty acids and 2-monoglycerides, subsequently form mixed micelles with bile salts and surfactants. However, there are *in vitro* and *in vivo* studies indicating that highly hydrophilic surfactants (HLB 12-17) can inhibit pancreatic lipase activity and therefore reduce the hydrolysis of triglycerides, resulting in lower oral bioavailability of the poorly water-soluble drugs [216–218]. In agreement between these studies and the current study, Formulation 3 contains no surfactants but has shown the most effective lipid digestion compared to other formulations based on the *in vitro* lipolysis results (Table 2-1, Figure 4-2 and Figure 4-3). Therefore, the addition of surfactants into sesame oil did not improve triglycerides hydrolysis in *in vitro* lipolysis system in this study.

4.4.3. *In vivo* pharmacokinetics and biodistribution

Based on the *in vitro* lipolysis results, sesame oil, Formulation 3 and Formulation 4 were selected to proceed further for *in vivo* pharmacokinetics study. However, unlike the *in vitro* lipolysis, the *in vivo* pharmacokinetic results have shown that the systemic bioavailability of CBD following oral administration of sesame oil formulation is higher than for Formulation 3, and similar to the Formulation 4 (Table 4-1).

There is, therefore, a lack of correlation between *in vitro* and *in vivo* results in this study. Potential explanation could be related to the fact that there are two steps that dominate the bioavailability of CBD, the lipid digestion in the intestinal lumen leading to intraluminal micellar solubilisation of CBD, and the intestinal lymphatic transport (which is related to the association of CBD with chylomicrons in the enterocyte). The *in vitro* lipolysis model simulates only one step out of these two - the lipid digestion in the intestinal lumen. Similar lack of *in vitro/in vivo* correlation have been previously reported for other drugs with substantial intestinal lymphatic transport component in the absorption process [67,188]. The lipid digestion and chylomicron formation in the enterocyte are both complex processes, and there is currently no appropriate single *in vitro* model that can simulate the entire process, including intraluminal and intracellular events. It has been suggested that a better prediction of bioavailability of highly lipophilic drugs administered in lipid-based formulations could be achieved by combining the *in vitro* lipolysis with microsome metabolism assay [219,220]. It is possible that in the future affinity to chylomicrons should

be added to *in vitro* lipolysis and microsomal stability assays for better prediction of bioavailability of highly lipophilic drugs administered in lipidic formulations [130,141].

The area under the curve (AUC) values indicate that Formulations 3 and 4 lead to lower variability in systemic exposure to CBD compared to the sesame oil vehicle (Table 4-1). A high proportion of Tri-C8 facilitated the digestion of lipids and enhanced the micellar solubilisation of CBD in the intestinal lumen. The Formulation 3 has a slightly faster t_{max} than the sesame oil formulation, which could be due to faster digestion of Tri-C8 compared to sesame oil. However, chylomicrons mainly consist of LCTs, whereas MCTs are mostly transported through the portal vein rather than packed into chylomicrons in the enterocytes [176,188,203]. Therefore, a rapid digestion of Tris-C8 could deliver a part of CBD into enterocyte at the earlier digestion stage, before long-chain lipids become available in the enterocyte for formation of chylomicrons. Consequently, this quickly absorbed part of CBD dose is likely to be delivered to the systemic circulation through portal vein with substantial hepatic first-pass metabolism, therefore, resulting in reduced overall systemic exposure.

Indeed, addition of Tris-C8 into sesame oil reduced the AUC variability compared to pure sesame oil formulation. However, the extent of CBD absorption *in vivo* was not improved. As stated above, the surfactants with HLB in a range of 12 to 17 may negatively affect the lipolysis process, and therefore, decrease the drug distribution into the mixed micelles in the intestinal lumen. The TPGS is a surfactant with antioxidant properties, which has been

reported to enhance the chylomicrons formation, as well as potentially being delivered into the intestinal lymphatic system by association with the chylomicrons [197]. However, it has also been reported that TPGS and oleic acid-containing mixed micelles have negative effect on the bioavailability and lymphatic transport of antiretroviral drug saquinavir in comparison to oleic acid microemulsions [221]. Another work suggested that TPGS was not hydrolysed by pancreatic lipase in GI tract and remains in the intact form until it transports to enterocyte [217]. Therefore, it is possible that addition of the TPGS restricted the drug association with chylomicrons in the current study.

There are no significant differences in the triglyceride levels in both serum and lymph fluid samples, when Formulation 3 and Formulation 4 are compared to sesame oil formulation (Figures 4-5 and 4-6). The triglyceride levels in serum and lymph fluid samples indicate that there might be an overload of LCT in these formulations for lymphatic transport, therefore resulting in the saturation in the process of triglyceride re-synthesis in the enterocytes. Even though both Formulation 3 and Formulation 4 contain approximately 50% of sesame oil compared to pure sesame oil vehicle formulation, the LCT uptake for these two formulations is similar to the sesame oil.

The overall results of the biodistribution studies suggest that sesame oil has more efficient performance in enhancing the intestinal lymphatic transport of CBD compared to both Formulation 3 and Formulation 4.

Taken together, the combined results of *in vivo* pharmacokinetics and biodistribution studies suggest that addition of surfactants and MCT to natural sesame oil does not improve CBD oral bioavailability and intestinal lymphatic transport compared to simple sesame oil-based formulation.

4.5. Conclusion

Sesame oil-based formulation has been previously reported to lead to substantial increase in bioavailability and intestinal lymphatic transport of CBD in rats, but with considerable variability. The MCT incorporated into sesame oil enhanced the micellar solubility of CBD *in vitro*, but surfactants used in this study may have reduced the lipolysis of triglycerides in comparison to other MCT-containing formulations. Even though the addition of MCT into lipid-based formulations reduce the viability, pure sesame oil vehicle was still superior in the extent of CBD lymphatic transport and bioavailability *in vivo*. There are multiple factors that dominate the lipid digestion, including the chain length of triglyceride, or lipid interaction with surfactants. Furthermore, the lymphatic transport requires a combination of a highly lipophilic compound (with affinity to chylomicrons) and the presence of lipids that eventually lead to chylomicrons formation (LCT or long-chain fatty acids). Further studies will be needed to assess alternative approaches to reduce inter-subject variability associated with pure sesame oil formulation of CBD without negatively affecting the extent of absorption of the drug.

5. NATURAL VEGETABLE OILS COMPOSITION AFFECTS THE INTESTINAL LYMPHATIC TRANSPORT AND SYSTEMIC BIOAVAILABILITY OF CO-ADMINISTERED LIPOPHILIC DRUG CANNABIDIOL

5.1. Introduction

Lipid-based formulations is an emerging oral drug delivery strategy to improve the absorption and bioavailability of lipophilic compounds, most commonly following oral administration [21,222,223]. The physicochemical properties of drugs, such as aqueous solubility and biological membrane permeability, are main variables affecting their oral bioavailability [224]. Lipid-based vehicles can lead to the increased solubility of lipophilic drugs in the intestinal lumen and eventually enhance their oral bioavailability [37,136,225].

When long-chain triglycerides (LCT) or long-chain fatty acids are present in an oral lipid vehicle, the co-administered highly lipophilic compounds have the potential for intestinal lymphatic transport through the mechanism of incorporation into chylomicrons in the enterocytes [136,138,222]. Chylomicrons are large lipoproteins that act as means of transport of dietary lipids from enterocytes to lymph lacteals, and eventually to the systemic circulation through intestinal lymphatic system [143,144,226]. Chylomicrons are mainly composed of LCT, as well as phospholipids, cholesterol, cholesterol esters and apoproteins

[77,79,227,228]. Diet is the primary source of these triglycerides (TG), and it has been reported that LCT-rich meal or formulations facilitate the production of chylomicrons [229–232]. Once consumed, dietary TG undergo series of transformations in the intestinal lumen and enterocytes before they are incorporated into chylomicrons. The first step is a hydrolysis of TG in the gastrointestinal (GI) tract on sn-1 and sn-3 positions to produce fatty acids and 2-monoglyceride. The LCT are then re-synthesised from these lipid digestion products in the enterocytes and packed into chylomicrons. Because of their large particle size (75 to 1200 nm), chylomicrons cannot penetrate into blood capillaries, and therefore are directly transported into the intestinal lymphatic system, bypassing the portal vein and liver. Avoidance of liver at the first pass makes lymphatic transport of drugs a promising route for drug delivery, especially for compounds with substantial hepatic first pass metabolic loss [144,233,234]. It has been reported that drugs with lipophilicity of $\log D_{7.4} \geq 5$ and TG solubility of >50 mg/g are good candidates for high association with chylomicrons and the intestinal lymphatic transport [141,235].

LCT are abundant in most natural vegetable oils [236,237]. The use of natural vegetable oils as a main component of lipid-based formulations for highly lipophilic compounds has been reported in several previous studies. Incomplete list of examples include soybean oil-induced enhancement of the oral absorption of Leukotriene B4 (LTB4) inhibitor ontazolast through lymphatic transport [238], peanut oil-induced improvement in the lymphatic transport and oral bioavailability of some highly lipophilic synthetic cannabinoids [156],

and olive oil-induced enhancement in absorption of two highly lipophilic immunomodulators following oral administration, in comparison to lipid-free vehicle [239].

Previous studies in our laboratory have reported that sesame oil vehicle leads to efficient transport of a highly lipophilic phytocannabinoid cannabidiol (CBD) through the intestinal lymphatic system following oral administration in rats. CBD concentrations in the lymph fluid were about 250-fold higher than in plasma and the overall systemic exposure was 2.8-fold higher compared to a lipid-free formulation [120,129]. However, a considerable variability in the concentrations of CBD in lymph fluid, lymph nodes and plasma has been found following the oral administration of sesame oil-based formulation [1,2,120,129]. The high lipophilicity of the drug [124], as well as incomplete digestion of sesame oil or variable efficiency of emulsification in the GI tract could potentially contribute to the variability in plasma and lymph concentrations of CBD [2,120].

Possible approaches to reduce the variability associated with lipid-based drug delivery of CBD have been recently investigated, including the use of pre-digested lipids for the formulation rather than undigested oil [1]. However, simple elimination of the lipid hydrolysis step in the GI tract using purified digested excipients did not reduce the variability and led to a reduction in the extent of the lymphatic transport and bioavailability in comparison to a natural sesame oil vehicle. This suggests that the hydrolysis of TG in the GI tract by lipases could be not a main factor associated with variability in CBD concentrations in plasma and the lymphatic system. Therefore, more recently a different

approach has been attempted utilising medium-chain triglyceride (MCT) and/or surfactants combined with sesame oil lipid-based formulations to reduce variability [2]. This approach enhanced the CBD solubility in the mixed micelles *in vitro* and reduced the systemic blood concentrations variability *in vivo*. However, these mixed lipid-based formulations did not reduce the inter-individual variability of CBD concentrations within the intestinal lymphatic system, and overall led to lower extend of lymphatic transport and bioavailability in comparison to a pure sesame oil vehicle [2].

Vegetable oils differ substantially in their composition and it is likely that these differences could lead to variable performance as a vehicle for delivering lipophilic drugs to the lymphatic system and systemic circulation following oral administration [240–244]. Evaluating these differences might offer an insight into the factors and ingredients in vegetable oils that are crucial for the enhancement of the lymphatic transport and bioavailability of lipophilic drugs. Therefore, the aim of this study was to compare the composition and drug lymphatic transport and bioavailability enhancement properties of various vegetable oils using CBD as a model compound.

5.2. Experimental design

CBD was formulated in natural vegetable oils as described in Section 2.2.4. Vegetable oil-based formulations were assessed by *in vitro* lipolysis and *in vivo* pharmacokinetic and biodistribution studies as described in Sections 2.3. and 2.4., respectively. The fatty acid

compositions in vegetable oils and the chylomicrons after administration of corresponding vegetable oil-based formulations were assessed as described in Sections 2.5.4. and 2.6.3. Samples from *in vitro* lipolysis and *in vivo* experiments were prepared for HPLC analysis as described in Sections 2.5.1. to 2.5.3. The HPLC conditions are described in Sections 2.6.1. and 2.6.2. The triglyceride levels were determined in *in vivo* samples as described in Section 2.5.5. The experimental design is summarised in the general scheme flowchart and presented in Figure 5-1.

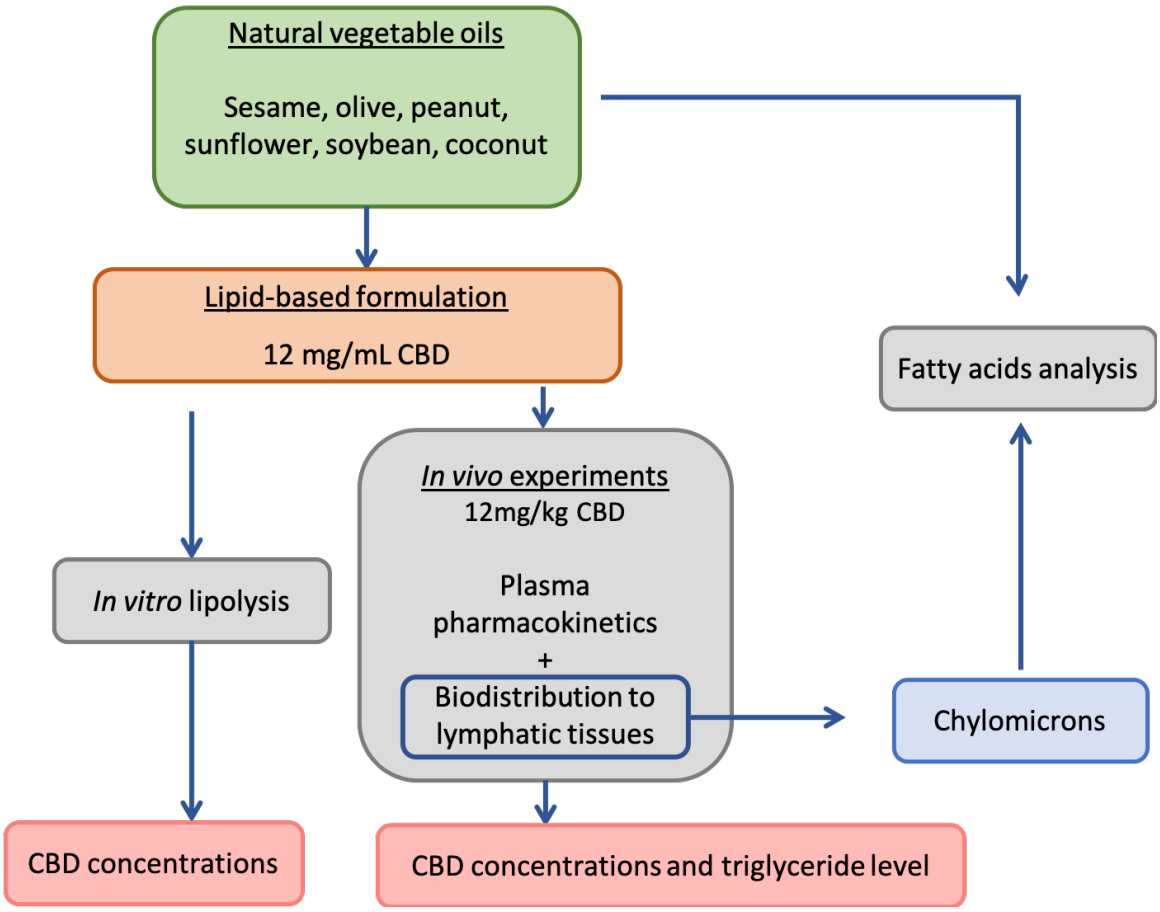


Figure 5- 1 **. Flowchart of experimental design of comparing the bioavailability and lymphatic targeting of CBD in co-administered natural vegetable oils. CBD, cannabidiol.**

5.3. Results

5.3.1. Distribution of CBD into lipolysis fractions following *in vitro* digestion of vegetable oil formulations.

The distribution of CBD into the sediment, aqueous micellar and oil phases following *in vitro* lipolysis of different vegetable oil formulations is presented in Figure 1. No statistically significant differences were observed in CBD distribution into undigested oil phases between different vegetable oil vehicles (Figure 5-2A). The distribution of CBD into the micellar aqueous phase was higher for sesame and sunflower oil-based formulations than for olive, peanut and coconut oil vehicles (Figure 5-2B). The distribution of CBD into the sediment phase was higher for peanut and coconut oil vehicles compared to the rest of the vegetable oil formulations (Figure 5-2C).

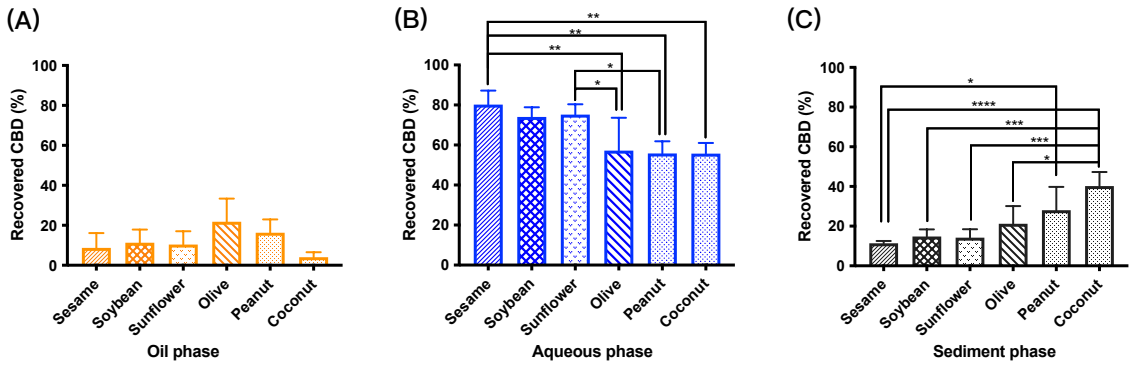


Figure 5- 2 . The distribution of CBD into oil, aqueous micellar and sediment fractions following *in vitro* lipolysis of 12 mg/mL CBD in sesame, soybean, sunflower, olive, peanut and coconut oils. The volume of formulations was 0.1 mL in all cases, lipolysis reaction was set up to run for one hour. (A) Distribution of CBD into oil phase. (B) Distribution of CBD into aqueous micellar phase. (C) Distribution of CBD into

sediment phase. All data are presented as mean ± SD (n = 4-6). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. *, p <0.05, **, p <0.01, ***, p <0.001, ****, p <0.0001.

5.3.2. *In vivo* pharmacokinetics of CBD following oral administration in different vegetable oils and lipid-free formulation

Pharmacokinetics of CBD was assessed following oral gavage administration of 6 natural vegetable oil-based formulations and the lipid-free formulation in rats. The plasma concentration-time profiles of CBD are presented in Figure 5-3, the pharmacokinetic parameters are summarised in Table 5-1. The maximum concentrations (C_{max}) of CBD in rat plasma following oral administration of sesame oil and olive oil-based formulations are 2.6-fold (p <0.01) and 3.2-fold (p <0.001) higher than for lipid-free formulation, respectively. The oral administration of sesame oil, soybean oil, peanut oil and olive oil-based formulations resulted in statistically significantly higher (p <0.05) area under the plasma concentration-time curve ($AUC_{0-\infty}$) compared to lipid-free formulation.

Table 5-1. Plasma pharmacokinetic (PK) parameters of CBD following oral administration in lipid-free and lipid-based formulations (mean ± SD).

Formulations	$t_{1/2}$ [a] (h)	t_{max} [b] (h)	C_{max} [c] (ng)	$AUC_{0-\infty}$ [d] (h×ng/mL)	n
Lipid-free	2.3 ± 0.6	2	81 ± 30	356 ± 83	6
Sesame	1.5 ± 0.2	3	209 ± 118**	865 ± 342***	6
Soybean	2.2 ± 0.3	3	165 ± 41	775 ± 164**	6
Peanut	2.3 ± 0.4	3	153 ± 36	737 ± 130**	6
Olive	2.0 ± 0.3	2	258 ± 66***	835 ± 130***	6
Sunflower	2.3 ± 0.4	3	112 ± 57	551 ± 136	6
Coconut	1.5 ± 0.4	5	96 ± 36	413 ± 164	6

[a] half-life

[b] time to maximum concentration in plasma

[c] the maximum concentration in plasma

[d] AUC from 0 to infinity

, * compared to lipid-free formulation (**, p <0.01, ***, p <0.001)

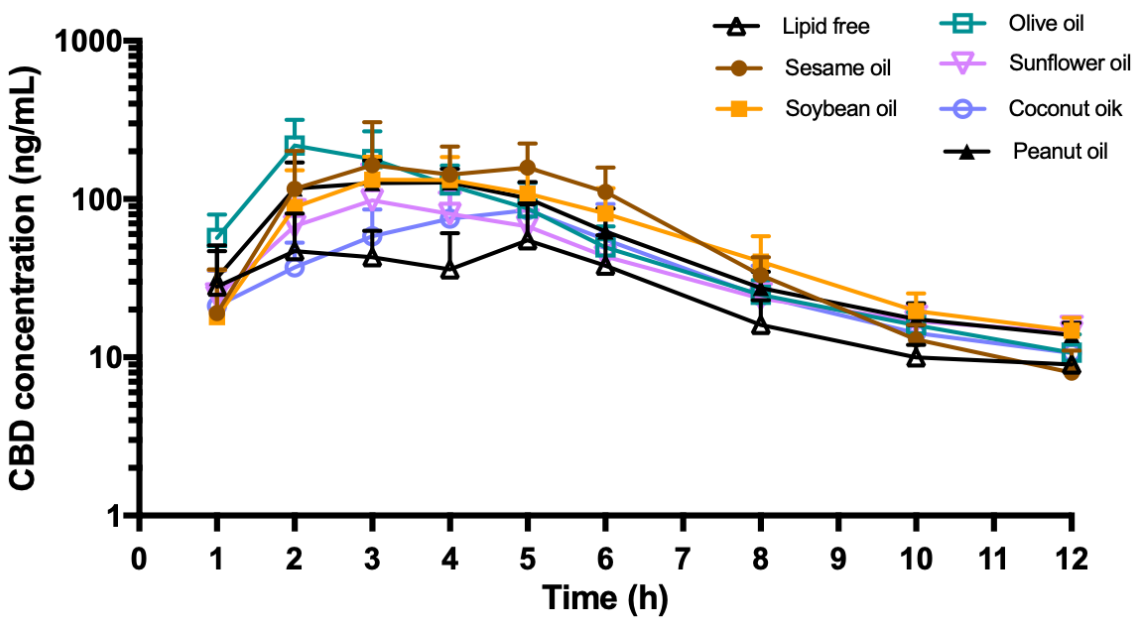


Figure 5- 3 . Plasma concentration-time semi-logarithmic plot of CBD (mean ± SD, n = 6) following oral administration at a dose of 12 mg/kg (12 mg/mL) CBD in lipid-free (propylene glycol:ethanol:water, 80:10:10, v/v/v) formulation, sesame oil, soybean oil, peanut oil, olive oil, sunflower oil and coconut oil.

5.3.3. Lymphatic targeting of CBD following oral administration in natural vegetable oil-based vehicles.

Rat serum, mesenteric lymph and mesenteric lymph nodes (MLN) have been collected at pre-selected time points (plasma t_{max} and 1 h prior to t_{max}), determined based on the pharmacokinetic parameters (Table 5-1), and assessed for CBD (all samples) and TG levels (lymph fluid and serum). There were no statistical differences in CBD and TG levels in serum among six vegetable oil formulations at both t_{max} and t_{max} – 1 h (Figure 5-4).

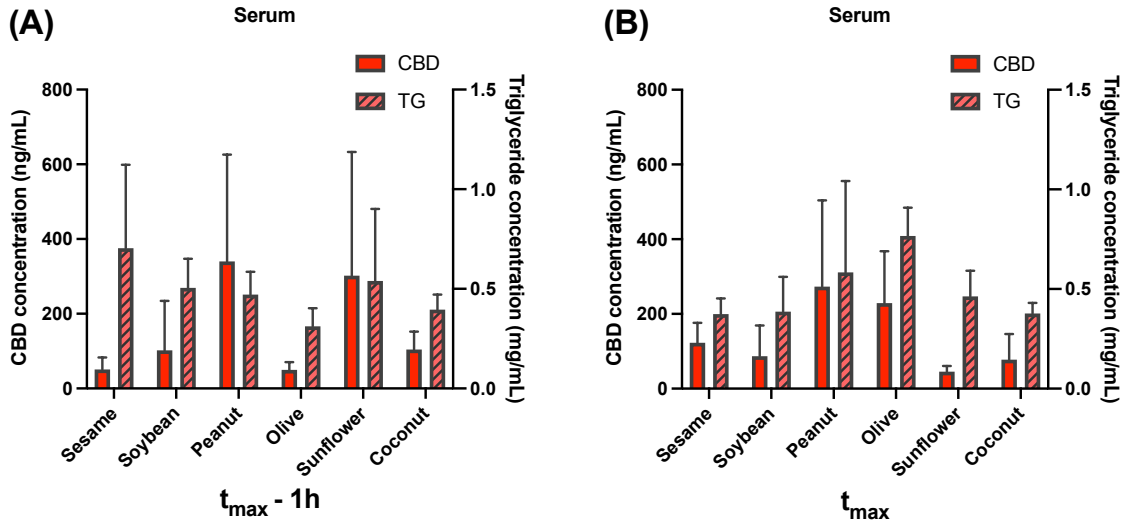


Figure 5- 4 . CBD and TG concentrations in serum. CBD was orally administered in sesame, soybean, peanut, olive, sunflower and coconut oil vehicles at a dose of 12 mg/kg. The concentration of CBD was 12 mg/mL in all formulations. (A) The concentrations of CBD and TG in rat serum at t_{max} – 1 h (one hour prior to plasma t_{max}). (B) The concentration of CBD and TG in rat serum at t_{max}. All data are presented as mean ± SD, n = 4-6. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

Figures 5-5 and 5-6 show the concentration of CBD in mesenteric lymph fluid and MLN. Figure 5-5 also shows TG levels in lymph fluid samples. There were no statistically significant differences in CBD concentrations in mesenteric lymph fluid at t_{max} – 1 h between all lipid-based formulations. However, oral administration of coconut and soybean oil formulations resulted in higher TG levels in lymph fluid at one hour before plasma t_{max} (t_{max} – 1 h) (Figure 5-5A). Olive oil formulation led to 3.7-fold, 2.2-fold, 3.1-fold and 5-fold higher levels of CBD in mesenteric lymph fluid at plasma t_{max} compared to soybean, sesame, sunflower and coconut oil-based formulations, respectively (Figure 5-5B). The TG levels in lymph fluid were highest in the olive group compared to other lipid-based

formulation groups at plasma t_{max} (Figure 5-5B). No differences in CBD distribution into MLN were observed between various vegetable oils formulations at plasma t_{max} – 1 h and t_{max} (Figure 5-6).

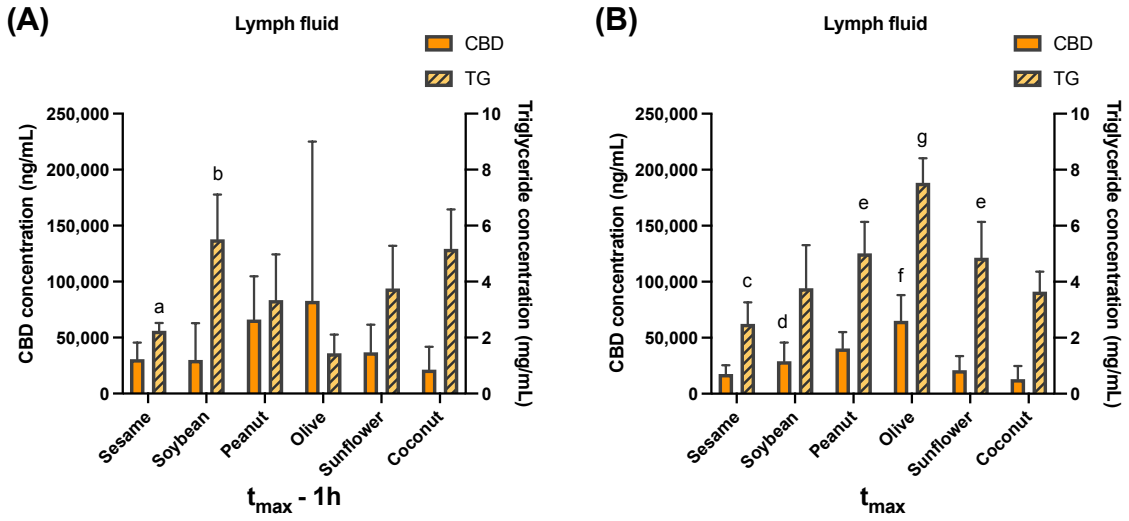


Figure 5- 5 . CBD and TG concentrations in the mesenteric lymph fluid. CBD was orally administered in sesame, soybean, peanut, olive, sunflower and coconut oil-based formulations at a dose of 12 mg/kg. The concentration of CBD was 12 mg/mL in all formulations. (A) The concentrations of CBD and TG in lymph fluid at one hour prior to plasma t_{max} (t_{max} – 1 h). Differences in TG levels: a, *p* <0.05, *vs.* coconut oil; b, *p* <0.01, *vs.* sesame and olive oils. (B) The concentrations of CBD and TG in lymph fluid at plasma t_{max}. Differences in TG levels: c, *p* <0.001, *vs.* olive oil; e, *p* <0.01, *vs.* olive oil; g, *p* <0.01, *vs.* soybean and coconut oils. Differences in CBD concentrations: d, *p* <0.05, *vs.* olive oil; f, *p* <0.01, *vs*. sesame oil. All data are presented as mean ± SD, n = 4-6. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

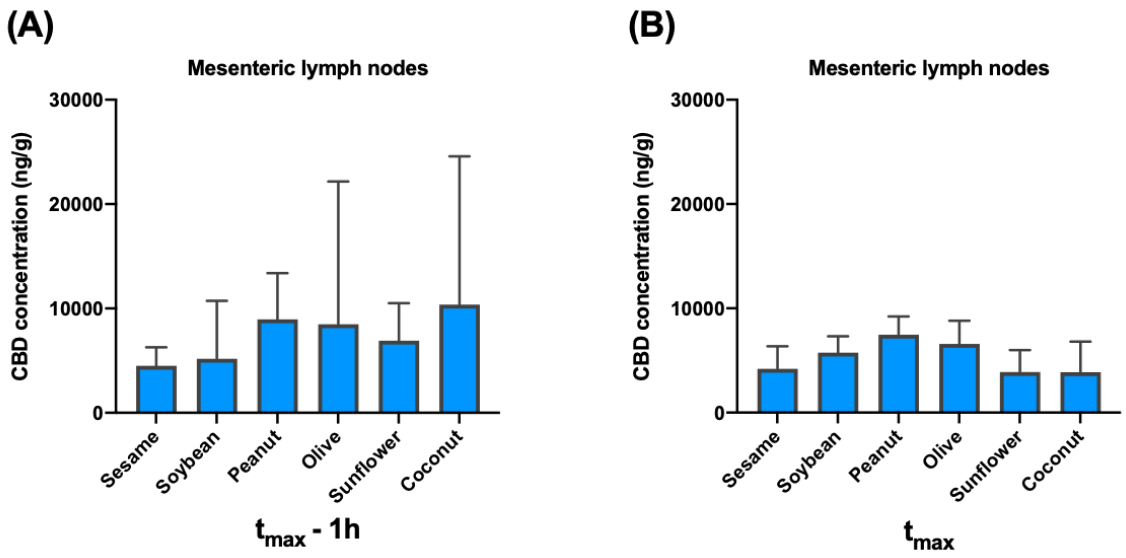


Figure 5- 6 . Distribution of CBD into mesenteric lymph nodes (MLN). CBD was orally administered in sesame, soybean, peanut, olive, sunflower and coconut oil-based formulations at a dose of 12 mg/kg. The concentration of CBD was 12 mg/mL in all formulations. (A) The concentrations of CBD and TG in MLN at one hour prior to plasma t_{max} (t_{max} – 1 h). (B) The concentrations of CBD and TG in MLN at plasma t_{max}. All data are presented as mean ± SD, n = 4-6. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test.

5.3.4. Fatty acids composition of vegetable oils and the corresponding rat chylomicrons

Fatty acid composition of vegetable oil vehicles has been assessed and compared to the composition in the corresponding lymph chylomicrons following administration of these vehicles to rats (Table 5-2). The composition of the fatty acids in the chylomicrons was overall similar to the fatty acids in the administered vehicles in each case. Coconut oil contains a higher proportion of saturated medium-chain fatty acids (C6-C12), as well as

C14:0 (myristic acid) in comparison to other vegetable oils. The only medium-chain fatty acid (capric acid, C10:0) found in rat chylomicrons was following oral administration of coconut oil vehicle. In the case of coconut oil administration, no fatty acids longer than 22 carbons were identified in chylomicrons. Intriguingly, a considerable amount of arachidonic acid (C20:4) was found in rat chylomicrons following administration of all vegetable oil vehicles, while it is not present in vegetable oils themselves.

Table 5-2. Fatty acid composition of natural vegetable oils and the corresponding rat lymph chylomicron samples. Chylomicrons were isolated from lymph fluid samples following oral administration of corresponding vegetable oil-based formulations.

	Fatty acid (% total fatty acid)									
	Saturated fatty acid [b]									
	C8:0	C10:0	C12:0	C14:0	C16:0	C17:0	C18:0	C20:0	C22:0	C24:0
Sesame										
Oil					12.9±0.3	0.2±0.02	8.1±0.6	1.2±0.01	1.0±0.04	0.9±0.2
CM [a]					13.1±0.3		6.3±0.3*			
Soybean										
Oil					15.9±0.2	0.3±0.1	7.9±0.4	0.9±0.02	1.4±0.1	0.7±0.1
CM [a]				0.3±0.01	10.9±0.4*		6.2±0.9*			
Peanut										
Oil					11.9±0.02	0.2±0.02	5.1±0.1	2.7±0.02	7.3±0.3	4.2±0.1
CM [a]				0.6±0.2	13.7±2	0.7±0.2*	5.6±0.5	0.9±0.1***	1.7±0.3****	
Olive										
Oil					12.3±0.2	0.2±0.02	7.8±1.2	1±0.1	0.5±0.1	0.01±0.01
CM [a]				0.3±0.1	12.2±0.6	0.5±0.1	5±0.3*	0.5±0.1*	0.5±0.2	
Sunflower										
Oil					9.5±0.2	0.2±0.03	8±0.1	0.8±0.03	2.3±0.02	1.1±0.3
CM [a]				0.3±0.03	14.4±1.9*		6.8±0.5*			
Coconut										
Oil	3.7±0.1	2.6±0.02	28.5±0.3	21.1±0.4	18.3±0.3	0.2±0.02	8.1±0.2	0.5±0.1	0.6±0.1	
CM [a]		0.6±0.2**	26.5±7.3	14±4.4	18±1		7.7±1.7			

	Unsaturated fatty acid [c]							
	C16:1	C17:1	C18:1	C18:2	C18:3	C20:1	C20:4	C22:1
Sesame								
Oil	0.2±0.01	0.1±0.01	44.3±0.1	30.4±0.6	0.3±0.1	0.4±0.02		0.2±0.02
CM [a]			39.5±2.1	38.3±2.3*			2.7±0.7	
Soybean								
Oil	0.1±0.02	0.2±0.01	29.7±0.3	37.9±1	4.1±0.2	0.4±0.1		0.6±0.8
CM [a]			29.8±0.2	47.3±0.3**	0.4±0.1***	2.6±0.3*	2.6±0.3	
Peanut								
Oil	0.1±0.01	0.1±0.04	50.8±0.5	15.2±0.1	0.2±0	2±0.1		0.2±0.1
CM [a]	0.7±0.2*		47.6±4.2	23.5±1**	0.5±0.1*	1.4±0.3	3.1±0.9	
Olive								
Oil	0.7±0.04	0.2±0.02	71.6±1	4.8±0.2	0.5±0.01	0.4±0.02		0.1±0.03
CM [a]	1.2±0.1**	0.1±0.01*	67.8±1.4*	8.6±0.6**	0.6±0****	0.4±0.03	2.3±0.4	
Sunflower								
Oil	0.1±0.01	0.1±0.03	37.6±0.3	39.8±0.1		0.3±0.1		0.3±0.04
CM [a]			24.4±2.9*	45.4±2.3	3.2±2.6	2.7±1.4	2.9±0.7	
Coconut								
Oil		0.1±0.1	13.8±0.3	2.5±0.2				
CM [a]			11±0.9*	9.4±1.6*		3.5±3.3	10.5±10.5	

[a] CM: chylomicrons

[b] C8:0 = caprylic acid; C10:0 = capric acid; C12:0 = lauric acid; C14:0 = myristic acid; C16:0 = palmitic acid; C17:0 = heptadecanoic acid; C18:0 = steric aid; C20:0 = arachidic acid; C22:0 = behenic acid; C24:0 = lignoceric acid
[c] C16:1 = palmitoleic acid; C17:1 = cis-10-heptadecenoic acid; C18:1 = oleic acid; C18:2 = linoleic acid; C18:3 = linolenic acid; C20:1 = eicosenoic acid; C20:4 = arachidonic acid; C22:1 = erucic acid
*, **, ***, ****; are statistically significantly different from oil group (*, p <0.005; **, p <0.01; ***, p <0.001; ****, p <0.0001)

5.4. Discussion

Although natural vegetable oils have long been investigated as the formulation vehicles to stimulate chylomicrons formation and enhance the intestinal lymphatic transport and oral bioavailability of lipophilic drugs, little research has focused on whether various natural vegetable oil vehicles have similar lymphatic transport and bioavailability enhancement capabilities for lipophilic drugs [245]. Therefore, in this work we have compared different natural vegetable oils for their ability to promote intestinal lymphatic transport and systemic bioavailability of co-administered CBD. In addition to the list of vegetable oils containing LCT known to facilitate intestinal lymphatic transport, an oil with shorter triglyceride chains (coconut oil) has been also included for comparison.

The intraluminal digestion and processing of six vegetable oils has been assessed using the *in vitro* lipolysis system. The extent of the distribution of CBD into the aqueous micellar phase was higher for olive, peanut and coconut oil in comparison to other experimental groups (Figure 5-2B). The lipolysis of vegetable oils and micellar solubilisation of CBD following the completion of the lipolysis process could be affected by the nature of fatty acids of these oils. It has been shown before that oral administration of another highly

lipophilic drug halofantrine in trilinolein (tri C18:2) vehicle led to higher intestinal lymphatic transport and bioavailability than administration in triolein (tri C18:1) vehicle [246]. This suggests that degree of saturation of fatty acids may impact the lipids digestion and micellar solubility of lipophilic drugs in the GI tract during and following lipid digestion. Therefore, high oleic acid (C18:1) versus linoleic acid (C18:2) ratio in vegetable oils, such as in olive and peanut oils, could be a reason for lower CBD distribution into the aqueous micellar phase in *in vitro* lipolysis experiment compared to other formulations (Figure 5-2B and Table 5-2).

It has been reported that triglycerides containing unsaturated fatty acids are hydrolysed more rapidly than those containing saturated fatty acids [247,248]. Moreover, rate and extent of lipid digestion are also affected by fatty acid chain length, with MCT being hydrolysed more efficiently and rapidly in comparison to LCT [131,188,249,250]. However, despite the fact that coconut oil contains a higher proportion of medium-chain and saturated fatty acids, such as myristic acid (C14:0) and palmitic acid (C16:0), it leads to lower distribution of CBD into the aqueous micellar phase in *in vitro* lipolysis compared to other vegetable oil vehicles (Figure 5-2B and Table 5-2). Furthermore, peanut oil contains about 10% of very-long-chain fatty acids, such as arachidic (C20:0) and eicosenoic acids (C20:1), which may delay the lipolysis process. As a result, CBD distribution into the aqueous micellar phase following *in vitro* lipolysis of peanut oil-based formulation was lower than for other formulations. Despite the fact that there were no statistically significant differences in the distribution of the CBD into lipid phase, olive and

peanut oils showed a trend of higher CBD distribution into the undigested lipid phase when compared to other formulations in *in vitro* lipolysis (Figure 5-2A).

Six lipid-based formulations and lipid-free formulation were then assessed *in vivo* in rats. With the exception of sunflower and coconut oil-based formulations, the rest of the formulations significantly enhanced the plasma AUC of CBD in comparison to the lipid-free formulation (Table 5-1). It is not surprising that the oral bioavailability of CBD following oral administration of the coconut oil-based formulation was comparable to that of the lipid-free formulation. According to the GC-MS/MS assessment, coconut oil contains 30% MCT (Table 5-2), which have lower participation in chylomicrons assembly process in comparison to LCT. A proportion of CBD dose associated with digested MCT was most probably absorbed through the hepatic portal vein transport pathway with hepatic first-pass metabolism, resulting in a lower overall bioavailability of CBD in co-administered coconut vehicle in comparison to other oil vehicles. In addition, a prolonged t_{max} (5 hours) was observed in the coconut oil group. Such prolonged drug absorption might be related to the fact that coconut oil contains more than 80% saturated fatty acids, which are hydrolysed slower than unsaturated fatty acids (Table 5-1) [248].

The *in vivo* PK results show that most lipid-based formulations significantly improved the bioavailability of CBD in comparison to the lipid-free formulation, particularly sesame and olive oil-based formulations outperforming other lipid-based vehicles. These *in vivo* findings are not entirely consistent with the results of the *in vitro* lipolysis assay. As was

previously reported, one of the limitations of *in vitro* lipolysis system is that it only simulates the lipid digestion step in the GI tract rather than the entire absorption and lymphatic transport process [67,188].

In biodistribution studies rat serum, lymph fluid and MLN samples were collected at predetermined time points, and analysed for CBD (serum, lymph fluid and MLN) and TG (serum and lymph fluid) levels. There were no statistically significant differences in the levels of TG or CBD in serum and MLN samples between all lipid-based formulation groups, which might be attributed to substantial variability (Figures 5-4 and 5-6). Interestingly, lower TG levels in lymph at one hour before plasma t_{max} (t_{max} – 1 h) in olive oil group indicated that the olive oil-based formulation did not produce as many chylomicrons as other lipid-based formulations at that early time point (Figure 5-5A). However, at t_{max}, olive oil efficiently promoted the chylomicrons production and showed the highest CBD levels, which suggests that olive oil can deliver higher levels of co-administered CBD through the lymphatic system in comparison to other natural lipids (Figure 5-5B). The overall biodistribution results suggest that olive oil was superior in promoting drug transport through the enterocytes and association with the chylomicrons in comparison to other vegetable oils.

To understand better the effect of lipid profile of various natural vegetable oils on the enhancement of chylomicron formation and drug association with chylomicrons, fatty acid composition has been assessed for lipid vehicles and the corresponding lymph

chylomicrons (Table 5-2). The data suggest that natural vegetable oils consisting of mainly oleic acid (C18:1) and linoleic acid (C18:2) promote the intestinal lymphatic transport of CBD by stimulating chylomicron formation (Figure 5-5 and Table 5-2). In agreement with these data, it was previously shown in Caco-2 cells that increased oleic acids and linoleic acids concentration in the medium stimulates the synthesis and secretion of chylomicrons [251,252]. Both sesame and olive oils are rich in unsaturated C18 fatty acids, therefore they promote the intestinal lymphatic transport and overall systemic exposure of CBD more efficiently than other vegetable oils.

Interestingly, olive oil has shown lower variability in lymphatic transport and bioavailability of CBD in comparison to sesame oil. The variability in absorption of lipophilic drugs administered in vegetable oil vehicle could be associated with oxidation of the oil. It has been found that photo-oxidation and auto-oxidation are major issues affecting the stability of edible oils [253,254]. The formation of fatty acids hydroperoxides is the sign of photooxidation, and the main peroxidation product is produced from linoleic acid [255,256]. Recent oils stability study has shown a decline in linoleic acid levels in nut oils following 14 days of continuous light exposure. However, fewer oxidative changes were observed in the oleic acid composition [257]. According to the fatty acid profile of vegetable oils described in Table 5-2, sesame oil contains higher proportion of C18:2 than olive oil, whereas olive oil is rich in C18:1. Thus, sesame oil could more prone to oxidation, which may explain higher variability in absorption of CBD observed with sesame oil vehicle than with olive oil formulation.

Moreover, the data of fatty acids composition in vegetable oils and in the corresponding lymph chylomicrons (Table 5-2) suggest that chylomicrons composition is primarily dictated by the composition of the administered oil. Similar conclusions were reached in other studies where size, density and fatty acid profiles of human and animal chylomicrons have changed in accordance to changes in lipid consumption [258–262]. Even though dietary lipids affect the fatty acids composition in chylomicrons, it appears that in all cases palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) are major fatty acids that could be found in chylomicrons at different proportions [263,264]. These fatty acids in various proportions may lead to differences in drug loading into chylomicrons. Previously published works reported that MCT appears in human chylomicrons after oral administration of MCT [265]. Indeed, the current study has also shown that MCT were detected in the chylomicron samples after administration of coconut oil (Table 5-2).

A trace amount of odd chain fatty acids such as heptadecanoic acid (C17:0) and cis-10-heptadecenoic (C17:1) has been found in both oil and chylomicron samples (Table 5-2). According to previous research, a proportion of heptadecanoic acid is directly supplied from diet, and the other part of C17:0 is synthesized from stearic acid (C18:0) through α -oxidation [266,267]. It has been suggested that odd chain fatty acids are associated with health benefits such as metabolic regulation. However, there is little research on the lymphatic transport aspect of these fatty acids [268]. Rat lymph chylomicrons have been

found to contain a certain amount of arachidonic acid (C20:4) following oral administration of all vegetable oil-based formulations. It appears that this fatty acid was added from nondietary source because it is absent in natural vegetable oils (Table 5-2). According to previous reports, arachidonic acid is synthesized from linoleic acid (C18:2) during the absorption/lymphatic transport process, and its metabolites are associated with the regulation of lymphatic contractility [269–271].

Moreover, arachidonic acid is a major component of phospholipids, which are located on the cell membranes [272,273]. A class of phospholipids, phosphatidylethanolamine, can be converted to N-acylphosphatidylethanolamine (NAPE), a precursor of two major endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG) [274,275]. These endocannabinoids are neurotransmitters that regulate the immune system and other pharmacological effects of cannabis by binding to cannabinoid receptors [276–279]. In addition to their interaction with CB1 and CB2 receptors, anandamide and 2-AG also interact with non-cannabinoid receptors, such as GPR55 and TRPV1 [280,281]. CBD is a phytocannabinoid that also interacts with these receptors of endocannabinoid system [281,282]. Furthermore, there are studies that demonstrated that intake of unsaturated dietary fats could significantly enhance anandamide and 2-AG concentrations in the brain and intestine [283,284]. Therefore, the endogenous addition of arachidonic acid to lymph chylomicrons could suggest potential enhanced activation of the endocannabinoid system following oral administration of CBD with lipids, beyond the interaction of CBD itself with relevant receptors.

It should be noted, that in addition to fatty acids composition investigated in this study, it is also possible that other small molecule ingredients in vegetable oils, such as lignans, vitamin E and phospholipids can contribute to their optimal performance as vehicles facilitating the lymphatic transport of lipophilic drugs [164,166,167]. The involvement of these substances in regulating chylomicron production or lipophilic drug lymphatic transport needs to be further investigated in future studies.

5.5. Conclusion

In this study, six different natural vegetable oils were evaluated as lipid vehicles to facilitate the intestinal lymphatic transport and oral bioavailability of a lipophilic model drug CBD. The fatty acid composition in oils and corresponding lymph chylomicrons following oral administration of lipids was assessed. According to fatty acid analysis and *in vivo* studies, coconut oil-based formulation has higher MCT content than other oils, which leads to lower lymphatic transport and systemic bioavailability of CBD in comparison to other lipid-based formulations. In contrast, sesame and olive oil vehicles led to higher CBD concentrations in lymphatic tissues and systemic circulation than other natural vegetable oils. This is most likely due to the fact that sesame and olive oils contain higher proportion of oleic (C18:1) and linoleic acids (C18:2), which are known to promote intestinal lymphatic transport. However, sesame oil has higher variability of CBD absorption in comparison to olive oil, most probably because it is more prone to oxidation due to higher linoleic acid content.

6. PLANT DERIVED INTACT OIL BODIES FACILITATE SYSTEMIC BIOAVAILABILITY AND LYMPHATIC TARGETING OF CANNABIDIOL

6.1. Introduction

Plant derived oils have been extensively investigated as a lipid excipient in formulations to improve drug oral absorption for many years, primarily for poorly water soluble or low permeability compounds [16,21,25,285]. It is due to the fact that the oral drug delivery system is a safer and preferable drug administration route for patients, particularly those with chronic diseases who have to take long-term medicines [286]. However, most lipophilic compounds have limited oral bioavailability because of their low water solubility and membrane permeability [224,287]. Therefore, lipid-based formulation attempts to assist lipophilic drugs in overcoming these problems.

Lipophilic compounds are mainly absorbed in the small intestine due to their physicochemical characteristics [288,289]. The mixed micelles are produced in the gastrointestinal (GI) tract. These mixed micelles, which are composed of lipids, bile salts and phospholipids, can deliver these lipophilic compounds through the unstirred water layer (positioned in front of the brush border) and passively diffuse to the enterocyte [290,291]. Therefore, the co-administered lipid vehicles, especially triglycerides, provides

substantial lipids to enhance the solubility of these lipophilic compounds in the mixed micelles.

Furthermore, a highly lipophilic compound, with logD7.4 more than 5, has the potential to associate with chylomicrons in the enterocyte [141]. Chylomicrons are large lipoproteins that can be uptaken directly to the lymph lacteals, thus drugs carried in chylomicrons can bypass the portal vein to avoid first-pass hepatic metabolism [177,178]. Therefore, drug association in chylomicrons can avoid first-pass effect loss and enhance overall systemic exposure. Moreover, the primary component of chylomicrons are long-chain triglycerides, which can be either obtained from the external diet or generated endogenously [292–294]. Because most vegetable oils are rich in long-chain triglycerides, they have been used as lipid-based carriers to facilitate the lymphatic transport and oral bioavailability of co-administered highly lipophilic drugs [242,295,296].

Previous studies have demonstrated that natural vegetable oil-based vehicle can successfully deliver lipophilic compounds to the systemic circulation following oral administration [1,23,120,156]. However, vegetable oil-containing lipid-based formulations have stability issues and substantial variability in drug absorption [1,2,129,297,298]. Therefore, many researchers are keen on investigating more stable formulations such as adding surfactants into oils to prevent oil oxidation, or developing commercial lipid mixtures composed of various types of fatty acids and triglycerides [23,299,300]. The primary objective of these works was to attempt to employ these pre-emulsified or self-

emulsified drug delivery systems to achieve higher drug distribution into mixed micelles in the GI tract. However, even though some of these experiments have effectively improved the oral bioavailability of lipophilic drugs, the addition of surfactants or co-solvent to oils did not outperform a single type of natural vegetable oil [2,301]. In particular, some vegetable oils are rich in long-chain triglycerides to produce chylomicrons, facilitating the lymphatic transport of these lipophilic compounds.

Moreover, it has been proposed that natural ingredients in vegetable oils might contribute to the transport of fatty acids in enterocytes or the formation of chylomicrons therefore improving lymphatic transport and the oral bioavailability of lipophilic drugs [302]. Interestingly, vegetable oils have been found to be stored in plant seeds or fruits in a form similar to human or animal lipoproteins [303]. This biosynthesised structured intracellular small spherical particle is identified as the oil body, also named as oleosome [304]. Intriguingly, oil bodies are found to have a similar size (0.2 to 2.5 μm in diameter) and structure to the human or animals chylomicrons [305,306]. Triglycerides are the primary component of oil bodies, which is surrounded by unilaminar phospholipid and alkaline hydrophobic proteins [307,308]. These proteins, known as oleosin, caleosin and steroleosin, are embedded on the surface of oil bodies and play a crucial role in stabilising the oil body structure to avoid flocculation and coalescence during plant growth [307,309].

Oil bodies have been suggested as a lipid carrier for formulation applications due to many advantages: 1) All components in oil bodies are natural ingredients and safe to ingest; 2)

The extraction technique of oil bodies is simple with high yield; structure of oil bodies are similar to pre-emulsified o/w emulsion, therefore oil bodies can be used as excipients with no further homogenization; 3) Proteins are found on the surface of oil bodies to maintain the structure stable [308,310]. The current method has already been demonstrated to extract intact oil rapeseed bodies by aqueous extraction using bicarbonate-based soaking [311]. In addition, there are publications for oil bodies applied to transdermal drug delivery systems such as gel and cream [312–314]. However, oil bodies have never been investigated as an oral drug carrier since they were discovered.

Thus, plant derived intact oil bodies will be employed as a lipid vehicle for delivering lipophilic drug cannabidiol (CBD) through the lymphatic system to the systemic circulation and compared to the corresponding vegetable oil in this study. Cannabidiol is a non-psychoactive phytocannabinoid extract that has been shown more than 60% association with both human and rat chylomicrons and artificial emulsion, therefore it has been chosen as a model drug [120]. The rapeseed has been selected for the oil body extractions. Facilitation of systemic bioavailability and lymphatic targeting of CBD by oil bodies and rapeseed oil will be investigated by means of *in vitro* lipolysis and *in vivo* rat model.

6.2. Experimental design

CBD was formulated in natural rapeseed oil as described in Section 2.2.4. The preparation of oil bodies-based formulation is described in Section 2.2.5. Both rapeseed oil- and oil

bodies-based formulations were assessed by *in vitro* lipolysis and *in vivo* pharmacokinetic and biodistribution studies as described in Sections 2.3. and 2.4., respectively. The fatty acid compositions in rapeseed oil and the oil bodies were assessed as described in Sections 2.5.4. and 2.6.3. Samples from *in vitro* lipolysis and *in vivo* experiments were prepared for HPLC analysis as described in Sections 2.5.1. to 2.5.3. The HPLC conditions are described in Sections 2.6.1. and 2.6.2. The triglyceride levels were determined in *in vivo* samples as described in Section 2.5.5. The experimental design is summarised in the general scheme flowchart and presented in Figure 6-1.

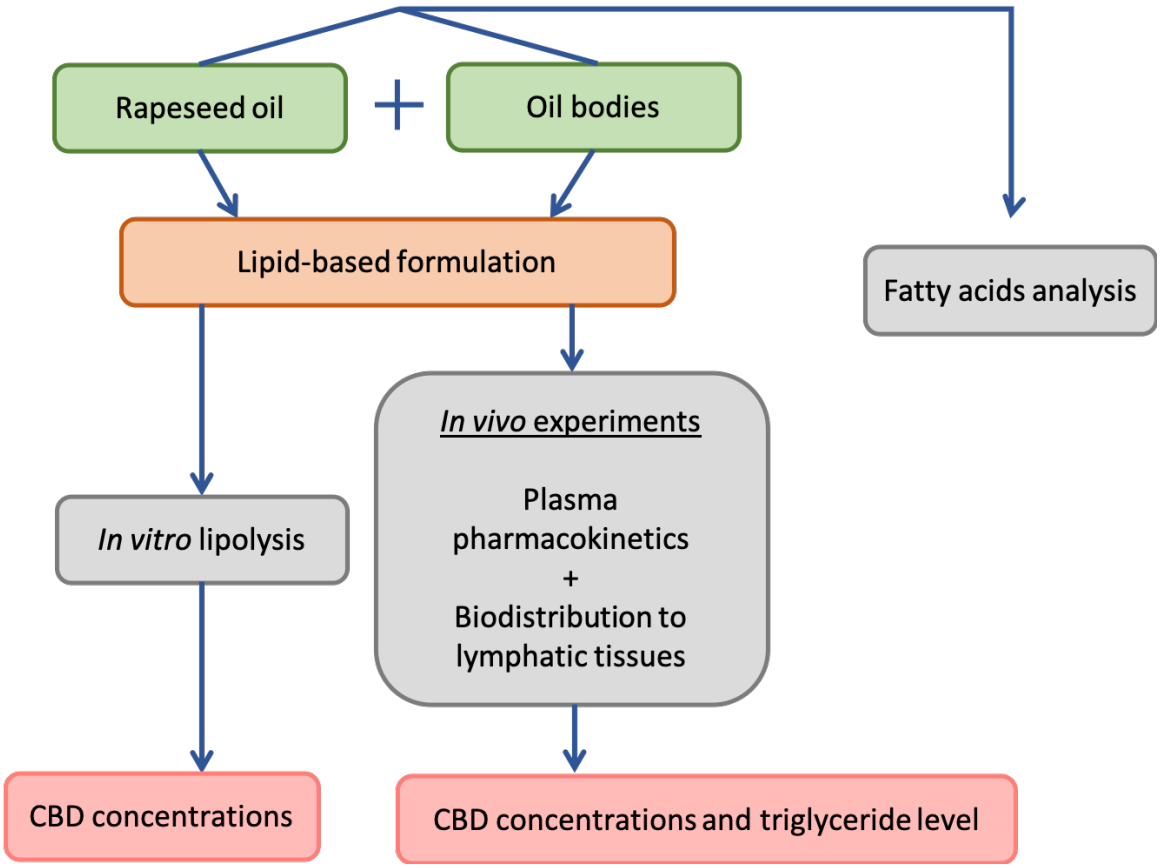


Figure 6- 1 . Flowchart of experimental design of comparing the bioavailability and lymphatic targeting of CBD in co-administered rapeseed oil and oil bodies. CBD, cannabidiol.

6.3. Results

6.3.1. Fatty acid composition of lipid vehicles

Because the batch plant sources of commercially obtained rapeseed oil and oil bodies are different, the oil from the rapeseed oil bodies has been extracted and the fatty acid composition has been subsequently measured for both oils (Table 6-1). The fatty acid contents of rapeseed oil and oil bodies were comparable, with saturated, monounsaturated and polyunsaturated fatty acids present. The differences between rapeseed oil and oil bodies were found in the amount of behenic acid (C20:0), oleic acid (C18:1) and linolenic acid (C18:3), and there was no erucic acid (C22:1) presented in the oil body samples, but present in the commercial rapeseed oil.

Table 6-1. Fatty acid composition in rapeseed oil and the oil extracted from oil bodies.

Fatty acid (% of total fatty acids)		Oil	
		Rapeseed (commercial)	Oil bodies (oil extracted from oil bodies)
Saturated [a]	**C14:0**	0.09 ± 0.01	0.10 ± 0.00
	C16:0	5.10 ± 0.06	5.11 ± 0.09
	C17:0	0.06 ± 0.00	0.06 ± 0.01
	C18:0	0.19 ± 0.04	0.17 ± 0.07
	C20:0	0.44 ± 0.02**	0.37 ± 0.01
	C22:0	0.31 ± 0.01	0.28 ± 0.02
	C24:0	0.13 ± 0.01	0.12 ± 0.02
Unsaturated [b]	**C16:1**	0.28 ± 0.00	0.25 ± 0.02
	C17:1	0.08 ± 0.01	0.12 ± 0.04
	C18:1	67.21 ± 0.21****	64.20 ± 0.11
	C18:2	18.2 ± 0.09	19.39 ± 0.19
	C18:3	6.65 ± 0.2****	8.84 ± 0.09
	C20:1	1.14 ± 0.04	1.11 ± 0.02
	C22:1	0.19 ± 0.00	

[a] C14:0 = myristic; C16:0 = palmitic; C17:0 = heptadecanoic; C18:0 = steric; C20:0 = arachidic; C22:0 = behenic; C24:0 = lignoceric

[b] C16:1 = palmitoleic; C17:1 = cis-10-heptadecenoic; C18:1 = oleic; C18:2 = linoleic; C18:3 = linolenic; C20:1 = eicosenoic; C22:1 = erucic

, **; are statistically significantly different from the oil body group (**, p <0.01; ****, p < 0.0001)

6.3.2. Distribution of CBD in lipolysis fractions following *in vitro* lipolysis of rapeseed oil- and oil bodies-based formulations

The simulated intestinal fluid at the fasted stage was used to determine the digestion efficacy of lipid-based formulations by *in vitro* lipolysis system (Figure 6-2). There were no differences in the recovered dose of CBD in the aqueous phase between rapeseed oil and oil body formulations. The proportion of the dose of the drug distribution into the micellar phase following digestion represent the drug theoretically available for absorption into enterocyte [50,315]. There was a higher (4.3-fold) undigested formulation remaining in the oil phase for the rapeseed oil-based formulation than the oil bodies-based formulation. CBD in the sediment phase was 1.6-fold less in the rapeseed oil group when compared to the oil body group.

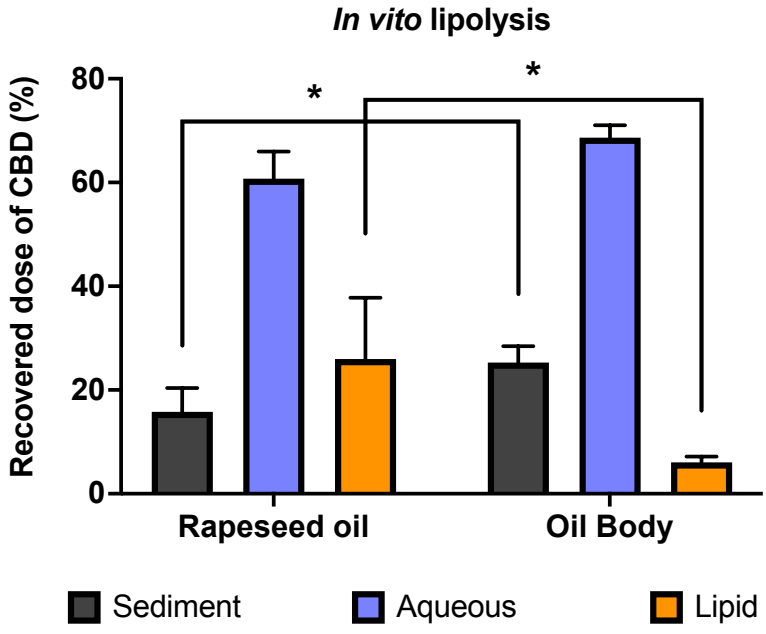


Figure 6- 2 . Distribution of CBD in sediment, aqueous and lipid phases following *in vitro* lipolysis of rapeseed oil- and oil bodies-based formulations. The concentration of

CBD was 12 mg/mL in rapeseed oil and 6 mg/mL in oil bodies. The experiment was terminated after one-hour reaction. The two-tailed unpaired student t-test was used for statistical analysis (n=3, mean ± standard deviation (SD)). *, p <0.05.

6.3.1. *In vivo* pharmacokinetics of CBD following oral administration of rapeseed oil- and oil bodies-based formulations

Rapeseed oil- and oil bodies-based formulations were administered orally to rats for the *in vivo* pharmacokinetic studies and rat plasma concentration-time profiles of CBD are presented in Figure 6-3. Table 6-2 is summarizing of the pharmacokinetic parameters, which are half-life ($t_{1/2}$), time to maximum concentration in plasma (t_{max}), the maximum concentration in plasma (C_{max}) and plasma concentration-time curve (AUC). The oil body group showed a more rapid digestion time and higher drug concentration (C_{max} and AUC) compared to the rapeseed oil group.

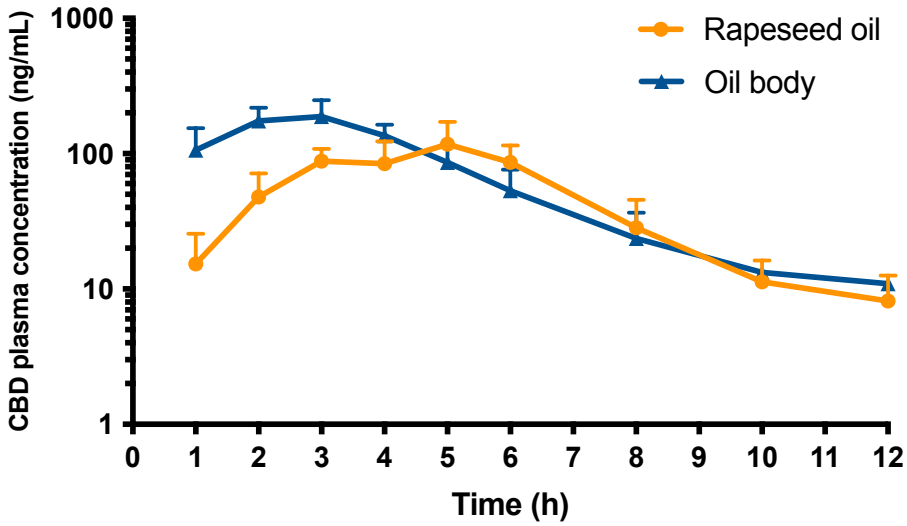


Figure 6-3. Plasma concentration-time profiles of CBD following oral administration of rapeseed oil- and oil bodies-based formulations. The concentration of CBD in rapeseed oil was 12 mg/mL and in oil bodies-based formulations was 6 mg/mL, the administered dose of CBD was 12 mg/kg in all treatment groups (n=6, mean ± standard deviation (SD)).

Table 6-2. Pharmacokinetic parameters of CBD derived from plasma concentration-time profiles following oral administration of rapeseed oil- and oil bodies-based formulations (mean ± standard deviation (SD)).

Formulation	$t_{1/2}$[a] (h)	t_{max}[b] (h)	C_{max}[c] (ng/mL)	$AUC_{0-\infty}$[d] (h×ng/mL)	n
Rapeseed oil	1.6 ± 0.4	5	124 ± 47	521 ± 140	6
Oil bodies	2.2 ± 0.1	3	209 ± 57*	882 ± 137**	6

[a] half-life;

[b] time to maximum concentration in plasma;

[c] the maximum concentration in plasma;

[d] AUC from 0 to infinity;

*, ** are statistically significantly different from rapeseed oil (*, p <0.05; **, p <0.01).

6.3.1. Lymphatic targeting of CBD following oral administration of rapeseed oil- and oil bodies-based formulations

The serum and lymph samples were harvested in the biodistribution study following oral administration of rapeseed oil- and oil bodies-based formulations. The CBD concentrations and the triglyceride levels were analysed in the corresponding samples. The pre-determined time including plasma t_{max} and one hour before t_{max} (t_{max} – 1 h) for tissue collection were selected based on the pharmacokinetics results (Table 6-2). CBD concentration in serum at t_{max} – 1 h following oral administration of oil bodies-based formulation was higher than the rapeseed oil-based formulation (Figure 6-4A). The triglyceride concentration in lymph fluid has been found to be 2.5-fold higher level for rapeseed oil than for oil bodies group at plasma t_{max} (Figure 6-5B). There were no statistically significant differences in CBD concentrations in both lymph fluid and mesenteric lymph node (MLN) samples between rapeseed oil and oil bodies groups (Figures 6-5 and 6).

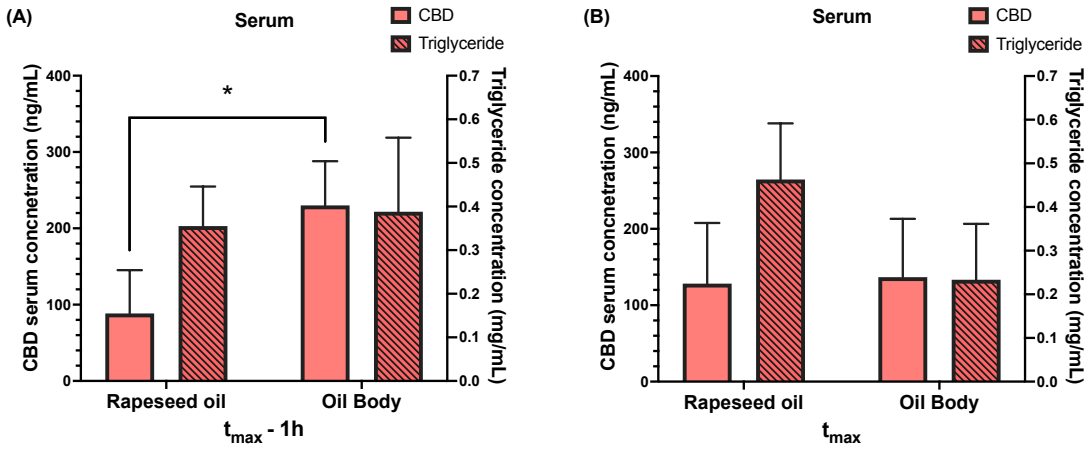


Figure 6-4. Concentration of CBD and triglyceride in rat serum. CBD was orally administration in rapeseed oil (12 mg/mL) and oil bodies (6 mg/mL) at a dose of 12 mg/kg to rats. (A) The concentration of CBD and triglyceride level in rat serum at one-hour prior to t_{max} (t_{max} – 1 h). (B) The concentration of triglyceride and CBD in rat serum at t_{max}. All data are shown as mean ± standard deviation (SD), n=4. The two-tailed unpaired student t-test was used for statistical analysis. *, p <0.05.

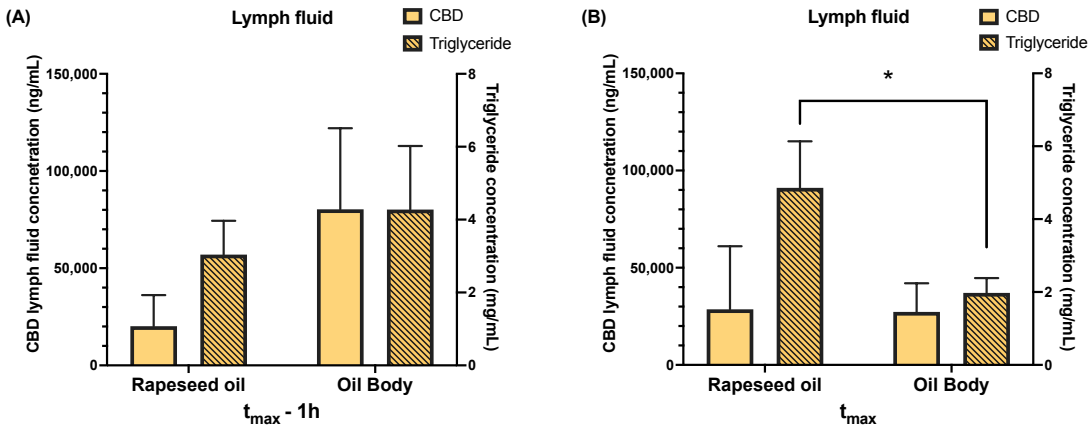


Figure 6-5. Concentration of CBD and triglyceride in lymph fluid. CBD was orally administration in rapeseed oil (12 mg/mL) and oil bodies (6 mg/mL) at a dose of 12 mg/kg to rats. (A) The concentration of CBD and triglyceride level in lymph fluid at one-hour prior to t_{max} (t_{max} – 1 h). (B) The concentration of triglyceride and CBD in

lymph fluid at t_{max}. All data are shown as mean ± standard deviation (SD), n=4. The two-tailed unpaired student t-test was used for statistical analysis. *, p <0.05.

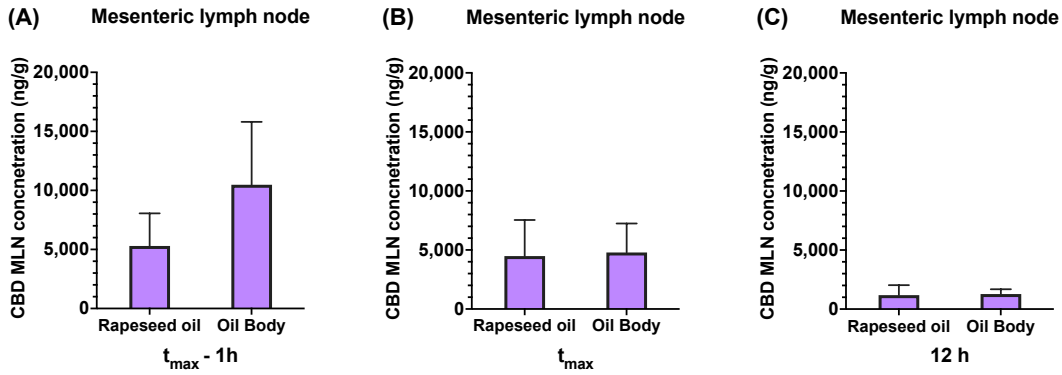


Figure 6- 6 . Concentration of CBD in mesenteric lymph nodes (MLNs). CBD was orally administration in rapeseed oil (12 mg/mL) and oil bodies (6 mg/mL) at a dose of 12 mg/kg to rats. (A) The concentration of CBD MLNs at one-hour prior to t_{max} (t_{max} – 1 h). (B) The concentration of MLNs at t_{max}. (C) The concentration of CBD MLNs at 12 h. All data are shown as mean ± standard deviation (SD), n=4-6. Two-tailed unpaired student t-test was used for statistical analysis.

6.4. Discussion

The lipid-based drug delivery is a promising technique to improve the oral absorption of lipophilic compounds [16,21,25]. The primary lipids used in the lipid-based formulations are fatty acids, natural oils and other triglycerides [21]. Such selection of lipids is inspired by the digestion and absorption mechanisms of triglycerides in humans and animals [77,292]. Vegetable oils primarily consist of triglycerides extracted from seeds [316]. The oil bodies, also known as oleosomes, has been discovered in plant seeds and nuts to store

these triglycerides [304]. The physical features of oil bodies are comparable to the unilaminar spherical lipoproteins mentioned above, thus oil bodies can be thought of as a natural drug carrier [308,310,317]. Rapeseed oil bodies were chosen in the current study as a lipid vehicle for delivering CBD through the lymphatic system to the systemic circulation and compared to the rapeseed oil.

Because of the drug loading capacity, the optimum concentration of CBD in oil bodies was 6 mg/mL, which was half the value of the CBD concentration in rapeseed oil. The dose of CBD for *in vitro* and *in vivo* studies was same for both formulations, respectively. The digestion of the lipid-based formulations in small intestine were assessed by means of *in vitro* lipolysis model. Less CBD reminding in the undigested lipid phase following *in vitro* lipolysis of oil bodies-based formulation compared to rapeseed oil-based formulation (Figure 6-2). This may due to the surrounding phospholipids on the surface of oil bodies aiding in formulation digestion, allowing more lipid to be hydrolysed to produce mixed micelles in the aqueous phase when compared to rapeseed oil.

However, the oil bodies-based formulation had also more CBD recovered in the sediment phase following *in vitro* lipolysis in comparison to rapeseed oil-based formulation. A considerable quantity of proteins is embedded in the phospholipid layer on the surface of oil bodies [305]. An *in vitro* gastric and intestinal digestion of walnut oil bodies has previously been reported, with proteins in oil bodies first being hydrolysed by pepsin in the gastric environments and then further digested by the gastrointestinal proteases [318].

However, the current study did not include pepsin or intestinal proteases. As a result, a portion of protein may be denatured and precipitate in the sediment phase during *in vitro* lipolysis experiment.

In addition, a previously reported *in vitro* study has indicated that hydrolysed and non-hydrolysed proteins could remain on the surface of oil bodies in the stomach until they reach the small intestine [318,319]. Under the gastric conditions, the capacity of the protein to stabilise the oil body structure diminishes following enzymatic digestion, and size of oil bodies has increased [320]. However, the droplet size of oil body emulsion is smaller in the GI tract before forming mixed micelles than in the gastric conditions [321]. Because oleosin is still functional in stabilising the oil body structure, these oil bodies will not swell more than in stomach environment [322]. Then fatty acids are released from hydrolysed triglycerides in oil bodies to form the multiple-phase emulsion, with a water-protein-rich phase situated in the core of this emulsion [318].

As a result, the structure of the oil body emulsion is more stable and could have reduced variability in formulated drug absorption compared to simple oil emulsion during the digestion process. Both *in vitro* and *in vivo* experiments also corroborate this viewpoint. There were lower variations for the CBD distributed into three phases after *in vitro* lipolysis (Figure 6-2) and higher CBD oral absorption in rat studies for the oil bodies-based formulation than for rapeseed oil-based formulation (Figure 6-3 and Table 6-2).

However, oil bodies and rapeseed oil supply sources were different, which might impact lipid digestion in the GI tract and thus influence the CBD absorption. Even though fatty acids analysis results (in rapeseed oil and oil bodies have shown statistically significant differences in oleic acid (C18:1), linolenic acid (C18:3) and behenic acid (C20:0)) composition, the actual changes were less than one-fold (Table 6-1). Therefore, in the current study, the fatty acid profile differences in the formulations may not be a substantial factor in the differences in drug absorption.

Moreover, findings in Chapter 5 suggested that when oils consist of high proportion of oleic acid, the chylomicron production and drug associates to chylomicrons might be promoted. There was a higher level of triglyceride in lymph fluid at t_{max} following oral administration of rapeseed-oil based formulation than the oil body group, which might be attributed to the higher oleic acid content in rapeseed oil than oil bodies (Figure 6-5 and Table 6-1).

Although the triglyceride levels in rat serum and lymph fluid have shown a higher trend in the rapeseed oil group than the oil body group, the drug distribution into tissues was opposite to the triglyceride concentration results (Figures 6-4 to 6-6, Appendices Figure 7-1 and Table 7-1). Oil bodies can deliver more CBD into intestinal lymphatic tissues and systemic circulation, which might be due to the unique structure of oil bodies, allowing oil body emulsion to remain structurally stable during the digestion in the GI tract in comparison to the oil. Furthermore, the additional phospholipids in oil bodies can aid in

the formation of mixed micelles with bile salts and cholesterol in the intestinal lumen, increasing the drug solubility in these mixed micelles and resulting in more CBD absorption through the intestinal lymphatic transport compared to rapeseed oil-based formulation.

However, different batches of oil bodies produced might have different impact on drug absorption. The preliminary study for oral administration of oil bodies-based formulation has shown that the overall CBD AUC were no less than 1200 h×ng/mL, whereas the average AUC from another batch of oil bodies-based formulation was 882 ± 137 h×ng/mL (Appendix Table 7-1 and Table 6-2). Such differences might arise due to differences introduced in the manufacturing and purifying steps, leading to different moisture content of oil bodies [311]. Consequently, although the CBD concentrations in two batches of oil bodies-based formulations were the same, the triglyceride levels were different and the drug absorption through the intestinal lymphatic system was affected. It is worth noting that the spray dried oil bodies-based formulation powder can be obtained to eliminate the batch to batch variability in further investigations [323–325]. Overall, it seems that the oil bodies-based vehicle enhances the CBD lymphatic transport and oral bioavailability in rats in comparison to the rapeseed oil. However, future experiments will be needed to confirm these preliminary data.

6.1. Conclusion

In this study, the natural rapeseed oil bodies were used as lipid-based vehicles to facilitate CBD oral bioavailability and lymphatic transport. Both *in vitro* and *in vivo* studies have suggested that oil bodies can be digested more efficiently than their natural oil counterparts (rapeseed oil in the current study). In rat pharmacokinetics experiments, oil bodies led to 1.7-fold higher total oral absorption of co-administered CBD when compared to rapeseed oil. The oil bodies-based vehicles facilitate delivery of CBD to the lymphatic tissues, including lymph fluids and mesenteric lymph nodes, at one hour prior to t_{max} when compared to rapeseed oil. Despite the fact that oil bodies were produced from rapeseed seeds, the sources of rapeseed oil and rapeseed were different. There were discrepancies in fatty acid profiles of two lipid vehicles in arachidic (C20:0), oleic (C18:1), linolenic (C18:3) and erucic (C22:0) acids, which might affect the drug absorption in this study. Oil bodies have never been investigated as an oral drug carrier, and the current study shows that employing this natural plant-based emulsion could have a great potential to improve lipophilic drug systemic exposure and lymphatic targeting in comparison to simple oils. Further research will be required to confirm these preliminary findings and to determine if other plant seeds or fruit-derived oil bodies have a comparable impact compared to rapeseed oil bodies. Moreover, the safety and long-term stability of oil bodies need to be assessed in further investigations.

7. GENERAL THESIS DISCUSSION, IMPLICATIONS, LIMITATIONS AND FUTURE WORK

7.1. General discussion

This PhD project has confirmed that oral lipid-based drug delivery approaches increase the intestinal lymphatic transport and systemic bioavailability of a model highly lipophilic drug cannabidiol (CBD). The primary aim of this project is to address the shortcomings of sesame oil as a lipid vehicle, which is associated with considerable variability in intestinal lymphatic transport and systemic bioavailability of co-administered CBD.

Sesame oil-based formulation has been shown in previous studies to lead to a high concentration of CBD in the intestinal system and higher systemic bioavailability than lipid-free formulation [120,129]. However, both lymphatic transport and bioavailability of CBD were also associated with substantial variability following oral administration of sesame oil-based formulation. Therefore, the first tested approach in this work was to try to reduce the variability associated with the formulation by substituting sesame oil with purified lipids or pre-digested lipids. The selection of such lipids was based on the hypothesis that the intraluminal digestion is a possible rate-limiting step in the processing of lipidic vehicles and the absorption of CBD through the lymphatic system, therefore the digestion of lipids in the gastrointestinal (GI) tract could be a source of variability. Such hypothesis is also supported by the fact that sesame oil contains a variety of saturated,

monounsaturated and polyunsaturated triglycerides, which could influence the lipid digestion rate and extent in the intestinal lumen [247,326].

However, as described in Chapter 3, CBD administered in pre-digested lipid vehicles exhibited comparable variability of intestinal lymphatic transport and systemic bioavailability to administration in sesame oil formulation. In addition, sesame oil-based formulation led to around 3-fold higher area under the curve (AUC) of CBD compared to pre-digested lipid formulations (Chapter 3, Table 3-1). Therefore, the intraluminal digestion step does not appear to be a main cause of variability in intestinal lymphatic transport and systemic bioavailability if CBD.

Sesame oil contains a variety of triglycerides and small bioactive molecules that may have a synergistic effect on the absorption of lipids and co-administered highly lipophilic compounds, resulting in higher CBD distribution into the intestinal lymphatic system and systemic circulation compared to pre-digested and purified lipids. Therefore, investigating additional ingredients or the diverse forms of fatty acids in sesame oil could aid in the development and design of lipid excipients with more predictable performance for lipid-based formulations. Commercial excipients such as glyceryl monolinoleate (Maisine® CC) and glyceryl monooleate (Peceol^{TM}) have previously been shown to improve the *in vitro* solubility and *in vivo* oral bioavailability of highly lipophilic compounds [327,328]. However, natural sesame oil appears to be superior in promoting the lymphatic targeting of lipophilic drugs, particularly those with immunological properties against cancer,

inflammation and human immunodeficiency virus (HIV) compared to other type of lipid-based formulations [129,155,329,330]. This emphasises the importance of natural sesame oil as a vehicle for facilitating the intestinal lymphatic transport of CBD. Moreover, avoiding the hydrolysis of triglycerides in the intestinal lumen has little effect on the absorption rate and extent of lipids and co-administered CBD.

Medium-chain triglyceride (MCT) and surfactants were then added into the sesame oil-based formulation to facilitate the emulsification and aqueous micellar solubilization of CBD in the GI tract (Chapter 4). The hypothesis was that increased emulsification and micellar solubilisation efficiency would lead to lower variability and higher extent of lymphatic transport and oral bioavailability of CBD. Surfactants, including Tween® 85, Tween® 80, Span® 20 and d-α-tocopherol polyethylene glycol 1000 succinate (TPGS) as a solubilizer, have previously been extensively employed in oral lipid-based drug delivery to enhance the encapsulation of poorly water-soluble drugs in mixed micelles during intraluminal digestion [217,331–336]. The MCT have been shown to be digested more rapidly and thoroughly compared to long-chain triglycerides (LCT) in *in vitro* lipolysis model [131]. Indeed, the inclusion of Tween®85, TPGS and MCT in the lipid-based formulation has increased the aqueous micellar solubility of CBD in the simulated intestinal digestion phase following *in vitro* lipolysis (Chapter 4, Figure 4-2), but sesame oil formulation still outperformed concerning the extent of CBD lymphatic transport and oral absorption *in vivo* (Chapter 4, Figures 4-4 to 4-7). However, addition of surfactants

and MCT to sesame oil contributed to a reduction of AUC variability and to shortening the t_{max} of co-administered CBD (Chapter 4, Table 4-1).

Similar formulation approaches have been investigated by other researchers but using self-nano-emulsifying drug delivery systems (SNEDDS) focusing on increasing CBD overall oral bioavailability, where they predominantly used a high proportion of surfactant with LCT to reduce the particle size at the nanoscale following self-emulsification of formulations [128,201,337]. Even though oral administration of SNEDDS was shown to substantially improve the bioavailability of CBD in comparison to CBD powder, equivalent drug absorption was observed when compared to sesame oil-based or other natural oils formulations. However, the current project suggests that using pure sesame oil as the lipid vehicle results in higher levels of CBD being distributed into the intestinal lymphatic system compared to formulations containing additional surfactants and MCT in sesame oil. In addition, CBD was previously found to have an effective chylomicron association of at least 67% to rat and human chylomicrons [120]. Accordingly, pure sesame oil with a high LCT content may stimulate the production of chylomicrons more efficiently than formulations containing MCT or surfactants, further resulting in enhanced CBD chylomicron association during intestinal lymphatic transport.

In addition, a lack of correlation between *in vitro* and *in vivo* results were shown when delivering CBD using lipid-based formulations in this thesis as described in Chapter 4. This discrepancy between *in vitro* lipolysis and *in vivo* rat model could be explained by the fact

that the *in vitro* test only focuses on the digestion step in the GI tract rather than an entire absorption and transport (portal or lymphatic) process [50]. As a result, lipid-based formulations with a high LCT proportion may have lower drug levels in the aqueous micellar phase following *in vitro* lipolysis, but higher lymphatic transport *in vivo* in comparison to formulations with a high surfactant or MCT content [338]. The vehicle containing high levels of LCT can efficiently enhance the lymphatic transport and oral bioavailability of highly lipophilic compounds with relatively high affinity to chylomicrons. Therefore, advanced and sophisticated lipid-based formulations might not be in fact needed for targeting highly lipophilic drugs, with high affinity to chylomicrons, to intestinal lymphatic system. In this project, it becomes clear that mechanisms other than hydrolysis and emulsification steps also dominate CBD absorption rate and extent following oral co-administration of sesame oil-based formulation during intestinal lymphatic transport.

A new hypothesis was that the inter-subject CBD variability associated with co-administered sesame oil could be diminished by the alternative approaches, which replace sesame oil with other natural vegetable lipids (Chapter 5). Six natural vegetable oils, including sesame, soybean, peanut, olive, sunflower and coconut oils, were chosen because they are commonly employed as lipid vehicles facilitating lymphatic transport or oral bioavailability of highly lipophilic drugs [156,238,239,339]. According to the *in vivo* pharmacokinetic study in rats, sesame- and olive-based formulations have significantly increased CBD systemic exposure by 2.4- and 2.3-fold, respectively, compared to the lipid-free formulation (Chapter 5, Table 5-1). Interestingly, only sesame oil formulation showed

high variability in CBD absorption in comparison to other dietary oils in this project. Such high variability might be attributed to the higher C18:2 in sesame oil, which causes photooxidation compared to olive oil, as discussed in Chapter 5. In addition, the *in vivo* biodistribution study has confirmed that olive oil has provided sufficient fatty acids to produce chylomicrons and resulted in delivery of higher levels of CBD to the intestinal lymphatic system than other oils (Chapter 5, Figure 5-5).

Gas chromatography-tandem mass spectrometry (GC-MS/MS) was applied to analyse the fatty acid profile of different vegetable oils and rat chylomicrons, which were collected after the oral administration of corresponding lipid-based formulations, to help in understanding of the performance of natural vegetable oils as a vehicle facilitating the lymphatic transport of CBD (Chapter 5, Table 5-2). Coconut oil contains 30% medium-chain fatty acids (MCFA) of the total fatty acid composition, all of which are saturated fatty acids, leading to a lower lymphatic transport and oral bioavailability of co-administered CBD compared to other oils. On the other hand, sesame, soybean, peanut, olive and sunflower oils are rich in long-chain fatty acids (LCFA), in particular oleic acid (C18:1) and linoleic acid (C18:2). Following oral administration of dietary lipids, the fatty acid composition in chylomicrons is remarkably similar to that of the corresponding natural vegetable oils. It was found that arachidonic acid (C20:4) was present in rat chylomicrons, but was absent in natural vegetable oils, suggesting that C20:4 was added endogenously in the intestinal mucosa. C20:4, which could be biosynthesised from C18:2, have been shown

to be involved in the modulation of lymphatic contractility during lymphatic transport [269–271].

Interestingly, arachidonic acid (C20:4) is a crucial compound important for endocannabinoid system as discussed in Chapter 5. The phytocannabinoid CBD in this study is also interacts with endocannabinoid receptors [281,282]. In addition, it has been found previously that ingestion of gamma-linolenic acid in diet enhances the C20:4 concertation in plasma and serum [340]. Intake of unsaturated dietary fats could significantly enhance anandamide and 2-AG concentrations in the brain and intestine [283,284]. Therefore, the endogenous addition of C20:4 to lymph chylomicrons could suggest potential enhanced activation of the endocannabinoid system following oral administration of CBD with lipids, beyond the interaction of CBD itself with relevant receptors.

Another important metabolic pathway of arachidonic acid is that phospholipids on the cell membrane are hydrolysed by phospholipase A2 (PLA2) to release free arachidonic acid [341,342]. Arachidonic acid can be further metabolised in two major eicosanoid pathways through the involvement of the enzymes cyclooxygenases (COXs) and lipoxygenases (LOXs) to mediate the immunoregulation. Arachidonic acid can be converted to prostacyclin (PGI2) and prostaglandins by COX-1 and COX-2 (also known as prostaglandin H synthase) [343–345]. In the LOXs pathway, arachidonic acid can be oxidised to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by the catalysation of 5-LOX

and then generate various leukotrienes (LTs) [343,344,346]. Interestingly, multiple sclerosis (MS), one of the autoimmune diseases for which CBD could be indicated, is associated with an elevated level of PGI2, prostaglandins and LTs in both animals and human studies [347–350]. In the current study, there is lower amount of arachidonic acid in chylomicrons following oral administration of olive oil-based formulation compared to other formulations, which may be related to the fact that olive oil contains lower levels of linoleic acid (Table 5-2). CBD has been shown to alleviate symptoms of MS in animals models and clinical studies [351–353]. Therefore, CBD may be beneficial for the treatment of auto-immune diseases when co-administered with a lipid vehicle that produces lower levels of C20:4 following oral administration.

Overall, CBD in olive oil improves the intestinal lymphatic transport and oral bioavailability with lower variability than other formulations, and with production of lower levels of arachidonic acid *in vivo*. Thus, olive oil seems to be a preferred lipid vehicle for CBD, particularly for treatment of related auto-immune diseases.

The fatty acid profile in chylomicrons is mainly dependent (with the exception of C20:4) on exogenously administered lipids. Even though these fatty acids vary in proportions in chylomicrons following administration of various natural vegetable oils, C16:0 (palmitic acid), C18:0 (steric acid), C18:1 and C18:2 were essential and constantly present. There are lower levels of C20:4 in rat chylomicrons following oral administration of olive oil-based formulation compared to other formulations, which may be related to olive oil

containing less linoleic acid. Overall, the major fatty acids in olive oil are C18:1 (71.6% of total fatty acids), and olive oil-based formulation delivers the highest quantity of CBD to the intestinal lymphatic system and systemic circulation, with minimal variability, compared to other vegetable oils.

The last approach of this PhD project in Chapter 6 was to use plant derived oil bodies to facilitate lymphatic targeting and systemic exposure of CBD. The oil bodies are tiny spherical particles (0.2 to 2.5 μm in diameter) surrounded by a single phospholipid layer with embedded proteins on the surface, and the core mainly consist of LCT [304,306]. The oil bodies, which is abundantly deposited in the cytoplasm of plant seeds or fruits, is also a natural pre-emulsified oil-in-water emulsion that could be exploited as a possible lipid excipient for formulation development [308,310,314,317]. Therefore, oil bodies were used as a lipid carrier to develop an oral lipid-based formulation to facilitate lymphatic transport and bioavailability of CBD. The *in vitro* lipolysis and *in vivo* studies were subsequently conducted to compare the performance of oil bodies to the corresponding natural (rapeseed) oil.

Following the *in vitro* lipolysis experiment, the rapeseed oil-based formulation had a comparable CBD distribution into the aqueous micellar phase, but a higher drug level remained in the oil phase than the oil body group, indicating that the rapeseed oil was less digestible than the oil body (Chapter 6, Figure 6-2). Both *in vivo* pharmacokinetics and biodistribution studies suggested that using the oil body as a lipid vehicle enhanced overall

lymphatic transport and systemic exposure (1.7-fold) of CBD, and drug absorption was more rapid (t_{max} at 3 h) when compared to its oil form (Chapter 6, Figures 6-3 to 6-6 and Table 6-2). There are potentially some benefits for using oil bodies as lipid excipients: 1) all of the ingredients are natural and edible. 2) natural o/w emulsions with stable structure; 3) simple extraction procedure and economic advantage at cheap cost and 4) enhancing digestion rate and extent for highly lipophilic drugs [308,310,317]. However, to the best of our knowledge, there are currently no publications focusing on oil bodies applied to oral drug delivery. Therefore, this study combines pharmaceutical and food science fields in order to develop a promising lipid-based formulation that uses the plant derived oil body.

Overall, this PhD thesis discusses the optimisation and investigation of the oral lipid-based drug delivery primarily using natural vegetable oils to facilitate lymphatic targeting and systemic exposure of a highly lipophilic compound CBD. The primary approaches focused on the modification of sesame oil-based formulation and the alternative selection of excipients. The intraluminal digestion step in the GI tract does not appear to significantly affect the rate and extent of lipid-based formulations absorption because the variability and absorption of CBD in lymphatic transport and systemic circulation are not improved by co-administered pre-digested or purified lipids when compared to sesame oil. Even though incorporating MCT and surfactants to sesame oil can enhance the aqueous micellar drug solubility and minimise the variability in CBD absorption, the overall drug exposure in the lymphatic system and bioavailability was not superior to pure sesame oil-based formulation. Olive oil and plant derived oil bodies have shown great potential as lipid vehicles for

facilitating intestinal lymphatic targeting and systemic exposure of co-administered CBD. These approaches imply that pure natural vegetable oil, particularly olive oil, and potentially plant derived oil bodies can be beneficial in advancing lipid-based formulations to optimize the oral drug delivery of highly lipophilic compounds with affinity to chylomicrons.

7.1. Further work

7.1.1. Minor components in natural vegetable oils can regulate the lymphatic transport of highly lipophilic drugs

This PhD project demonstrated that natural vegetable oils could deliver high levels of CBD into systemic circulation via the intestinal lymphatic system. Even though the fatty acid profiles in both natural vegetable lipids and chylomicrons have been investigated to understand better how dietary lipids affect chylomicron production, which is related to lymphatic transport of highly lipophilic compounds, other factors may also play a role in this process. The hypothesis to be tested is that minor components in natural vegetable oils could regulate the lymphatic transport of highly lipophilic drugs. These factors could include small components in natural plants seed oils, such as fat soluble antioxidants tocopherols and lignans, which may affect the absorption of oils and co-administered compounds due to their bioactive characteristics [164,166,167].

Therefore, further research to identify the involvement of natural plant derived lignans in lymphatic transport could be designed. Lignans are the minor component of natural plant oils that have been primarily identified in olive and sesame oil, with up to 100 mg/kg in olive oil and around 1 to 2% in sesame oil [354–357]. The *in vitro* metabolism studies have established that plant lignans could be digested in the intestinal lumen and converted to mammalian lignans [358–360]. In addition to their antioxidant properties, lignans have been found to improve the thermal stability of vegetable oils [171,361]. Furthermore, plant lignans have also been shown to modulate lipid metabolism during digestion, reducing the serum and liver cholesterol levels [362]. However, there was no convincing evidence on the lignans involvement in the chylomicron formation process and lymphatic transport.

The current study has suggested that olive oil as a lipid vehicle performed better than other vegetable oils in facilitating lymphatic transport of highly lipophilic drug CBD. However, the main challenge for natural lipids is oxidation, which requires a strict storage condition and thus increases the limitations of their manufacturing as the commercial formulations [363]. In addition, natural vegetable oils from diverse origins or strains may have different lipid content. Regardless of the fact that dietary lipids have been shown to enhance CBD lymphatic targeting and bioavailability more efficiently than purified and pre-digested lipids, plant lignans may have contributed to the differences caused by these formulations. Understanding the participation of lignans in the lymphatic transport during lipid digestion could thus be beneficial for future lipid-based formulation development. Therefore, further research should be undertaken to investigate the range of substances in natural vegetable

oils, in addition to the fatty acid profile, in regulating the intestinal lymphatic transport of highly lipophilic drugs.

7.1.2. Further investigations for the plant derived oil bodies as a lipid-based vehicle

Another future work direction derived from this PhD research is the continued study of the oil body as a lipid-based vehicle promoting intestinal lymphatic transport and systemic exposure of co-administered highly lipophilic drugs. The current PhD project has suggested that a plant derived oil bodies could perform better than the corresponding vegetable oil in delivering CBD via the lymphatic system. However, the present study was unable to identify if the participation of oil body-associated proteins in the lymphatic transport of co-administered CBD plays a role, nor to assess if LCT-rich spherical structures covered with a single phospholipid layer may as efficiently deliver CBD to the lymphatic system and systemic circulation as the oil bodies.

Major oil body-associated proteins, including oleosins, caleosins, and steroleosins, are embedded on the oil body surface and play an essential part in particle structural stabilisation [320,364]. It has been shown that one of the functions of oleosins is the production and mobilisation of triglycerides and phospholipids in plant seeds [365,366]. However, the metabolism of such oil body proteins and their digestion in the GI tract, particularly in terms of contributing to lymphatic transport of lipids and highly lipophilic

drugs, has yet to be explored. Therefore, a phospholipid and LCT-containing and protein-free emulsion as a control lipid vehicle to assess the lymphatic delivery of CBD will be required for further investigation, in order to identify the engagement of these oil body proteins in drug absorption.

In addition, oil bodies in the current study was derived from the rapeseed and apparently performed better in drug delivery than its corresponding vegetable oil. Sesame and olive oils have been found to enhance CBD lymphatic transport and bioavailability effectively, therefore their oils can be used for production of artificial emulsion, which could be explored for further investigation as a drug carrier. However, there may be potential risks in using oil bodies as an excipient as some people are allergic to their proteins, such as oleosins in peanut and sesame [367,368]. Thus, the safety of consuming plant-derived oil bodies should be considered in further research. Moreover, the oil bodies could be preserved in a powder form by a spray drying technique [323–325]. This approach may also be applied to advance the manufacturing of oil bodies-based formulations to minimise the bench to bench variability, as solid dosage formulations have a longer shelf life than the liquid form.

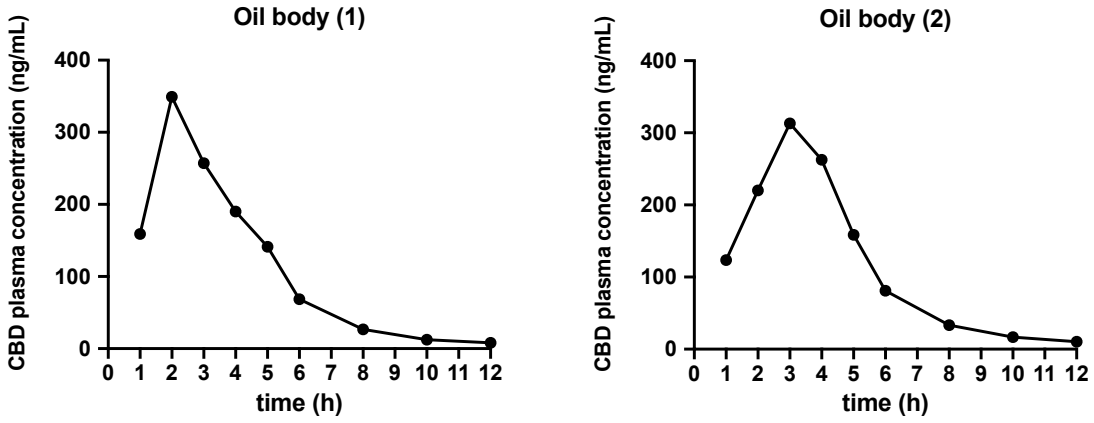
7.1.3. Applying advanced formulations to other lipophilic drugs

The current study suggested that some lipid-based vehicles, including olive oil and rapeseed derived oil bodies, could promote intestinal lymphatic transport and oral

bioavailability of co-administered CBD. As a highly lipophilic drug, CBD has already been shown to have a high association to rat and human chylomicrons [120]. Meanwhile, delivering CBD into the intestinal lymphatic tissues is the primary objective for designing the lipid-based formulation, because CBD has been shown to have anti-inflammatory, anticancer and immunosuppressive properties when accumulated in lymph nodes and immune cells [129,186,369,370]. In addition to CBD, other highly lipophilic compounds (such as delta-9-tetrahydrocannabinol (Δ^9-THC)) and lipophilic prodrugs (such as prodrugs of lopinavir and bexarotene) were also confirmed to have a high affinity to chylomicrons by the *in silico* and *in vitro* chylomicron association assays [120,155,329,330]. These drugs have been shown to have a therapeutic effect on human immunodeficiency virus (HIV), cancer and immunosuppression, which all have high relevance for the delivery to the mesenteric lymph nodes (MLN) [129,155,371]. Therefore, the findings of this work, despite being obtained with a model lipophilic drug CBD, are likely to be translatable for the delivery of many other lipophilic drugs and prodrugs.

APPENDICES

Individual *in vivo* PK results for oil bodies-based formulation in the preliminary study



Appendix Figure 7- 1 . Individual plasma concentration-time profile of CBD following oral administration of oil bodies-based formulations in the preliminary study. The concentration of in oil bodies-based formulations was 6 mg/mL and the administered dose of CBD was 12 mg/kg.

Appendix Table 7-1. Individual pharmacokinetic parameters of CBD derived from plasma concentration-time profiles following oral administration of oil bodies-based formulations in the preliminary study.

Formulation	$t_{1/2}$ [a] (h)	t_{max} [b] (h)	C_{max} [c] (ng/mL)	$AUC_{0-\infty}$ [d] (h*ng/mL)
Oil body (1)	1.8	3	303	1301
Oil body (2)	1.8	2	337	1273

[a] half-life;

[b] time to maximum concentration in plasma;

[c] the maximum concentration in plasma;

[d] AUC from 0 to infinity;

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