

Contribution of Intestinal Lymphatic Transport to the Antiproliferative Effect of Δ^9 - Tetrahydrocannabinol.

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Abstract

Δ^9 -tetrahydrocannabinol (THC) is the major lipophilic cannabinoid in the cannabis plant and is responsible for many of its pharmacological effects. Cannabis and cannabinoids are often administered orally in foods or lipidic formulations. Marinol® is an oral lipidic formulation containing THC dissolved in sesame oil, and is approved to treat nausea associated with cancer chemotherapy. THC exerts its pharmacological effects by binding to cannabinoid receptors 1 and 2, both of which are involved in a large variety of downstream signalling pathways. Some of these pathways regulate cell death. In agreement with this involvement, THC has been shown to be immunosuppressive *in vitro* and *in vivo*. Previous work on synthetic cannabinoids has shown that they can be transported in the lymphatic system in significant amounts when administered orally.

The lymphatic system is a passive circulatory network of vessels and lymphocyte-rich nodes that has three major functions. The first function is to maintain body fluid balance. The lymphatic system is open-ended, unidirectional, and collects excess fluid at blood capillaries and tissues. Large molecules that cannot re-enter the blood also enter the lymphatic system. The second function of the lymphatic system is to mount immune responses: after lymphocytes are produced in bone marrow and thymus, they migrate to the lymphatic system and are organised into lymph nodes, which are immunological checkpoints that screen passing fluid for antigens, where innate or adaptive immune responses can be mounted onto them. The third function of the lymphatic system is the absorption of dietary lipids. Lipids are a heterogeneous group of molecules that include lipids, triglycerides and vitamins which are found in food. Lipophilic lipids are emulsified in the gastrointestinal tract by bile and phospholipids, forming mixed micelles. The micelles provide an interface for pancreatic lipase to digest triglycerides into fatty acids and glycerol. These micelles dissociate when they approach the unstirred water

layer of the intestinal wall, and the contents are absorbed into specialised enterocytes. Absorption of long-chain fatty acids triggers the production of chylomicrons (CM), a large triglyceride-rich lipoprotein which transports digested fats through the lymphatic system. Triglycerides can then be released from CM by the action of lipoprotein lipase, which is expressed in a variety of cells and tissues. Lipophilic molecules and drugs can associate with CM, and share their fate in the body. Transport through the lymphatic system associated with CM bypasses the liver, which could reduce the first-pass metabolism of a drug. Lipophilic drugs with a high association with CM are prime candidates for lymphatic transport if administered orally.

The use of Marinol® by cancer patients receiving chemotherapy could target THC to the lymphatic system, further suppress the immune system of those taking it and possibly making them more vulnerable to secondary infection. Therefore, it is important to confirm whether THC is transported by the intestinal lymphatic system, and whether it is selectively targeted there at immunosuppressive concentrations. To address this research question, analytical assays needed to be developed and optimised to detect THC in a variety of different biological matrices. HPLC-UV methods were successfully developed and validated to determine concentrations of THC in plasma, CM emulsions, and simulated intestinal fluid.

The first step to determine the lymphatic transport of THC is to assess the fraction available for absorption in the small intestine. As mentioned previously, Marinol® is a lipidic formulation of THC, and the emulsification and digestion of lipids are not taken into account with traditional drug dissolution techniques. However, an *in vitro* lipolysis model is a technique that mimics lipid digestion and can reliably predict the absorption of a drug dissolved in a lipidic formulation. After establishment and optimisation of the model, up to 45% of THC dissolved in a lipidic formulation (sesame oil) was solubilised and made readily available for

absorption in simulated intestinal fluid. As the concentration of THC in formulation increased, the fraction of drug solubilised decreased.

The lymphatic transport potential of THC was then assessed using three techniques – association with CM, pharmacokinetic and biodistribution studies. THC had a high association with CM (72.5%), and its absolute oral bioavailability was improved three-fold when administered in a lipidic formulation (18.5%) compared to a lipid-free formulation (5.73%). At times of peak plasma concentration, the level of THC in mesenteric lymph node homogenate was 30 times higher than plasma, and was at immunosuppressive levels shown in previous *in vitro* studies (8.6 µg/mL). The mesenteric lymph nodes drain the small intestine, and would be the first nodes that dietary fats pass after being absorbed from the small intestine.

Finally, the antiproliferative effect of THC was assessed by exposure to peripheral blood mononuclear cells isolated from healthy individuals and cancer patients recovering from chemotherapy. THC produced a dose-dependent reduction in cell proliferation, with significant differences from vehicle above 10 µg/mL. THC had a reduced effect on cells isolated from cancer patients compared to cells isolated from healthy individuals. THC associated with CM emulsion also had a similarly reduced effect to cells isolated from healthy individuals compared to the effect of THC dissolved in organic solvent.

Collectively, this work confirms that THC is transported via the intestinal lymphatic system, and can be concentrated there at extremely high levels, shown to be immunosuppressive in previous *in vitro* studies.

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List of Abbreviations

AIC	Akaike Information Criterion
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen-Presenting Cells
ATP	Adenosine Triphosphate
AUC	Area under the curve
BCS	Biopharmaceutics Classification System
BrdU	5-bromo-2'-deoxyuridine
cAMP	Cyclic Adenosine Monophosphate
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CL	Clearance
CM	Chylomicrons
C _{max}	Maximal Plasma Concentration
CMC	Critical Micellar Concentration
D	Dose
DC	Dendritic Cells
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulphoxide
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2'-deoxyuridine
ELISA	Enzyme-Linked Immunosorbent Assay
F	Absolute Bioavailability
FAE	Follicle-Associated Epithelium
FBS	Fetal Bovine Serum
FDA	United States Food and Drugs Administration
FRB	Freely Rotatable Bonds
GALT	Gut-Associated Lymph Tissues
HBA	Hydrogen Bond Acceptors
HBD	Hydrogen Bond Donors
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency Virus
HLB	Hydrophilic-Lipophilic Balance
HPLC-UV	High-Performance Liquid Chromatography Ultraviolet
HQC	High Quality Control
HSV2	Herpes Simplex Virus Type 2
IFN- γ	Interferon-Gamma
ILF	Isolated Lymphoid Follicles
IM	Intramuscular
Io	Ionomycin

IS	Internal Standard
IV	Intravenous
IVIVC	In vitro-in vivo correlation
k_{01}	Absorption Rate Constant
k_{10}	Elimination Rate Constant
k_{12}	Distribution Rate Constant between compartment 1 and compartment 2
k_{21}	Distribution Rate Constant between compartment 2 and compartment 1
LBDDS	Lipid-Based Drug Delivery System
LC/MS/MS	Liquid Chromatography with Tandem Mass Spectroscopy
LCT	Long Chain Triglyceride
LDL	Low-Density Lipoprotein
LLOQ	Lower Limit Of Quantification
Log $D_{7.4}$	Distribution Coefficient at pH 7.4
Log P	Octanol-Water Partition Coefficient
LpL	Lipoprotein Lipase
LQC	Low Quality Control
MALT	Mucosa-Associated Lymph Tissues
MAPK	Mitogen Activated Protein Kinase
MCT	Medium Chain Triglyceride
MDCK	Madin Darby Canine Kidney
MLN	Mesenteric Lymph Nodes
MLR	Mixed Lymphocyte Reaction
MQC	Medium Quality Control
MS	Multiple Sclerosis
NK	Natural Killer
PAMPA	Parallel Artificial Membrane Permeability Assay
PBL	Peripheral Blood Leukocyte
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG400	Polyethylene Glycol 400
Pen-Strep	Penicillin-Streptomycin
PHA	Phytohaemagglutinin
PLS	Partial Least Squares
PMA	Phorbol Myristate
PO	Oral Administration (Per Os)
POD	Horseradish Peroxidase
PP	Peyer's Patches
PSA	Polar Surface Area
RE	Relative Error
RSD	Relative Standard Deviation
SC	Subcutaneous
SCT	Short Chain Triglyceride
SEDDS	Self-Emulsifying Drug Delivery System

SLN	Solid Lipid Nanoparticles
SMEDDS	Self-Microemulsifying Drug Delivery System
SNEDDS	Self-Nanoemulsifying Drug Delivery System
$t_{1/2}$	Terminal Half-life
TG	Triglycerides
THC	delta-9-tetrahydrocannabinol
THCV	Tetrahydrocannabivarin
$T_{\max}$	Time of maximal plasma concentration
$TNF\alpha$	Tumour Necrosis Factor Alpha
TRL	Triglyceride-Rich Lipoproteins
USP	United States Pharmacopeia
UWL	Unstirred Water Layer
V_1	Volume of Distribution of Compartment 1
V_d	Apparent Volume of Distribution
VLDL	Very Low-Density Lipoprotein
V_{ss}	Volume of Distribution at Steady State

Chapter 1: Introduction and Scientific Background

1.1 The Lymphatic System

The lymphatic system was first described by Hippocrates in one of his treatises, '*On Glands*' as vessels that attract and receive fluids, and contained white blood [2, 3]. This system was revisited in the 16th century by the Italian scientist Gasparo Aselli, who described lymphatic vessels as “milky veins” in the “well-fed” dog [4, 5]. Aselli named these vessels containing this milky fluid “lacteal vessels”, and these vessels drained to the liver. Then the French physician Jean Pecquet added to this work by investigating and discovering the correct route that lymph travels and leads back to general circulation, and Thomas Bartholin published the first full description of the human lymphatic system in 1652 [6], around a similar time that the Swedish scientist Olaus Rudbeck also independently discovered the lymphatic system [7]. Since then, more areas of the system were discovered, including the locations of glands or nodules in the mucosa of the small intestine, and specialised lymphoid follicles later named Peyer’s Patches (PP) [8, 9]. The modern understanding of the lymphatic system is largely based on ink injection studies performed by Florence Sabin [10, 11].

The lymphatic system was initially thought of as a drainage system for fluids and proteins contained in the interstitial spaces in tissues to return to the blood [2]. However, the lymphatic system was also rightly recognised as a major and critical part of the immune system, where foreign and/or toxic agents are screened, presented to appropriate immune cells that mount an immune response to eliminate them [4]. A specialised section of the lymphatic system (the section that was discovered first by the Hippocratic Treatise) is also responsible for the absorption of dietary lipids from food [12].

However, the system has also been involved in disease states like cancer, autoimmune disorders, and viral or parasitic infections. It is also the major pathway by which tumours metastasise in the body, more notably of breast, lung, prostate and colon cancers [13].

This section will focus on each of these aspects of the lymphatic system in more detail, to highlight the importance of the lymphatic system.

1.1.1 Anatomy of the lymphatics

As mentioned above, the lymphatic system was initially discovered as a drainage system for tissue fluid to drain back to general circulation to maintain fluid balance [6]. Unlike the blood circulatory system, the lymphatic system is open-ended, unidirectional and does not have a pump [14]. A schematic of the different components of the lymphatic system are shown in Figure 1-1.

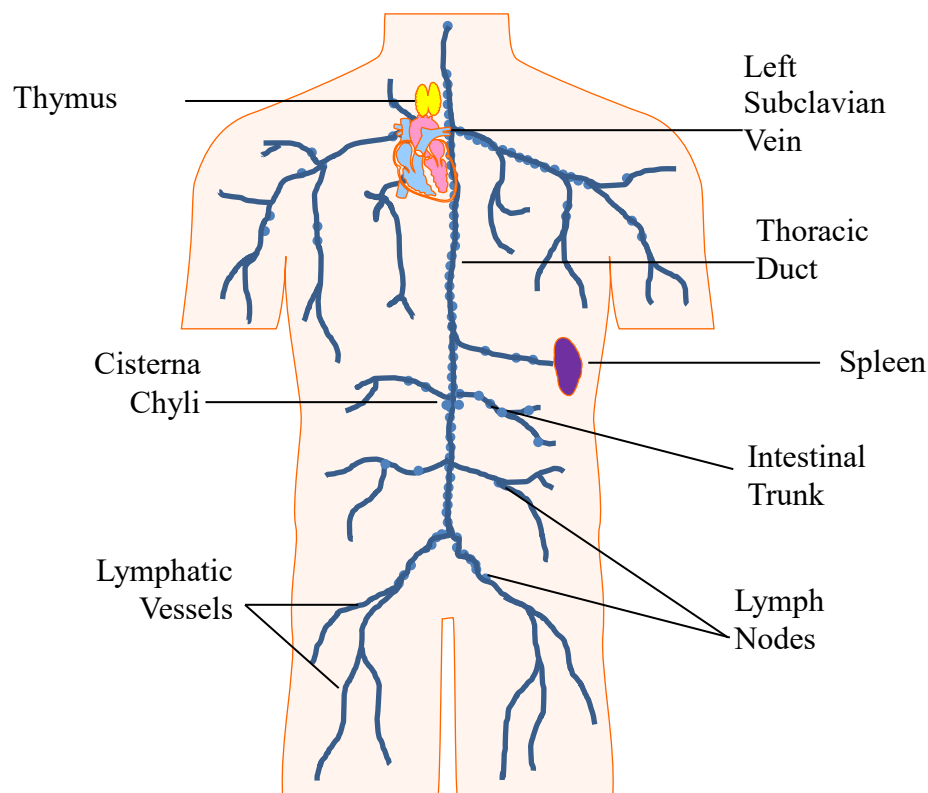


Figure 1-1. Schematic representation of the lymphatic system.

1.1.1.1 Lymphatic Vessels

The first structures that lymph drains into are lymph capillaries. These are in close proximity to blood capillaries, and have walls of single-layer endothelial cells arranged like a pressure valve [15]. The hydrostatic pressure in the interstitial space is greater than that in the lymphatic

capillary, so that fluid enters on this pressure gradient, but then cannot escape back into interstitial space. Lymph capillaries eventually drain into larger lymphatic vessels which also have valves, are anchored to surrounding tissue and have smooth muscle walls [16]. Lymph vessels drain lymph into and out of lymph nodes (described in more detail below). The vessels that lead into a node are called afferent lymph vessels, and the vessels that lead out of a node are called efferent lymph vessels.

Many efferent lymph vessels drain into even larger lymph trunks. There are four pairs of lymphatic trunks in the body that collect lymph from efferent lymph vessels originating in both halves of the body: the jugular, subclavian, bronchomediastinal, and lumbar lymph trunks [17]. There is also an unpaired intestinal lymph trunk, which specifically drains the small intestine [18]. These lymph trunks then drain into one of two lymph ducts – the thoracic duct or the right lymphatic duct. The right lymphatic duct drains lymph from the bronchomediastinal, subclavian and jugular trunks, and the thoracic duct collects lymph from the intestinal trunk and lumbar trunks via the cisterna chyli. The thoracic duct finally connects to the general circulation at the left subclavian vein, and the right lymphatic duct connects to the right [17].

1.1.1.2 Primary Lymphatic Organs

There are two primary lymphoid organs, bone marrow and the thymus. These organs produce mature lymphocytes that can identify antigens. The bone marrow produces almost all the white blood cells in the body, including the progenitors of macrophages, B-cells, T-cells, monocytes, natural killer cells and red blood cells [19]. The progenitors of T-cells (thymocytes) mature and differentiate in the thymus. Secondary lymphatic organs are the lymph nodes, spleen, and mucosa-associated lymph tissues (MALT) [20-22]. Immune responses are mounted in these secondary lymph organs. MALT includes a large variety of tissues that provide defence for a wide variety of foreign antigens. A specialised component of the MALT is the gut-associated

lymphoid tissues, which includes the tonsils, mesenteric lymph nodes (MLN) and Peyer's Patches (PP).

1.1.1.2.1 Peyer's Patches

Peyer's Patches are specific lymphoid tissues that are present in the lowest portion of the small intestine, the ileum and is part of the gut-associated lymphoid tissue. Since the gastrointestinal tract is often filled with food, it requires constant screening for potentially harmful pathogens such as bacteria, fungus and viruses [8, 23]. The PP consists of a sub-epithelial dome area and a T-cell area dispersed with B-cell follicles. A single layer of specialised epithelial cells cover the dome area, called the follicle-associated epithelium (FAE) and separates the lymphoid area from the intestinal lumen. Microfold (M) cells are dispersed across the FAE, which enable uptake of antigens from the lumen into PP [8, 9, 21, 23]. PP and the associated intestinal lymphatic tissues are shown in Figure 1-2.

1.1.1.2.2 Lymph Nodes

Lymph nodes are large structures containing a variety of lymphocytes and macrophages. There are two different regions, the medulla and cortex. The cortex consists of an area of T-cells and B-cell follicles. The T-cell area processes antigens presented by circulating dendritic cells (DC), and the B-cell follicles are stimulated when exposed to an antigen to begin mounting an adaptive immune response to it [21, 24]. Lymph fluid containing cells, macrophages, pathogens, and proteins passes through the cortex to the medulla, and flows onward out of the node. Lymph nodes are where the majority of the lymphocytes in the body reside [20]. A schematic of a typical lymph node is shown in Figure 1-3.

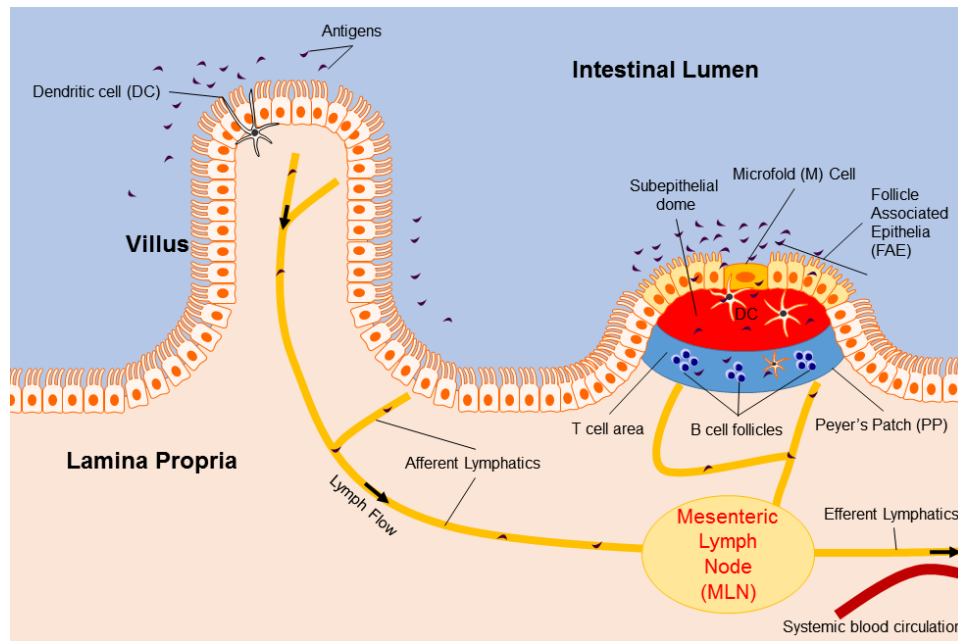


Figure 1-2. Schematic representation of the intestinal lymphatic tissues. Dendritic cells (DC) sample luminal antigens across Microfold (M) cells in Peyer's Patches (PP) and transport them to the lamina propria by receptor-mediated mechanisms. DCs can also straddle the epithelia and sample antigens directly from the lumen by trans-epithelial projections. DCs can then present these antigens to lymphocytes in the PP, or in mesenteric lymph nodes (MLN).

1.1.1.2.3 Spleen

The spleen is a lymphatic organ which plays an important role in the screening of red blood cells, and production of antibodies. These two major functions are conveniently split by the two different areas of the spleen – the red pulp, and white pulp which are separated by a marginal zone [25]. Red pulp mechanically filters passing blood for red blood cells that have reached senescence, antigens and microorganisms [20]. Blood contents that are filtered out this way (red blood cells in particular) are phagocytosed by macrophages contained in the Cords of Billroth [26]. White pulp contains T-cells, B-cells and antigen-presenting cells (APC), which work in concert to mount immune responses – T-cells are presented with antigens by APC, and stimulate B-cells to produce specific antibodies to eliminate the threat [27, 28].

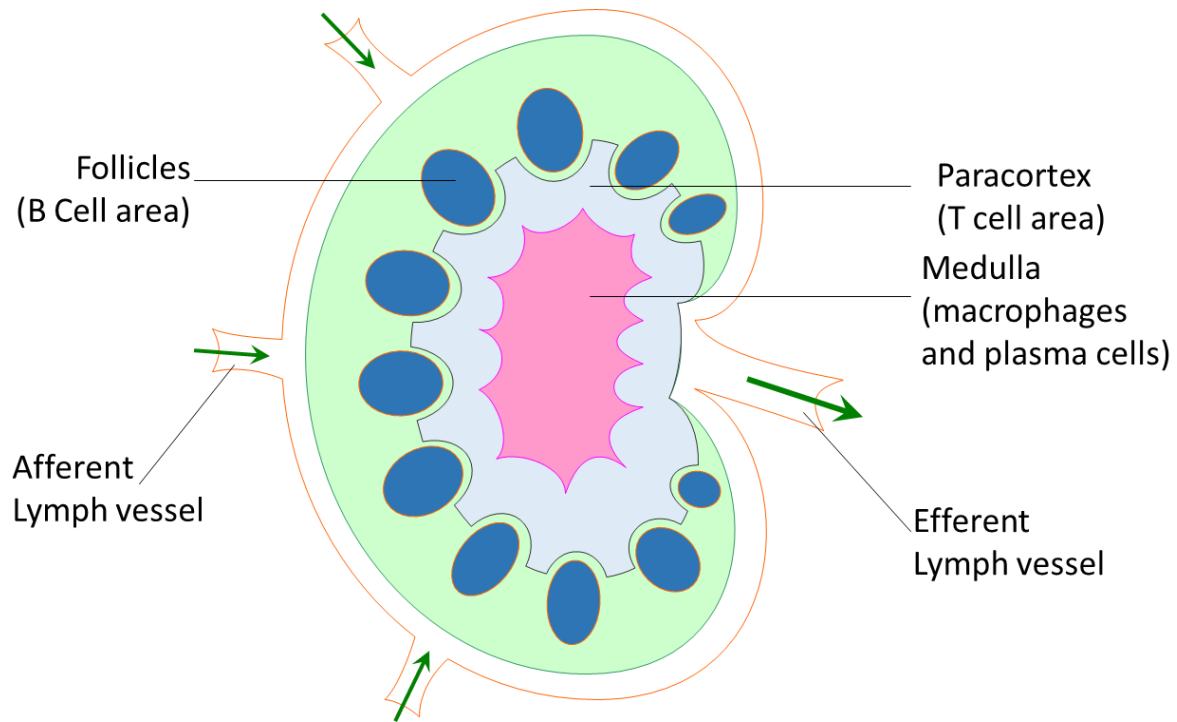


Figure 1-3. Schematic of a typical lymph node.

1.1.2 Functions of the lymphatic system

1.1.2.1 Removal and Recovery of Interstitial Fluid

The first discovered function of the lymphatic system was to attract and receive liquid from tissues [2]. When fluid escapes the arterial side of blood capillaries to reach tissue, not all of the liquid is able to be reabsorbed into the venous side. Molecules such as proteins or cell debris also would not be able to be easily reabsorbed into blood capillaries due to the pore sizes of around 30 nm [15]. Indeed, up to four litres of fluid and half of all small plasma proteins is extravasated into tissue every day. If this fluid is not recycled and returned to general circulation efficiently, fluid can accumulate in tissues and cause lymphedema [29]. The lymphatic system therefore maintains the fluid balance of the body by providing an open-ended unidirectional route for interstitial fluid to return to general circulation [30].

1.1.2.2 Trafficking of white blood cells and mounting of immune responses

The organs of the lymphatic system are responsible for the production, maintenance and distribution of the body's defence system. After their creation in the bone marrow, progenitor

cells travel to their appropriate target tissues – progenitor T-cells travel to the thymus to develop into mature T-cells and progenitor B-cells travel to the spleen to mature. These cell types are the most important types of immune cells involved in defence, and the vast majority of lymphocytes are found in some part of the lymphatic system, either residing in the many lymph nodes in the body, the spleen or the thymus [31]. Lymph nodes act as immunological checkpoints that can screen passing lymph fluid for antigens. Antigens are presented to T-cell areas of the lymph node cortex by APC such as DC, B cells or macrophages. This interaction between T-cells and APC continues until a specific T helper cell is able to recognise this antigen. The T helper cell is then able to stimulate the maturation of B-cells into plasma cells, to produce antibodies specifically recognising that antigen [27]. The mounting of immune responses in the lymphatic system is critical in fighting infection and disease of the body.

1.1.2.3 Absorption of Dietary Lipids

The third function of the lymphatic system is absorption of dietary lipids. More specifically, the intestinal lymphatic system has an integral role in their absorption [6]. This will be discussed in more detail in the section below.

1.2 Lipid Digestion and Absorption

Lipids are a large group of molecules which include fats, vitamins, triglycerides and sterols. Many of these molecules are found in food, and are digested and absorbed in the small intestine. Upon arrival to the small intestine, stomach contents are mixed with bile. Lipids are generally not soluble in water, and require a transport vehicle to prevent precipitation. Salts such as taurocholic acid from bile create colloid structures collectively called mixed micelles that facilitate dissolution of lipids [32]. Triglycerides (TG) are a major hydrophobic component of lipids, molecules consisting of three fatty acids attached to a glycerol backbone by ester linkages. TG and other lipids emulsified by the contents of bile are then able to be digested by

the pancreatic lipase/co-lipase complex [33]. Pancreatic lipase requires an oil-water interface to function [34], but its activity is inhibited when the concentration of bile salts is above a critical micellar concentration (CMC) when micelles are able to form. Co-lipase is able to anchor pancreatic lipase to the oil-water interface above CMC over the pH range found in the small intestine, thus enabling the efficient digestion of triglycerides [35].

In order for mixed micelles to be absorbed, they must pass a barrier adjacent to it, the unstirred water layer (UWL). The epithelium of the small intestine is composed of enterocytes, which have a critical role in the packaging and transport of the products of lipid digestion [36]. Once absorbed into the enterocyte, the fate of fatty acids varies with their chain length. Short- (less than 6 carbon) and medium- (6 – 12 carbon) chain fatty acids are sufficiently soluble in the cytoplasm, and are absorbed into the portal blood circulation by passive diffusion through the enterocyte [37]. Long-chain (above 12 carbon) fatty acids are not soluble in the cytoplasm, and instead are resynthesized to TG in the smooth endoplasmic reticulum [38]. These TG are incorporated into a carrier with a lipoprotein called apolipoprotein B-48, forming a large lipoprotein ranging from 80 – 1000 nm in diameter, called chylomicrons (CM). The exact mechanism by which CM are made is currently unknown, but it is generally thought to be triggered by the absorption of long-chain TGs [39]. Other components usually found in CM are fat-soluble vitamins, cholesterol, cholesteryl esters, and small amounts of phospholipids [40]. It is also believed that many lipophilic drugs associate with CM during or after their assembly in the enterocyte, and is an essential part of their absorption [41].

After their production, CM leave the enterocyte by exocytosis via the Golgi apparatus, and are too large to enter the portal vein (which has a pore size of around 30 nm [15]) and enter the lacteal and intestinal lymphatics instead [42]. Upon entry to the lacteal, CM follow the lymphatic route, and pass the MLN to the mesenteric lymph duct, cisterna chyli and thoracic

duct to reach the general circulation at the left jugular and subclavian vein [18]. When circulating in the bloodstream, CM interact with other lipoproteins to exchange components. CM interfaces with high-density lipoproteins (HDL) and exchanges apolipoproteins C2 and E for cholesterol [43, 44]. Apolipoprotein C2 activates the enzyme lipoprotein lipase (LpL), a cell-surface protein which enables the hydrolysis of TG and thus a cell's supply of fatty acid [45, 46]. It is assumed that associated vitamins and drugs are also released in this exchange, but this has not been confirmed. LpL is expressed on a wide variety of tissues involved in the life cycle of CM, including adipose [47], skeletal muscle, [46], liver [48], spleen [49], and on a variety of macrophages [43, 50, 51]. It is currently unclear if LpL is also expressed on T-lymphocytes, but it has been shown to be expressed on B-lymphocytes [52]. Once the majority of TGs and other lipid components have been donated to all of these tissues, CM returns apolipoprotein C2 to HDL and are degraded in the liver [53]. CM are essential for the absorption of dietary fats and the failure to synthesise apolipoprotein B-48 (such is the case for those with Anderson's disease) causes intestinal fat malabsorption with growth retardation and failure to thrive [54, 55]. A schematic representation of chylomicron formation is described in Figure 1-5.

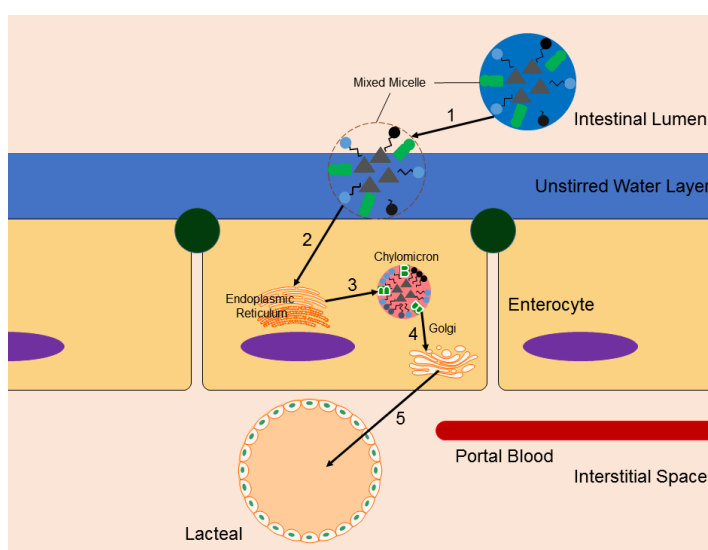


Figure 1-5. Schematic representation of lipid absorption, and chylomicron formation in the small intestine. (1) After their digestion by pancreatic lipase and solubilisation by bile salts, mixed micelles approach the unstirred water layer, and disintegrate into their components – triglycerides, bile salts and accompanying drugs – which can then enter the enterocyte. (2) Once inside the enterocyte, long-chain fatty acids are resynthesized to long-chain triglycerides (LCT) in the smooth endoplasmic reticulum. (3) Apolipoprotein B from the rough endoplasmic reticulum are combined with LCT to form large lipoproteins called chylomicrons (CM). (4) CM exit the enterocyte via the Golgi apparatus by exocytosis. (5) CM enter the interstitial space, are too large to enter the portal vein, and ultimately go to the lacteal instead.

1.2.1 Drugs Transported via the Lymphatic System

Targeting drugs through the lymphatic system has two unique benefits. Transport through the lymphatic system avoids many major organs involved in metabolism on the first pass, most notably the liver [56, 57]. Due to the abundance of immune cells in the lymphatic system, targeting drugs to the lymphatic system is also targeting them to the immune system which could be advantageous for antiviral agents such as the hepatitis B vaccine and didanosine, an antiretroviral medication used to treat HIV/AIDS [58, 59]. The lymphatic system is also the major pathway for the metastasis of intestinal tumours, so targeting cytotoxic drugs to the intestinal lymphatics would increase their effectiveness [60, 61].

When drugs are administered orally and absorbed into blood from the small intestine, its concentration is often reduced before reaching general circulation. This is because the next organ that the blood flows through after the small intestine is the liver [37]. Hepatocytes in the liver screen, metabolise and detoxify the material that passes through, including carbohydrates, lipids, vitamins, and many drugs [62]. This is generally referred to as first-pass metabolism [63]. Most oral drugs are significantly affected by first-pass metabolism and much research effort has been made to attempt to circumvent this metabolism so that administration of drugs can be more efficient, and targeting the lymphatic system has been one very attractive strategy [56, 64-66].

Drugs that are transported in the intestinal lymphatics have a number of common characteristics. Historically, drugs with a high octanol-water partition coefficient (Log P higher than 5), low water solubility and high solubility in TG (greater than 50 mg/mL) were thought to partition to the lymphatic system [67]. In particular, this was the case for two drugs, halofantrine and dichlorodiphenyltrichloroethane (DDT), both of which were found in the intestinal lymphatics [68, 69]. Since then, other features have been related to intestinal

lymphatic transport potential, the most prominent being a distribution coefficient at pH 7.4 (Log $D_{7.4}$), and association with CM [1, 70]. Log $D_{7.4}$ was shown to be a more appropriate physicochemical parameter than Log P to determine the lipophilicity of a drug since much of the pH conditions within the body range from 6.8 to 7.4 [71]. Association with CM was also found to be an important determinant of lymphatic transport, likely due to CM exclusively being transported in the intestinal lymphatic system after production. It is currently unknown where drugs associate with CM during their assembly, but it could be at any point in the process [1]. More recently, an *in silico* model and an *in vitro* model were also developed to predict a drug's association with rat CM [70]. The *in silico* model used a combination of eight physicochemical parameters multiplied to calculate a predicted CM association value, and the *in vitro* model assesses the association of a drug with an artificial lipid emulsion (Intralipid®) instead of CM, which is positively correlated to their CM association value.

1.2.2 Targeting drugs to the intestinal lymphatics via oral administration

Transport via the lymphatic system bypasses first-pass hepatic metabolism and has other unique effects that benefit drugs that are administered orally. Since the oral route is by far the most common route of drug administration, an effective strategy to target a drug to the intestinal lymphatic system is to co-administer a drug orally with long-chain TG or lipids [72]. These TG activate the lipid absorption pathway described above, stimulate CM production and intestinal lymphatic transport occurs [73]. Oral co-administration of lipids can change the pharmacokinetic and pharmacodynamic profiles of drugs [74] by reducing the rate of gastric emptying, and stimulating the release of bile (containing surfactants such as bile acids and phospholipids) from the gall bladder.

If a drug is a prime candidate for lymphatic transport, activating dietary fat absorption (and intestinal lymphatic transport) would provide an additional route for that drug to be absorbed,

increasing its effective dose with no change in administered dose [67], enhancing bioavailability as well. This was the case for two synthetic cannabinoids, PRS-211,212 and dexanabinol. These two synthetic lipophilic cannabinoids were targeted to the intestinal lymphatics by co-administration with lipids [75]. Their structures are similar to the major phytocannabinoid, delta-9-tetrahydrocannabinol described later (Figure 1-6). PRS-211,220 and dexanabinol have similar physicochemical properties, but different associations with CM and solubility in TG. In the study, each cannabinoid was administered orally in lipid-free or lipidic formulation, and the contribution of intestinal lymphatic transport to drug bioavailability was assessed. The work concluded that intestinal lymphatic transport played a different role in the absorption of each cannabinoid. Dexanabinol has low solubility in TG and moderate association with CM (7.9 mg/g and 31.6% respectively). It had similar bioavailability in lipid-free or lipidic formulation, and intestinal lymphatic transport contributed minimally to oral bioavailability. Despite this, the concentration of dexanabinol in lymph was still 50-fold higher than the concentration in plasma for eight hours after its administration. PRS-211,220 on the other hand, had high solubility in TG and high association with CM (95.8 mg/g and 66.1% respectively). It had three-fold higher bioavailability in a lipidic formulation compared to lipid-free, and intestinal lymphatic transport contributed a major portion to oral availability. Furthermore, the concentration of PRS-211,220 in lymph was 200 to 500-fold higher than the concentration in plasma for eight hours after its administration.

In a related study, Trevaskis *et al* investigated the lymphatic transport and recovery of JWH-015 in lymphocytes was assessed after co-administration with 4 or 40 mg of oleic acid, a long-chain fatty acid. JWH-015 is another lipophilic synthetic cannabinoid that suppresses the adhesion and migration of white blood cells to tissues where they are needed [76]. The authors concluded that co-administration of 40 mg of oleic acid enabled 9.4- and 31.6-fold higher levels of JWH-015 in lymphocytes and mesenteric lymph compared to co-administration of 4 mg

[31]. 40 mg of oleic acid also increased CD4⁺ and CD8⁺ T-lymphocytes flux into intestinal lymph up to 5-fold, a finding also observed by Miura *et al* in 1987 when olive oil was administered to rats by a stomach tube [77].

Drug association with CM only applies to drugs administered orally. There are two other routes to target a drug to the lymphatic system, which are by subcutaneous injection, intramuscular injection.

1.2.3 Subcutaneous and Intramuscular Injection

Drugs that are not sufficiently bioavailable orally or have potentially harmful high peak concentrations when administered intravenously can be administered subcutaneously or intramuscularly (SC or IM) [78]. After a drug is injected SC or IM, it relies on passive diffusion from the injection site to enter circulation. Small molecules (less than 2 kDa) are generally cleared from the injection site into blood capillaries, whereas larger molecules drain into lymph capillaries [79]. Due to the slower flow rate of the lymphatic system, drugs administered by SC or IM injection can take more than 24 hours to be completely absorbed [78]. This makes SC or IM administration suited for drugs that are long-acting [80]. Lipophilic drugs or proteins over 16 kDa such as antibodies that are not soluble in intravenous formulation are also good candidates for these routes of administration. Typical formulations for SC or IM administration are suspensions or aqueous mixtures, although more specialised polymers, nanoparticles or liposomes have found success in targeting to the lymphatic system [64, 65, 81, 82].

An example of a drug successfully being targeted to the lymphatic system after SC injection is a biological drug, human growth hormone [83]. Human growth hormone is a peptide hormone that stimulates cell growth, and is used in hormone therapy or to treat growth hormone deficiency [84]. The drug was injected to the interdigital space of the hind leg of sheep, and lymph and plasma were collected. The work found that 61.7% of the total administered dose

was recovered in peripheral lymph, which suggests that targeting of human growth hormone to the lymphatic system was successful.

An example of a drug successfully being targeted to the lymphatic system after IM injection is shown by the work of Kim and Han [85]. They incorporated methotrexate, a folic acid metabolism antagonist [86, 87] into liposomes (closed spherical structures consisting of at least one phospholipid bilayer) and administered to rats and drug concentration in plasma and regional lymph nodes were monitored. After IM administration, the concentration of free methotrexate in lymph nodes were 10-20 fold higher than plasma, but liposome-encapsulated methotrexate was 100-350 fold higher than plasma. The liposome formulation also decreased the accumulation of methotrexate in kidney and liver, improving the drug's specificity.

1.3 Delta-9-tetrahydrocannabinol (THC)

1.3.1 History and discovery of THC

The cannabis plant has had a long history of use by humans. The first recorded uses of cannabis was by the ancient Assyrians [88] and the ancient Chinese [89], as a ritualistic and psychoactive drug that 'takes away the mind'. Three thousand years later, cannabis is still one of the most widely used drugs in the world [90]. In modern times, cannabis is becoming accepted as a social and recreational drug, especially with the legalisation in Alaska, Colorado, Oregon and Washington, and decriminalisation in the USA and other countries in the world. The components of cannabis (phytocannabinoids) were isolated in 1964 by Raphael Mechoulam and Yechiel Gaoni [91]. This triggered more research on cannabis, which led to the discovery of the major phytocannabinoids [92], their synthesis [93], and discovery of the endogenous signalling pathway that these components use to exert their psychoactive effect [94, 95]. The major psychoactive component of cannabis is delta-9-tetrahydrocannabinol, THC.

THC is a lipophilic, aromatic terpenoid. It has very low solubility in water, and very high solubility in organic solvents, especially oils and alcohols [96]. Even though it is the main active phytocannabinoid, many other phytocannabinoids are present in the plant, including cannabichromene (CBC), cannabidiol (CBD), cannabigerol (CBG), and tetrahydrocannabivarin (THCV), the chemical structures are represented in Figure 1-6 [97]. THC and CBD are the most abundant and potent phytocannabinoids, so they have been the focus of the majority of cannabinoid research.

Since their discovery and characterisation, commercial formulations containing cannabinoids have been approved for medical use. The first was in 1986, when the Drug Enforcement Administration authorised the medical use of Marinol®, a soft gelatin capsule containing Dronabinol (synthetic THC) dissolved in sesame oil [98]. Dronabinol was approved for the treatment of nausea associated with cancer chemotherapy, and appetite loss in patients with Acquired Immune Deficiency Syndrome (AIDS) [99]. Another commercial formulation containing cannabinoids is a formulated extract of cannabis more recently approved as a drug in the United Kingdom in 2010 as an oromucosal spray to treat symptoms of multiple sclerosis, marketed as Sativex®. Sativex delivers THC and CBD in roughly equal proportion (achieved from mixing two different extracts of *Cannabis sativa* plant), other minor cannabinoids and other non-cannabinoid components [100, 101].

This section describes the mechanism of action, pharmacokinetics, *in vitro*, *in vivo* effects and in clinical trials using THC, and the rationale behind why it has potential to be transported by the lymphatic system.

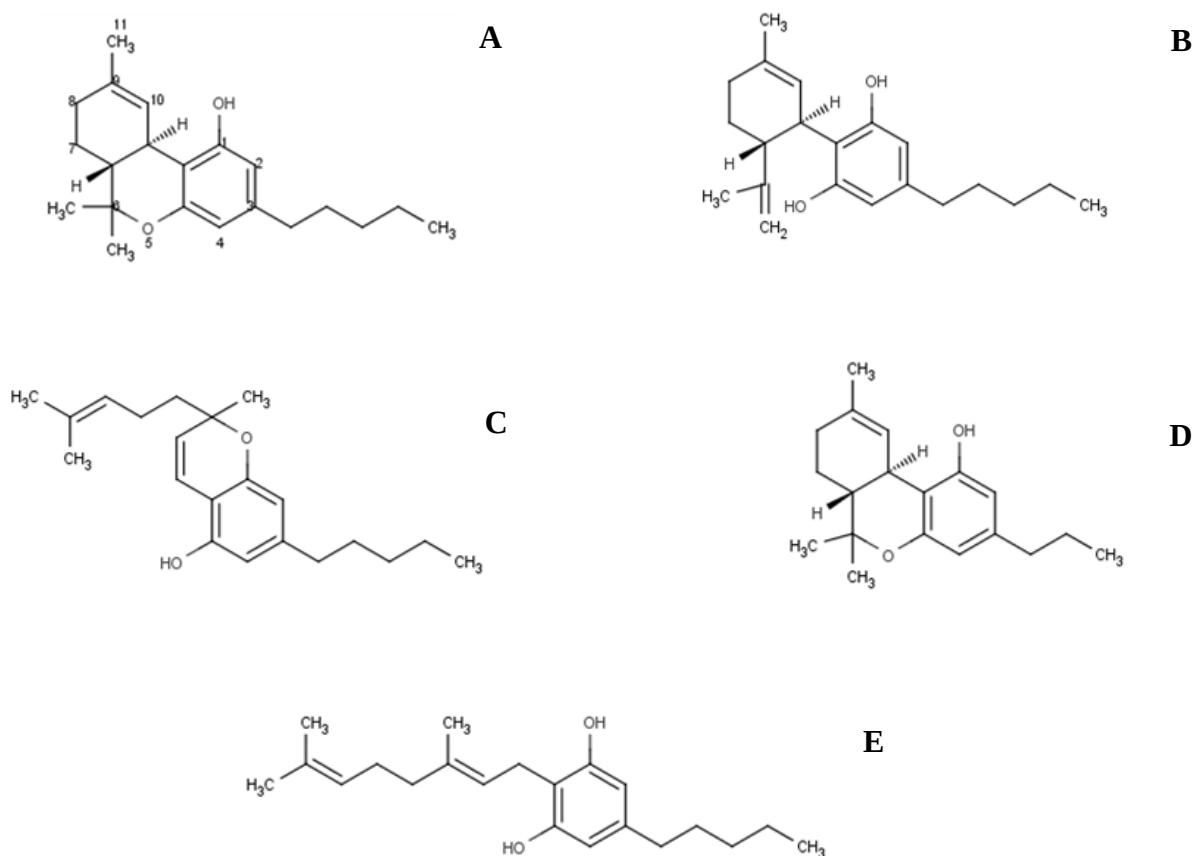


Figure 1-6. Chemical structure of major phytocannabinoids. Δ^9 -Tetrahydrocannabinol with ring carbons numbered according to the dibenzopyran numbering system [102] (A), cannabidiol (B), cannabichromene (C), tetrahydrocannabivarin (D) and cannabigerol (E).

1.3.2 Mechanism of action of THC

THC is the major psychoactive component of cannabis, and exerts its pharmacological effects by binding to cannabinoid receptors in the body. There are two major types of cannabinoid receptors, based on their localisation in the brain [103, 104], or peripheral tissues [105]. Since they were discovered first, the cannabinoid receptors found in the brain were named cannabinoid receptor 1 (CB1) and those found in peripheral tissues cannabinoid receptor 2 (CB2). Both receptors are part of the G-protein coupled receptor superfamily (GPCR), and have been extensively studied since [106-109]. In 1999, a novel “third” endocannabinoid receptor named GPR55 was discovered and was shown to be activated by THC, CBD and

endocannabinoids [110]. While the characterisation of this receptor is still ongoing, evidence suggests that it is involved in inflammation, neuropathic pain, bone physiology, diabetes and cancer [111].

Phytocannabinoids such as THC or CBD are not the usual ligands that bind the CB1 or CB2 receptors, as binding affinity studies of THC suggest that it is a partial agonist of CB1 and partial antagonist of CB2. The main endogenous agonist for the CB1 receptor is anandamide and the main agonist for the CB2 receptor is 2-arachidonoylglycerol, both of which bind at a much higher affinity than phytocannabinoids [104].

Even though CB1 and CB2 have different localisations, their downstream actions have some overlap [112, 113]. Activation of either receptor has shown to have a plethora of effects intracellularly. The first discovered response to receptor activation was the inhibition of adenylate cyclase, an enzyme which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) [114]. cAMP is a critical secondary messenger in many downstream signalling pathways [115]. Both receptors have also been involved in cell cycle arrest at G2/M phase [116, 117] and the induction of apoptosis in a variety of cell types [116-119]. This was attributed to the activation of mitogen activated protein kinase (MAPK), an enzyme that phosphorylates and regulates many cellular functions such as survival or death [120]. In addition, CB1 and CB2 are involved in the modulation of calcium ion channels [108].

1.3.3 Pharmacokinetics of THC

THC is metabolised in the liver, and is processed by cytochrome P450 CYP2C9 to 11-OH-THC and THC-COOH by oxidation at the 11 position (shown on Figure 1-6A), and conversion of that carbinol (CH₂OH) group to a carboxylic acid [121, 122]. THC is also metabolised by other enzymes in the cytochrome P450 family, such as CYP2C19, 2C11, and 3A4 [123, 124]. While 11-OH-THC is as psychoactive as THC, THC-COOH is relatively inactive. THC-

COOH is further glucuronidated as part of phase 2 metabolism, which leads to its excretion in urine [125].

Pharmacokinetic studies performed using THC can be categorised by the route THC is administered. Smoking is the main route of cannabis administration, and provides rapid absorption from the lungs through the blood to the brain. This is particularly attractive to users that are seeking the psychoactive effect of cannabis. A study in men between 18 and 35 given THC by inhalation and intravenous administration found that the drug concentration-time profile obtained from smoking was similar to profiles obtained after intravenous administration, but with slightly lower peak plasma concentrations [99]. In other studies, the maximal plasma concentration was found between 3 and 10 minutes after smoking [126, 127]. Bioavailability varied between individuals following smoking however, from 8 to 22% based on smoking technique, puff duration and depth of inhalation [128].

Oral bioavailability of THC has been studied less than the smoked route. Even though smoking offers a much quicker onset of action, it is offset by the harmful effects of the smoke itself. THC in oral formulation (such as Marinol®) reaches maximal plasma concentration over an hour after administration with lower peak THC concentrations [99]. The aforementioned study by Ohlsson *et al* that studied the smoked route administered 20 mg of THC added to a chocolate cookie, and obtained a bioavailability values from 4 to 12% [99]. Bioavailability values obtained in different studies also vary – another study by Wall *et al* administered 20 and 15 mg THC in soft gelatin capsules to men and women respectively, and found oral bioavailability values between 10 and 20% [122]. Other oral studies did not obtain an intravenous profile, which would have allowed calculation of absolute oral bioavailability [129, 130].

1.3.4 *In Vitro* effects of delta-9-tetrahydrocannabinol

As outlined above, THC has been shown to have a variety of effects on cells, demonstrating its involvement in multiple cell signalling pathways. After the discovery of the endocannabinoid signalling pathway, much work has been done to assess the effects of phytocannabinoids *in vitro*. The most common *in vitro* effect of THC is that it suppresses the proliferation and cell growth of a variety of immune cells by activation of CB2 [131]. CB2 is expressed on the surface of immune cells such as monocytes, macrophages, B-cells and T-cells [132].

Two studies by Klein *et al* found that THC suppressed responses of cultured T cells to a mitogen (8 µg/mL, [125]). Zhu *et al* found that THC triggered apoptosis in macrophages and T cells through suppression of the anti-apoptotic protein Bcl-2 (at concentrations of 5 and 10 µg/mL, [133]). In T helper cells, THC treatment at 5 µg/mL also inhibited the production of interferon-γ, and upregulated the expression of interleukin-4 and -5, decreasing the activation of Type 1 Helper T cells (which maximises the efficacy of macrophages and cytotoxic T cells) and increasing activation of Type 2 Helper T cells (which stimulates B cells to enhance antibody production) [134]. In addition, THC also suppresses the cytolytic activity of mature cytotoxic T cells (at 10 – 15 µg/mL, [131]).

This suppressive effect also applies to B cells that produce antibodies to fight infections in the body, although the concentrations of THC required to elicit these effects were considerably higher than those mentioned above. A study by Ngaoteprutaram *et al* found that THC significantly reduced the ability of naïve B cells to secrete immunoglobulin M (at 1.570 – 4.71 mg/mL, [135]). THC also affects the ability of splenocytes to respond to virus antigens. A study by Cabral *et al* assessed the effect of THC on the immune responsiveness to herpes simplex virus type 2 antigens in splenocytes, and found a dose-dependent suppression of a proliferative response to virus-infected cells (at 0.314 and 3.14 mg/mL [136]).

Dendritic cells and macrophages are specialised antigen-presenting cells of the immune system, a critical step in the mounting of immune responses. THC also induces apoptosis in these cell types at high concentrations, by activation of CB1 or CB2. Dendritic cells express both CB1 and CB2 [137], and macrophages primarily express CB2 [106]. Dendritic cells derived from bone marrow in mice exposed to THC 5 μ M and above (1.57 mg/mL) induced apoptosis by the caspase-dependent apoptotic pathway by activating caspases 2, 8 and 9, much like the findings of Zhu *et al* in macrophages described earlier [137].

There are also many other studies showing the involvement of THC in regulation of cytokines that also suppress the development and functionality of T-cell populations in particular, including cytotoxic T-cells [131, 138-140], helper T-cells [134, 139, 141] and regulatory T-cells [118]. The studies mentioned above provide ample evidence that THC is immunosuppressive *in vitro*, although the concentrations of THC at which some of these effects were seen may not be achievable *in vivo*. The highest concentration of THC achieved in human plasma after intravenous administration in previous studies are approximately 1 μ g/mL immediately after dosing [102, 142], and in the range of 1 – 50 ng/mL after oral administration [143, 144].

1.3.5 *In Vivo* effects of delta-9-tetrahydrocannabinol

Some studies cited previously also conducted *in vivo* work to prove that immunosuppression on the cellular level translates to a larger scale. This section provides examples where THC is able to compromise immune function in animal research models, decreasing their host's defence to bacterial, viral and fungal infection and the doses of THC administered to achieve this effect where appropriate.

One of the first studies done to demonstrate this reduction in immune function was conducted by Nahas *et al*, who isolated T cells from chronic marijuana smokers and assessed their mixed

lymphocyte response and proliferative response to mitogen compared to T cells isolated from control individuals, cancer patients, uremic patients and transplant patients. The response of T cells from marijuana smokers in both of these tests were lower than those from control individuals, and similar to those cancer patients with primary tumours [145].

Subsequent studies demonstrate the reduction of host resistances to bacteria and viruses after THC treatment. One study assessed the effect that THC has on mice infected with *Legionella pneumophila*, a gram-negative bacterium responsible for Legionnaires disease. Mice given one injection of THC at a dose of 1 – 5 mg/kg one day prior to a sublethal infection of *Legionella* and one more injection of THC one day after infection experienced acute collapse and died within 30 minutes of the second injection [146]. Subsequent work found that this suppression of immunity was by regulation of cytokines such as interferon- γ , interleukin-12 and tumour necrosis factor α [147]. THC also reduces host resistance to *Listeria monocytogenes* and herpes simplex virus type 2 (HSV2) in healthy mice. Half of the lethal dose of each of these pathogens was measured in cannabis extract-treated, THC-treated (both administered at doses of 200 mg/kg) and control mice. THC reduced the resistance of mice to *L. monocytogenes* 365-fold and resistance to HSV2 96-fold compared to control-treated mice. This effect was not observed in cannabis extract-treated mice [148]. Another study challenged healthy mice with a nonlethal dose of influenza virus after three consecutive days of THC administration by oral gavage at doses of 25, 50 and 75 mg/kg. Viral mRNA, inflammation, number of lymphocytes and macrophages recruited to the lungs were monitored after seven and ten days. THC decreased resistance to influenza in a dose dependent manner in the mice by increasing viral load and decreasing the magnitude of macrophage recruitment in response to infection [149]. Roth *et al* infected immunodeficient mice implanted with human PBL with HIV and 10 mg/kg THC, and assessed the number of HIV-infected PBL compared to saline-treated animals. While THC did not further decrease levels of T cells in response to HIV

infection, the percentage of HIV-infected PBL doubled (to 17% vs 7%) and HIV viral load in THC-treated animals increased 50-fold. Interferon- γ production was also decreased by THC treatment, and was additive to HIV infection [150].

Chronic THC treatment has also been shown to decrease mouse resistance to systemic *Candida albicans* infection. Mice were given 16 mg/kg of THC on days 1, 4, 8 through 11, and 15 through 18 over the course of three weeks, and then infected with *C. albicans* on day 19. Chronic THC treatment had no effect on the defence of mice to acute infection, but a secondary infection with candida significantly decreased survival and suppressed serum levels of IFN- γ compared to vehicle, suggesting that THC decreased the efficacy of the memory immune response to fungal infection [151].

Lastly, THC has also been shown to have immunomodulatory effects in larger mammalian hosts, such as Macaques. LeCapitaine *et al* demonstrated that chronic THC treatment (intramuscular injection twice daily for 12 months) did not alter the proliferation or apoptosis, lymphocyte subpopulation changes, but did upregulate the expression of CXCR4, a cell surface chemokine receptor involved in the homing of T cells to sites of infection, and a receptor that can also be used by HIV to infect T cells [152].

1.3.6 Clinical Trials using delta-9-tetrahydrocannabinol

Since the *in vitro* and *in vivo* data presented previously seem to agree in their assessment of THC's immunosuppressive potential, studies in humans have not been as clear cut, or numerous. Due to its more well-known effects for pain relief, many clinical trials using cannabis have focused on this property instead [153]. THC has also been found to be better than placebo in preventing vomiting caused by cancer chemotherapy, a finding that led to the approval of Marinol® as an antiemetic [154].

Of the studies that have been conducted, many do not find that THC has any immunosuppressive effect. For example, Bredt *et al* examined the short-term effects of smoked marijuana and Marinol® on HIV-infected patients. 62 Patients were given marijuana cigarettes containing 3.95% THC or 2.5 mg Marinol® capsules, and their immune phenotypes were monitored over 21 days. These phenotypes included quantitation of T cell, B cell and natural killer cell populations, immune function, lymphocyte proliferation and cytokine production. At the end of this study period, only a few statistically significant changes were observed (a decrease in one particular T cell population, but an increase in two others when comparing Marinol® and placebo patients) and no detrimental effects on the immune system were observed [155]. However, it is worth noting that there was no healthy human control group for this study, and plasma THC concentrations after oral administration were not assessed.

Other clinical studies did not assess the immune function after administration of THC, but rather its effect on patients suffering from multiple sclerosis (MS). A study by Wade *et al* assessed the effect of cannabis-based medicinal extract (Sativex®) on reducing spasticity, spasms, pain or tremors associated with MS. Patients were given 2.5 – 120 mg of Sativex daily over the course of 10 weeks. No significant changes in most symptoms of MS were observed, but Sativex treatment was found to reduce the spasticity associated with MS compared to placebo [156].

A larger scale clinical trial was conducted more recently in the UK which set out to investigate whether Marinol® might slow the course of progressive MS. Participants aged 18-65 received Marinol® starting at 3.5 mg capsules twice daily, up to a maximal dose of 28 mg per day, for up to four years. The expanded disability status scale (EDSS) progression of patients was monitored over the course of the study as a primary outcome, and found that Marinol® did not have a significant effect in slowing the progression of multiple sclerosis compared to placebo,

although one observation noted that Marinol® did seem to have an effect in slowing the progression of EDSS score in less disabled patients, but may have a slightly negative effect in patients with higher EDSS scores [157].

Unfortunately due to the nature of clinical trials, these studies have suffered from many subjects withdrawing before the end of the study period, especially the study by Zajicek where 29% of those selected for the study had failed to complete the study [157].

1.4 The case for intestinal lymphatic transport of THC

To string a thread through the previous subsections in this chapter, it is known that THC is a lipophilic phytocannabinoid that is soluble in lipids [92]. Commercial formulations containing THC have been approved for the treatment of vomiting and nausea associated with cancer therapy, and appetite stimulation in end-stage AIDS patients [98]. It is also known that it potentially has many physicochemical properties that lend it to be transported via the intestinal lymphatic system, an alternative absorption route facilitated by the presence of dietary lipids [67, 158]. The intestinal lymphatic system also contains the largest collection of resident white blood cells in the body, housed in lymph nodes that screen lymph fluid that passes through for antigens to mount immune responses to them [9]. Previous work on synthetic cannabinoids shows that they can be transported in the lymphatic system in significant amounts [75]. However, THC has also been shown *in vitro* and *in vivo* to suppress many facets of the immune response [118]. Although these effects have not been observed in humans as of yet, the clinical trials conducted using THC have not been in conditions facilitating intestinal lymphatic transport. This targeting of a potentially immunosuppressive agent such as THC to the lymphatic system may create high local concentrations that could have some of the suppressive effects listed previously.

1.5 Hypothesis and Aims

Therefore, the hypothesis of this PhD is that THC is transported via the intestinal lymphatic system when administered orally in conditions that facilitate dietary lipid absorption via association with CM, and that THC is sufficiently concentrated to be able to exert a suppressive effect on immune cells resident in lymph nodes while travelling through the lymphatic system.

To address this hypothesis specifically, the following experiments were performed:

1. Assess the dissolution of THC in the intestinal lumen, using an *in vitro* lipolysis model that is used for lipidic formulations of drugs like THC in Marinol® (sesame oil) [159].
2. Assess the lymphatic transport potential of THC, by a two different methods -First by using the previously developed *in silico* model using the physicochemical properties to calculate an association with rat chylomicrons [70], then obtain experimental association values with an artificial lipid emulsion, rat CM and human CM.
3. Assess the contribution of dietary lipids to the absorption of THC by conducting oral pharmacokinetic studies for THC administered in lipid-free and lipidic formulation.
4. Assess the distribution of THC in intestinal lymph nodes after oral administration.
5. To relate these concentration levels of THC to a cellular effect, assess the proliferative response of lymphocytes after exposure to THC, at the concentration range found in intestinal lymph nodes after oral administration.
6. Compare this response in healthy volunteers with lymphocytes from immunocompromised patients, and also compare the inhibitory concentrations when THC is exposed in a CM emulsion vehicle.

Chapter 2: Materials and Methods

2.1 Techniques used in the Assessment of the Lymphatic

Transport Potential of THC

2.1.1 *In vitro* Lipolysis

2.1.2.1 Materials

THC was donated by GW Pharmaceuticals (Cambridge, UK). Sesame oil, Tris maleate, taurocholic acid sodium salt hydrate, sodium hydroxide standard solution (1 M), L- α -phosphatidylcholine, porcine pancreatin powder and glyceryl trioctanoate were purchased from Sigma-Aldrich (Dorset, UK). Calcium chloride was purchased from Alfa Aesar (Lancashire, UK) and sodium chloride was purchased from Fisher Scientific UK (Leics, UK). The buffer solutions (pH4, 7, 10) used for calibration were obtained from YSI Incorporated (Ohio, USA). The water used was double distilled water. The chemicals used were of analytical grade or higher and used without further dilution unless specified otherwise.

2.1.2.2 Preparation of simulated intestinal fluid

Two types of intestinal fluid were used in this method, complete and incomplete. The compositions of the two buffers are most similar to those used by Dahan and colleagues [160] and is listed in Table 2-1 below:

Chemical	Weight (g)	Final Concentration in solution (mM)
Complete and Incomplete Simulated Intestinal Fluid		
Tris Maleate	11.86	50
Sodium Chloride	8.766	150
Calcium Chloride	0.5549	5
Complete Intestinal Fluid Only		
Taurocholic acid sodium salt hydrate	2.7785	5
L- α -phosphatidylcholine	0.96	1.25

Table 2-1. Chemicals used to prepare 1 litre of lipolysis buffers.

The pH of the buffers were then adjusted to 6.8 using a pH-stat titrator (T50 Graphix, Mettler Toledo Inc), protected from light with aluminium foil and stored at 4 °C till use.

2.1.2.3 Preparation of pancreatic lipase/colipase

Pancreatic lipase/colipase solution used in the lipolysis method was freshly prepared before each experiment. One gram of porcine pancreatic lipase was added to 5 ml of incomplete buffer and the solution was vortex mixed for 15 minutes. The mixture was then centrifuged at 5 °C and 1600 g for 15 minutes and the supernatant was collected and stored on ice until use.

2.1.2.4 Preparation of Lipidic formulation of THC

THC was accurately weighed and diluted in an appropriate volume of sesame oil, then mixed with a magnetic flea in a scintillation vial until fully dissolved. The formulation was then stored at -20 °C until use.

2.1.2.5 Calculation of dose for use in model

In order to best approximate the intraluminal processing of THC in the GI tract, it is important to simulate a biologically relevant condition. The *in vitro* lipolysis model uses a simulated intestinal fluid, water bath and pH monitoring to create an environment mimicking the small intestine. However, since the total volume of the *in vitro* lipolysis reaction is 40 mL, it is important to consider the volume of liquid *in vivo* and add the proportional amount of formulation. If we assume that the volume of liquid that accompanies a meal is 250 mL (the equivalent to one cup of liquid), and one typical dose of THC in sesame oil is 1 mL, then in a 40 mL *in vitro* lipolysis reaction vessel there should be 0.16 mL of oil. In the establishment and optimisation of this model, other volumes of formulation were attempted, but these formulations did not contain THC.

2.1.2.6 *In vitro* lipolysis procedure

A set volume of oil (160 μL) was added to 35.5 ml of complete buffer in a reaction vessel. The vessel was then attached to a pH-stat titrator (T50 Graphix, Mettler Toledo Inc), placed in a 37 $^{\circ}\text{C}$ water bath and mixed for 15 minutes to ensure even dispersion of the formulation. 3.5 mL of pancreatic lipase/colipase solution was added to the mixture, which initiates lipolysis. The mixture was then titrated with 1 M sodium hydroxide to maintain a pH environment of 6.8. The experimental apparatus is shown in Figure 2-1.

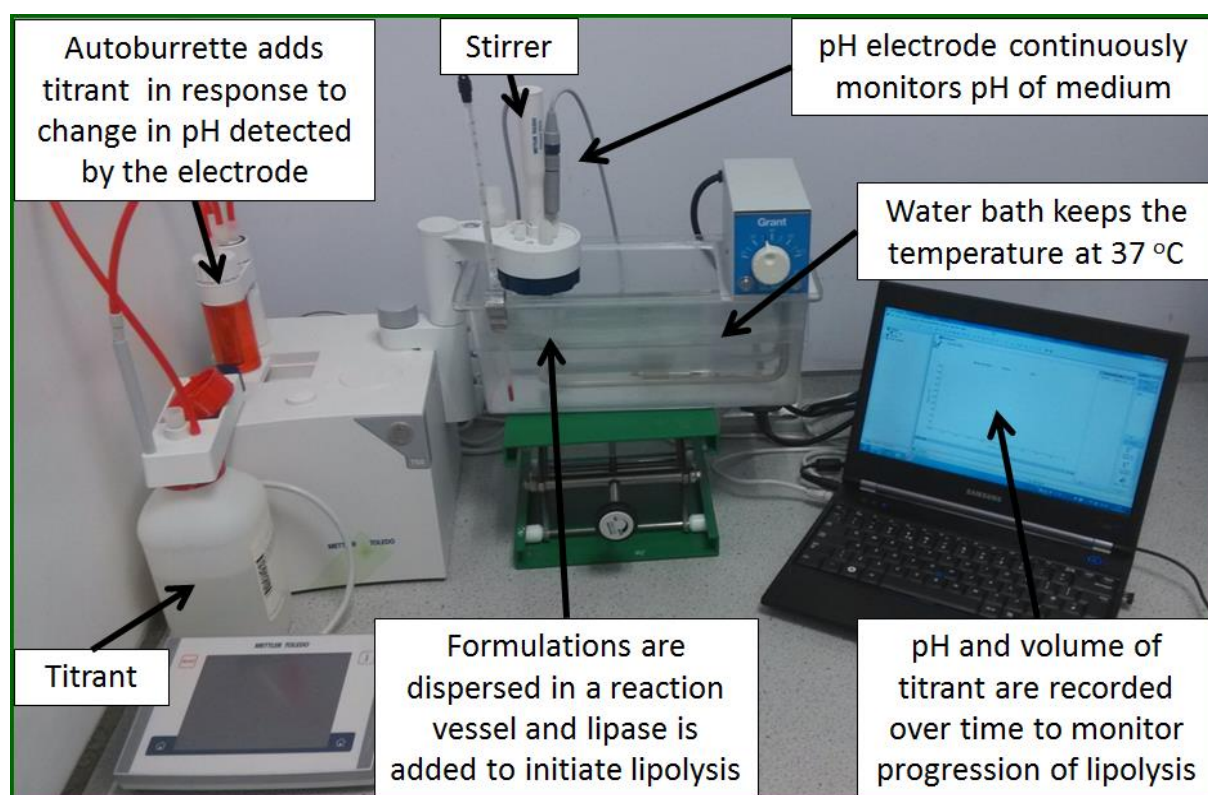


Figure 2-1. Experimental Setup of the *in vitro* lipolysis model.

Parameters of the pH titrator controlled by computer software (LabX light, Mettler Toledo) are stated below, and adapted from a previously used model in The Hebrew University of Jerusalem and modified by changing the set potential to pH 6.8, and minimum dosing rate to 3 $\mu\text{L}/\text{min}$ [161]:

Titrant: 1 M NaOH	Tendency: Positive
Electrode: DG111-SC pH	Maximum dosing rate: 1 mL/min
Stir speed: 35%	Minimum dosing rate: 3 µL/min
Set potential: 6.8 pH	Maximum volume: 30 mL
Control band: 0.05 pH	Minimum volume: 0.003 mL/min

Titration was stopped when the pH of the solution does not exceed the control band (pH 6.75 – 6.85) and minimum dosing rate is reached (3 µL/min). Data was recorded in LabX software.

After completion of lipolysis, the resulting reaction mixture was aliquoted into ultracentrifuge tubes (Beckman Coulter) and balanced for density gradient ultracentrifugation at 197000 *g* (*r*_{av} in SW 41 Ti Rotor, Beckman Coulter) and 33 °C for 90 minutes.

2.1.2.7 Separation of phases after completion of Lipolysis

The upper lipid layer, middle micellar layer and sediment were collected from each tube and stored at 4 °C until analysis (sediment was re-suspended in 1 mL of de-ionized water).

2.1.2.8 Calculation of the estimated fraction of THC available for absorption

The fraction of THC readily available for absorption post-lipolysis was assumed to be contained within the micellar phase, therefore the fraction of THC available was:

$$\text{Fraction Available} = \frac{\text{Amount of THC found in the micellar layer}}{\text{Total THC used in lipolysis}} \times 100\%$$

2.1.2.9 Statistical Analysis

The data was presented as means ± SEM unless specified otherwise. Differences were assessed using one-way ANOVA followed by the Tukey's correction for multiple comparisons. P-values of less than 0.05 were considered statistically significant.

2.2 Techniques for the assessment of lymphatic transport potential

2.2.1 Predicting intestinal lymphatic transport based on association with chylomicrons: *In silico* model

An *in silico* model was used as previously published [70]. Eight physicochemical properties for compounds were calculated from ACD/Labs software (online interface, <http://www.chemspider.com/>). These properties were, in order of importance, Log D_{7.4}, Log P, Polar Surface Area (PSA), Hydrogen Bond Acceptors (HBA), Freely Rotatable Bonds (FRB), Density, Molar Volume, and Hydrogen Bond Donors (HBD). The values for the properties were then multiplied by corresponding unscaled regression coefficients to produce a LOGIT value, and the predicted association value was reverse-calculated from this LOGIT value. These unscaled regression coefficients are listed in **Table 2-2** below:

Physicochemical Property	Unscaled Regression Coefficient
Log D 7.4	0.299879
Log P - Log D	-0.238127
PSA (Å ²)	-0.00855215
HBA	-0.184359
FRB	0.0805226
Density (g/cm ³)	1.45337
Molar Volume (cm ³)	0.00545912
HBD	0.0823094
Constant	-5.24138

Table 2-2. Physicochemical Properties used in in-silico prediction of chylomicron association and their unscaled regression coefficients. PSA = Polar Surface Area, HBA = Hydrogen Bond Acceptor, FRB = Freely Rotatable Bonds, HBD = Hydrogen Bond Donor.

2.2.2 Predicting intestinal lymphatic transport based on association with chylomicrons: *Ex vivo* model

The *ex vivo* chylomicron association model used in this work was similar to a previously used method, published by Gershkovich *et al* [1].

2.2.2.1 Materials

THC was provided by GW Pharmaceuticals. Phosphate-buffered saline, potassium bromide, sesame oil, ethylenediaminetetraacetic acid (EDTA), ethanol, propylene glycol, was purchased from Sigma-Aldrich UK. Water, HPLC solvents were purchased from Fisher Scientific UK. A serum triglyceride determination kit (catalogue number TR0100) was purchased from Sigma-Aldrich, and used following the manufacturer's instructions.

2.2.2.2 Preparation of density gradient solutions

Gradient solutions were prepared by adding potassium bromide to phosphate-buffered saline (PBS). The density solutions required for the isolation of chylomicrons were 1.063 g/mL, 1.019 g/mL and 1.006 g/mL. 1.006 g/mL was the density of native PBS solution, so to obtain 1.063 g/mL solution, 21.321 g of potassium bromide was added to 250 mL of 1.006 g/mL PBS solution, and 4.765 g of potassium bromide was added of 250 mL of 1.006 g/mL solution to obtain 1.019 g/mL solution.

2.2.2.3 Production and Isolation of Chylomicrons from blood

Rat Chylomicrons

The production of chylomicrons from the rat was performed as previously published with slight modifications [1]. The oral gavage procedures were performed by Pavel Gershkovich, and the plasma and chylomicron separation and isolation from blood samples was performed by myself. The protocol for this procedure was approved by the University of Nottingham Ethical Review Committee, in accordance with the Animals (Scientific Procedures) Act 1986, performed under PPL 40/3698. Four male rats weighing 330 – 350 g (Charles River Laboratories, UK) were used in this study, and were housed at the University of Nottingham Bio-Support Unit, kept in a temperature-controlled, 12 hour light-dark cycle environment with free access to water and food. Rats were fasted overnight the day before the procedure.

To begin the procedure, 0.5 mL of peanut oil and 1.5 mL of water was administered by oral gavage. One hour later, 0.3 mL of peanut oil and 1.5 mL of water was administered by oral gavage, and again one hour after that (a total of 1.1 mL of oil and 4.5 mL of water). 1.5 hours after the last administration, rats were placed under general anaesthesia (gas, isoflurane) and a terminal blood collection was performed from the abdominal vena cava using EDTA-coated syringes to prevent blood clotting. Plasma was then separated by centrifugation for 5 minutes at 3000 *g*. 4 mL of plasma was then added to an ultracentrifuge tube, and 0.57 g of potassium bromide was added and gently mixed to adjust its density to 1.1 g/mL. Other density solutions were then layered on top, in the following proportions:

1. 1.063 g/mL – 2.75 ml
2. 1.019 g/mL – 2.5 ml
3. 1.006 g/mL – 2 ml

The tubes were then ultracentrifuged at 39600 rpm (using a TH-641 rotor, to achieve 197'000 *g* at r_{av}) for 33 minutes, and the upper CM layer was collected from each tube, diluted as little as possible. The triglyceride content of the collected CM emulsion was then assessed using the Serum Triglyceride Determination Kit (Sigma Aldrich, UK) following the manufacturer's instructions, and diluted to 1 mg/mL of triglyceride by addition of 1.006 g/mL PBS solution. The emulsion can be kept overnight at 4 °C, or used immediately for association studies.

Human Chylomicrons

The protocol for CM production was approved by the University of Nottingham Ethics Review Committee, under study ID PG-HCM-10-15. Three healthy volunteers (aged 25-35) were instructed to eat a meal rich in TG (over 50 grams of fat, this was usually a McDonalds® Double Sausage Egg McMuffin meal with two hash browns accompanied with a coffee with sugar and milk, or eggs fried with olive oil accompanied with bread dipped in olive oil), and

20 mL of blood was collected by venepuncture 3 – 4 hours after the meal. Plasma and CM were then processed in a similar fashion to that described above.

2.2.2.4 Association of THC with isolated chylomicron and Intralipid® emulsion

Association with CM was based on a previously published method [1]. Stock solutions of THC were prepared in propylene glycol with 1% ethanol (v/v). An appropriate volume of this stock solution was added to 2 mL of CM emulsion (diluted to 1 mg/mL triglyceride content obtained previously) to reach a final molar concentration of 1.75×10^{-6} M. Intralipid® 20% was also used as an artificial lipid emulsion, and diluted to the same triglyceride concentration for use in association studies. The emulsion was incubated with THC at 37 °C for 60 minutes with constant mixing by magnetic stirrer. After incubation, the density of the solution was adjusted to a density of 1.1 g/mL by adding potassium bromide (0.285 g), placed in ultracentrifuge tubes and the following proportions of density gradient solutions gently layered on top:

1. 1.063 – 3.5 ml
2. 1.019 – 3.5 ml
3. 1.006 – 2 ml

The tubes were then ultracentrifuged at 39'600 rpm (using a TH-641 rotor, to achieve 197'000 g at r_{av}) for 33 minutes, and the upper CM layer was collected from each tube using a glass Pasteur pipette. The volume of the collected fraction was accurately measured and 100 μ L was used for drug content determination by HPLC-UV (described in a later section).

2.3 Determination of THC in different biological matrices

2.3.1 Determination of THC in Lipolysis Media by HPLC-UV

In order to determine the distribution of THC in the three phases of lipolysis, an analytical assay was developed, optimised and validated for use. Due to the differing nature of these three phases, each phase had to be regarded as a separate method.

2.3.1.1 Materials

THC was provided by GW Pharmaceuticals, Vitamin D3 (used as Internal Standard) was purchased from Sigma-Aldrich UK. Methanol, THF, n-Hexane and water were purchased from Fisher Scientific, and were of analytical grade.

2.3.1.2 Preparation of stock solutions

Standard solutions of THC and Internal Standard (IS, Vitamin D3) were prepared in methanol at concentrations of 50 and 10 mg/mL respectively and stored at -20 °C. Working standard solutions of THC and IS (5 and 1 mg/mL) were diluted in methanol and also stored at -20 °C.

The working solutions of THC and IS were diluted in methanol immediately before preparation of calibration curves to concentrations of 5, 10, 15, 20, 25, 30, 40, 50, 200, 400, 600, 800, 1000, 1500, 2000, 2500, 5000, 10000, 20000, 30000, 40000 and 50000 µg/mL. The calibration curve for micellar phase was prepared at concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 20, 40, 60, 80 and 100 µg/mL. The calibration curve for sediment phase was prepared at concentrations of 1, 2, 3, 4, 5, 20, 40, 50, 60, 80, 100, 150 and 200 µg/mL. The calibration curve for lipid phase was prepared at concentrations of 20, 40, 60, 80, 100, 200, 250, 500, 1000, 2000, 3000, 4000 and 5000 µg/mL. 20 µL of these solutions were spiked into 180 µL of micellar and sediment phase sample to make up to a total sample volume of 200 µL. 10 µL was spiked into 90 µL of lipid phase sample to make up a total sample volume of 100 µL.

2.3.1.4 Sample Preparation

Samples were prepared for HPLC-UV analysis by liquid-liquid extraction, based on a previously published method with slight modifications [75]. 200 µL of samples from the micellar, sediment or lipid phase were mixed with 60 µL of IS (100 µg/mL) and 600 µL of THF, and then vortex mixed for one minute. Three ml of n-hexane were then added to each tube and then mixed for five minutes. The tubes were centrifuged at 2800 g for ten minutes at 10 °C and

the upper organic layer was transferred to a fresh glass tube. The organic layer was evaporated to dryness under nitrogen at 35 °C, reconstituted in 200 µL of methanol, and 10 µL was injected for analysis. Due to the presence of lipids in the lipid phase, samples were reconstituted in 1.5 mL of methanol instead.

2.3.1.5 Chromatographic Conditions

Two HPLC-UV systems were used in this study. The first was an Agilent 1100 series HPLC, with a G1322A degasser, G1311A quaternary pump, G1313A autosampler, G1316A temperature-controlled column compartment, and G1315B diode array detector with micro cell. Data processing was carried out with ChemStation (Agilent Technologies) software.

The second system used was a Waters Alliance 2695 separation module with a 996 Polydiode array detector. Data processing was carried out with EmpowerTM 2 software.

In both cases, absorbance was monitored at 220 nm for THC and Vitamin D3. A Phenomenex Luna C18 (2) 5 µm 2.1 x 150 mm column was used, and elution was performed isocratically using methanol and water in a ratio of 90:10 (v/v). The flow rate was 0.2 mL/min, and the system was maintained at 43 °C.

2.3.1.6 Partial Method Validation

This method was partially validated for selectivity, accuracy, precision, sensitivity and linearity, and is detailed in Chapter 3.

2.3.2 Determination of THC in Chylomicron and Intralipid® emulsion using HPLC-UV

In order to accurately determine the concentration of THC in CM and Intralipid® emulsion for association studies, it was necessary to develop an analytical assay, also using HPLC-UV.

Preliminary work showed that THC had a very high association value to CM, but used a different analytical assay and apparatus to detect THC [162].

2.3.2.1 Materials

THC was provided by GW Pharmaceuticals and Probucol was purchased from Sigma-Aldrich UK. Intralipid® 20% was kindly provided by Snow Stolnik-Trenkic, and purchased from Fresenius Kabi (Cheshire, UK). Methanol, THF, n-Hexane and water were purchased from Fisher Scientific UK and were of analytical grade.

2.3.2.2 Preparation of stock solutions of THC

Standard solutions of THC and IS (Probucol) were prepared in methanol at concentrations of 50 and 2 mg/mL respectively and stored at -20 °C. Working standard solutions of THC and IS (5 and 50 µg/mL) were diluted in methanol and also stored at -20 °C.

2.3.2.3 Sample Preparation

10 µL of IS (50 µg/mL) and 300 µL of THF was added to 100 µL of sample in a glass tube, and was vortex mixed for 1 minute. Three mL of n-hexane was then added and vortex mixed for 5 minutes. The upper layer was transferred to a fresh tube and evaporated to dryness under nitrogen gas at 35 °C. The residue was reconstituted in 200 µL of methanol, and 20 µL was injected for analysis.

2.3.2.4 Chromatographic Conditions

A Waters Alliance 2695 separation module with a 996 Polydiode array detector was used; absorbance was monitored at 220 nm. A Phenomenex Luna C18 (2) 5 µm 2.1 x 150 mm column was used, and elution was performed isocratically using methanol and water in a ratio of 90:10 (v/v). The flow rate was 0.2 mL/min, and the column heater was maintained at 50 °C. Data processing was carried out with Empower™ 2 software.

2.3.3 Determination of THC in rat plasma by HPLC-UV

2.3.3.1 Materials

THC was provided by GW Pharmaceuticals, and DDT was purchased from Sigma Aldrich UK. Rat plasma was purchased from BD Biosciences UK. Acetonitrile, n-Hexane, THF, ethanol and water were purchased from Fisher Scientific, and were of HPLC grade.

2.3.3.2 Preparation of standard solutions

Standard solutions of THC and IS (DDT) were prepared in acetonitrile at concentrations of 1.32 and 0.5 mg/mL respectively, and stored at -20 °C. Working standard solutions of 200 and 50 µg/mL were prepared by appropriate dilution in acetonitrile and were also stored at -20 °C. Calibration curves and quality control samples were also prepared with CBD, which was also validated with this method, in equal collaboration with Atheer Zgair.

The working solution of THC was diluted in acetonitrile immediately before the preparation of calibration curves, to 0.15, 0.2, 0.3, 0.4, 1, 2, 4, 10, 20, 100 and 200 µg/mL. These were spiked into plasma by adding 7.5 µL (and also 7.5 µL of CBD stock solution) into 135 µL of rat plasma, to make up to 150 µL total.

2.3.3.3 Sample Preparation

Samples were prepared for analysis by a combination of protein precipitation and liquid-liquid extraction. Fifteen microliters of IS (50 µg/mL DDT) were added to 150 µL of rat plasma in a glass tube. Plasma proteins were precipitated by addition of 600 µL of cold acetonitrile and the mixture was vortex mixed for 1 minute. 600 µL of water was added and the mixture was mixed again for 1 minute. 3 mL of n-Hexane was added and mixed for 5 minutes. The tubes were then centrifuged at 1160 *g* for 15 minutes at 10 °C (Harrier 18/80R, UK) and the upper organic layer was transferred to a new glass tube by glass pipette. This organic layer was evaporated to dryness under nitrogen gas at 35 °C (Techne DRI-Block type DB-3D,

Cambridge, UK), then reconstituted in 150 μ L of acetonitrile, and 30 μ L was used for injection into the HPLC system.

2.3.3.4 Chromatographic Conditions

A Waters Alliance 2695 Separations module with a 996 photodiode array detector was used for analysis. Sample chiller and column heater were kept at 4 and 55 °C respectively. Separation was achieved using an ACE C18-PFP 150 mm x 4.6 mm, 3 μ m particle size column (Hichrom Ltd., Reading UK), protected by an ACE C18-PFP 3 μ m guard cartridge. The mobile phase was a mixture of acetonitrile and water in a ratio of 62:38 (v/v). The flow rate was set at 1 mL/min for 20 min. The absorbance was monitored at 220 nm. Data processing was carried out using EmpowerTM 2 software. Under these chromatographic conditions, THC eluted at 14.5 minutes and IS at 18 minutes.

2.3.3.5 Full Method Validation

This method was fully validated in accordance with the United States Food and Drugs Administration (FDA) guidelines on Bioanalytical Assays on Selectivity, Sensitivity, Stability, Recovery, Accuracy and Precision [163]. This validation and development of the method is described in Chapter 3.

2.3.3.6 Modification and Application in Biodistribution studies

This assay was also used for the determination of THC in homogenised rat mesenteric lymph node (MLN), brain, liver and spleen taken after oral administration of a lipid-based and lipid-free formulation containing THC. The solid samples were diluted three-fold (based on weight) in double-distilled water and then homogenised at 15000 rpm on Polytron PT 10-35 GT homogeniser for 3 minutes. Tissue homogenate was prepared for HPLC-UV analysis by the same method as plasma, and THC was detected in the same chromatographic conditions.

2.4 Techniques for the assessment of proliferation

2.4.1 Assessment of Proliferation of Peripheral Blood Mononuclear Cells

2.4.1.1 Materials

Flat clear-bottomed 96-well plates (Costar ®), RPMI-1640 culture medium with L-glutamine, Trypan Blue 20%, Histopaque®-1077, fetal bovine serum (FBS), penicillin-streptomycin (Pen-Strep), dimethyl sulphoxide (DMSO), phytohaemagglutinin (PHA), phorbol myristate (PMA), and ionomycin calcium (Io) were purchased from Sigma Aldrich UK. Colorimetric BrdU Cell Proliferation ELISA was purchased from Roche UK.

2.4.1.2 Human Subjects

Two types of peripheral blood mononuclear cells (PBMC) were required for this study, those isolated from healthy volunteers and cancer patients. Unfortunately, due to the action of chemotherapy agents, white blood cell counts are generally low in patients undergoing chemotherapy [164, 165]. It is therefore important to pick an appropriate type of condition that would yield sufficient numbers of immunocompromised PBMC. A collaboration with Dr. Ivo Hennig at Nottingham City Hospital and the Nottingham Health Science BioBank was successful in recruiting patients suffering from non-seminomatous germ cell tumours of the testicle, towards the end of their chemotherapy treatment [166]. All participants were male. Their diagnoses and treatment information is shown in Chapter 6.

2.4.1.3 Isolation of Peripheral Blood Mononuclear Cells

PBMC were isolated under the ethical approval of Professor Bruno Gran, for the Study “Role of the Innate Immune System in Multiple Sclerosis”, and the BioBank study “Immunosuppressive Effects of Cannabinoids on Human Lymphocytes and Implication in Cancer Treatment”. A competent phlebotomist (nurse at Dr. Hennig’s clinic) collected blood samples by venepuncture into heparinized vacuum glass tubes.

The Ficoll-Paque technique was used to isolate peripheral blood mononuclear cells (PBMC) from blood. Briefly, blood was gently layered on top of Histopaque®-1077 solution, and centrifuged at 400 x g for 30 minutes. Red blood cells present are aggregated by the polysucrose present in solution and rapidly sediment, resulting in pelleting at the bottom of the centrifuge tube. An opaque buffy coat layer containing PBMC was then extracted using a pipette to clean 50 mL conical tubes. Sterile isotonic PBS was added to the conical tube up to 50 mL, and this was centrifuged at 250 x g for 10 minutes to pellet cells at the bottom of the tube. Most platelets remaining in solution however, will not pellet. PBS is then discarded (along with these platelets) and the cell pellet is resuspended in 10 mL of RPMI cell culture medium containing L-glutamine, 10% FBS and 1% Pen-Strep. The cell density is then measured using 20% Trypan Blue solution and cell counting by a haemocytometer. The cell suspension is then diluted to 750'000 cells/mL for use in cell proliferation assays.

2.4.1.4 Proliferation Assay

The proliferation of PBMC was assessed as described by the manufacturer. PBMC were seeded into flat clear-bottomed 96-well plates at a density of 50000 cells/well, and THC was added in final concentrations of 0.01, 0.1, 1, 2.5, 5, 10, 15 and 20 µg/mL. Cells were incubated at 37 °C for 30 minutes to allow sufficient dispersion of THC (Vehicle and cell culture media was also included as treatments as negative control). PHA diluted in cell culture media was then added (some cells also received culture media instead and served as unstimulated controls), and the cells were left to grow for two days at 37 °C. BrdU was then added and incubated for an extra 12 hours. The cells were then centrifuged at 800 g for 10 minutes, and tapped dry. The cells were denatured and fixed onto the well plate, and Anti-BrdU-Peroxidase (Anti-POD) was then added and incubated for 90 minutes. After incubation, cells were washed three times with PBS, and POD substrate was finally added. The absorbance of these wells

was then observed at 370 nm, with reference wavelength at 492 nm using a plate reader (FL600, BIO-TEK Instruments Inc, USA).

2.4.1.5 Statistical Analysis

Cell proliferation values obtained was absorbance at 370 nm (reference wavelength 492 nm). For improved consistency between individual samples, absorbance values were normalised relative to the absorbance of vehicle-treated cells. Differences between proliferation values were assessed by one-way ANOVA with Dunnet's correction, differences less than $p < 0.05$ were considered significant.

2.5 Techniques used in Pharmacokinetic and Biodistribution Studies

2.5.1 Jugular Vein Cannulation

2.5.1.1 Animals

Rats were purchased from Charles River UK, and housed in the University of Nottingham Bio-Support Unit, and kept in a temperature- and humidity-controlled environment with 12-hour light/dark cycles and free access to water and food. All protocols for surgeries were approved by The University of Nottingham Ethical Review Committee in accordance with the Animals (Scientific Procedures) Act 1986.

2.5.1.2 Preparation of formulations

Formulations for intravenous administration were prepared by accurately weighing THC in a scintillation vial, then dissolving appropriately in ethanol, propylene glycol and water to a final proportion of 10:80:10 v/v/v. This formulation proportion was also used as the oral lipid-free formulation of THC (with a different final concentration). The lipid-based formulation for oral

administration was prepared by weighing THC in a scintillation vial, and dissolved in the appropriate volume of sesame oil.

2.5.1.3 Preparation for surgery

Two-part jugular vein cannulas were prepared and sterilised for insertion. The cannulas consisted of a PE-50 tubing roughly 15 cm, upon which two 2 mm segments of Tygon tubing attached 3 cm from each end. Silastic tubing was cut into a V shape at one end. Tygon tubing's were attached first, and silastic tubing was fitted on by loosening the segment in chloroform. The cannulas were completed by attaching a blunt needle (the sharp end disconnected with a plier) on the end without silastic tubing and washing out remaining chloroform with double distilled water. The finished cannula was then sterilised by the ethylene oxide sterilisation process for use in surgery along with other tools that contain plastic. Skin and muscle scissors, scalpel blades, cotton buds, needle holders and other metal or wood tools were sterilised by autoclaving before use in surgery. Rats were acclimatised for five days in the aforementioned living conditions before surgery.

On surgery day, an intraperitoneal injection of ketamine and xylazine was given at a dose of 90 mg/kg and 9 mg/kg respectively, as injectable anaesthetic agents. When the animal started to lose consciousness, its fur was shaved down the dorsal neck, right ventral neck and the upper part of thorax and sterilised with disinfectant. The animal's eyes were then treated with corneal protectant, and placed on a heat-pad that maintains smooth respiration. The depth of anaesthesia was then assessed by the pedal withdrawal reflex, and surgery can proceed if that reflex is absent or barely detectable.

2.5.1.4 Surgery procedure

The surgery procedure was performed by Dr. Pavel Gershkovich, assisted by my colleagues Atheer Zgair, Jong Bong Lee, and myself. While we did not perform the surgery itself, all the

surrounding the tasks of non-surgical animal handling, administration of injectable anaesthetic, injectable analgesic, animal monitoring, sequential administration of intravenous compounds by cannula and blood sampling, killing of animals, laparotomies to remove organs were split equally between Atheer, Jong Bong and myself to ensure smooth progression of surgery and experiment days. In return for assisting with my experiments, I also similarly assisted Atheer and Jong Bong with their pharmacokinetic studies.

To begin, a 1-1.5 cm skin incision (using scissors) was made over the locale on the ventral neck where the right jugular vein can be seen to pulsate below the skin surface. Then, using gentle tissue scissors a blunt dissection was performed until the jugular vein could be clearly seen. The jugular vein was cleaned from surrounding tissues using a pair of forceps and then cotton tips. A silk suture was placed under the jugular vein and the double suture was cut into one 10 cm piece (closer to animal's chest) and one piece long enough to tape down to the table corner (closer to the head of the animal). A 0.5 cm skin incision was made on the dorsal neck of the animal and the cannula was pulled subcutaneously (using plastic guide) from the dorsal neck to the ventral neck with the silastic end close to the jugular vein. A spatula was placed under the jugular vein. The jugular vein was ligated by silk suture closer to the animal's head and the suture was taped to the table corner. A semi-closed loop was made from the 10 cm piece of silk suture closer to animal's chest. A small incision was made in the vein using forceps and gentle iris scissors. The cannula was inserted 3 cm into the jugular vein using Catheter Introducer (BD™ 406999). To check the cannula had been correctly inserted, 0.1 ml sterile saline was injected and withdrawn and blood re-injected. The semi-closed loop was closed on the vein and cannula and 3 knots were made. The cannula was secured in place by performing an 8-shape suturing around a piece of Tygon tubing and 3 knots. Three additional knots were made around the cannula, and the cannula was checked with saline. The connective tissue above the jugular vein was sutured using Vicryl (Polyglactin 910) suture, making 3 knots

in every suture. The skin above the vein was sutured in a similar fashion. The cannula was checked again to see if it was working, then secured to muscle tissue on the dorsal neck by sewing and performing an 8-shape loop around a piece of Tygon tubing and three knots. The skin was sutured on the dorsal neck, making 3 knots each time. After a final check of the cannula, the end was cut using scissors to leave a 3-4 cm piece protruding from the back, and the end was plugged with a metal pin. An analgesic (ketoprofen 5 mg/kg) was then given subcutaneously (so it would take effect when the animal recovered from anaesthesia), and the animal was placed in an isolated cage kept at 27 °C until fully recovered. Pharmacokinetic experiments were done the day after surgery.

2.5.1.5 Intravenous pharmacokinetic study

For the preliminary intravenous pharmacokinetic study, two rats were administered an IV bolus of 5 mg/kg THC (in 10 mg/mL formulation as described above). Blood was sampled from the cannula before dosing, then at 5, 15, 30, then 2 and 7 hours following administration. For the subsequent IV study for calculation of bioavailability of orally administered THC, five rats were administered an IV bolus of 4 mg/kg THC (in 8 mg/mL formulation) and blood was sampled from the cannula at 5, 15, 30, minutes, then 1, 2, 4, 6, 8 and 12 hours following administration.

In both cases, 0.25 mL of blood was withdrawn at each sampling. To avoid the potential food interactions, rats were withheld from food until two hours after the start of the experiment. Plasma was separated by centrifugation (3000 *g*, 10 minutes, at 15 °C) and stored at -80 °C until analysis.

2.5.1.6 Oral pharmacokinetic study

Four rats were administered a bolus of 12 mg/kg THC in either a lipid-free or lipid-based formulation by oral gavage. 0.25 mL of blood was sampled from the cannula at 0.5, 1, 2, 3, 4,

5, 6, 8, and 12 hours after administration. To keep experimental conditions constant with the intravenous study, rats were also withheld from food until two hours after the start of the experiment. Plasma was separated by centrifugation (3000 *g*, 10 minutes, at 15 °C) and stored at -80 °C until analysis.

2.5.1.7 Biodistribution of THC following oral administration of THC

After determination of the time of maximal plasma concentration ($T_{\max}$), rats that did not have jugular vein cannulation surgery were allocated to either receive lipid-free or lipidic formulation. A dose of 12 mg/mL THC in either formulation was administered by oral gavage. At one hour before $T_{\max}$, $T_{\max}$ and 12 hours, rats were killed by asphyxiation and confirmation of death with cerebral dislocation. A laparotomy was then performed on the animal and the MLN, brain, liver and spleen were isolated and cleaned with double distilled water, patted dry and stored at -20 °C until analysis. Since the MLN are not commonly studied, their location in the rat was determined after personal communication with Dr. Wim De Jong, and a number of reference texts [167-170].

2.5.1.8 Pharmacokinetic data analysis

Plasma drug-time profiles for intravenous and oral studies of THC were analysed using Phoenix WinNonLin software version 6.3 (Certara, L.P./Pharsight Ltd) using non-compartmental and compartmental approaches [171].

Non-compartmental approach

For the intravenous study, the following pharmacokinetic parameters were calculated for the 12-hour period: terminal half-life ($t_{1/2}$), area under the curve (AUC) from 0 to 12 hours (AUC_{0-12}), AUC extrapolated to infinity (AUC_{inf}), clearance (CL), and apparent volume of distribution (V_d).

For the oral study, AUC_{inf} was also calculated, and data were used to estimate maximal plasma concentration (C_{max}), time of maximal plasma concentration (T_{max}).

The absolute bioavailability (F) of THC after a single oral dose was calculated using the AUC_{inf} and dose of oral (PO) and intravenous (IV) studies the following equation:

$$F = \frac{AUC_{inf\ PO} \times Dose_{IV}}{AUC_{inf\ IV} \times Dose_{PO}} \times 100\%$$

Compartmental approach

The models compared in this study were standard WinNonLin pharmacokinetic models.

In order to best describe the distribution and elimination of the intravenous study and absorption of the oral study, four kinetic models were compared. The first is a one-compartment model with bolus input and first-order elimination, the second model was a two-compartment model with bolus input and first-order elimination, the third model was a one-compartment model with first-order extravascular input (oral) and first-order output, and the fourth and final model was a two-compartment model with first-order extravascular input (oral) and first-order output. Relevant pharmacokinetic parameters were calculated by WinNonLin using curve stripping.

2.5.1.9 Comparison of models

To identify which model provides the best approximation of the data, the Akaike information criterion (AIC) for each model was used as a comparison figure. The models with the smallest AIC are considered the most adequate according to the parsimony principle.

2.5.1.10 Statistical Analysis

Data is presented as means $\pm$ SEM. Differences between association values were assessed using one-way ANOVA. Differences were assessed by one-way ANOVA with Fisher's LSD

test for comparisons between concentrations of THC in organ homogenate. P values below 0.05 were considered statistically significant.

Chapter 3: Development, Optimisation and Validation of Analytical Assays for detection of Δ^9 -tetrahydrocannabinol

3.1 Optimisation of the *in vitro* lipolysis model

In vitro lipolysis is a technique which mimics the intestinal lipid digestion process, and is therefore a suitable method to determine the likely fate of drugs delivered by lipidic formulations. It was developed because of the growing number of poorly water-soluble drug candidates, and lipid-based drug delivery systems were being developed to effectively solubilise them. Conventional drug dissolution systems were not as appropriate in evaluating the efficacy of lipidic formulations due to their potential to being digested [160]. This led to the development of a model that could incorporate addition of lipid digesting enzymes.

The first version of an *in vitro* lipolysis model was developed in 1988 by Reymond and Sucker to predict the *in vivo* performance of a lipid formulation of cyclosporine [172]. A number of lipolysis models have been in use around the world. Of note are the researchers at the University of Copenhagen in Denmark, Monash University in Australia and Hebrew University in Jerusalem, which have used the model to assess the intraluminal processing of drugs formulated in lipid-based drug delivery systems [173]. All three of these research groups use pancreatic lipase as their lipase source, but differ in how much enzyme is used, the set pH condition, and choice of phospholipid in the intestinal fluid [160, 174, 175]. The version used in this project is most similar to the one described by Dahan *et al* [176].

3.1.1 Optimisation of experimental conditions

The pH conditions used by other research groups were deemed not representative of the *in vivo* pH of the small intestine where dietary lipid absorption is likely to occur, and was therefore set at pH 6.8 to more closely reflect these conditions [170]. There were also a number of technical difficulties initially encountered when attempting *in vitro* lipolysis. Since the volume of formulation was lower than previously used (in other laboratories from previous research the typical volumes of formulation were 0.2, 0.5 and 1 mL), there was an issue with inconsistent

titration volumes of NaOH, and premature cessation or overshooting of lipolysis before the theoretical amount of triglycerides added had been digested (Figure 3-1).

Two parameters were changed to attempt to address this problem. The first was to reduce the concentration of NaOH titrant to 0.5 M instead of 1 M. Unfortunately this increased the titrant volumes for higher lipid loads, and the concentration of other buffer components changed significantly as a result. Therefore, this approach was deemed not optimal to digest all lipid loads. For the same reason, changing the amount or concentration of enzyme added was not pursued as a potential solution. The second parameter that was changed was the minimum rate of NaOH addition, which increased the accuracy of titration. Reducing this rate from 5 $\mu\text{L}/\text{min}$ to 3 $\mu\text{L}/\text{min}$ solved this titration problem for low volumes of sesame oil without any drawbacks, allowing consistent analysis of lipidic formulations using the model.

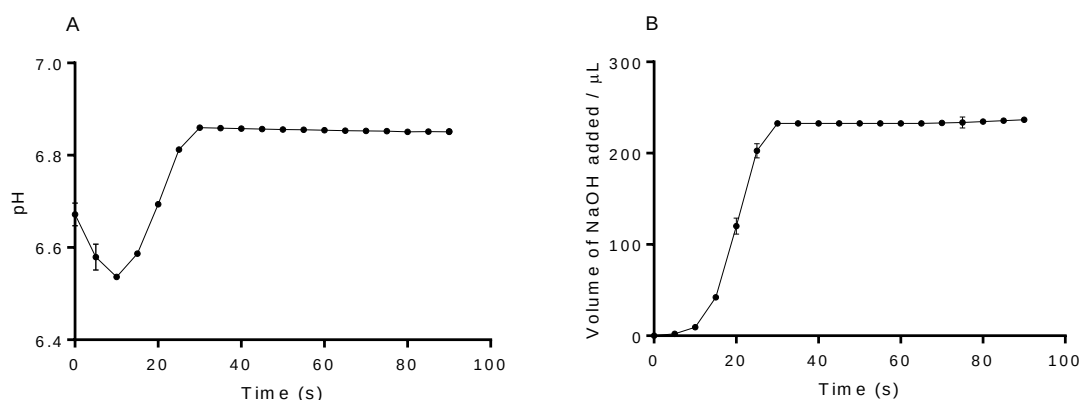


Figure 3-1. Premature termination of lipolysis at 200 μL of peanut oil. Data presented as means $\pm$ SD, $n = 5$.

3.1.2 Optimisation of lipid load

Based on previous lipolysis studies, the doses of formulation used varied from 200 mg to 1 mL or 1 gram of formulation [174-176]. The most commonly seen doses were 200, 500 and 1000 μL of formulation, and blank samples of these lipid loads were tested with the model for consistency of titration (Figure 3-2). There were significant differences between the volume of titrant and lipid load, as expected. Larger lipid loads released more fatty acids, which

required more sodium hydroxide to titrate. Likewise, larger lipid loads also took a longer time to digest, as lipolysis reaction neared completion.

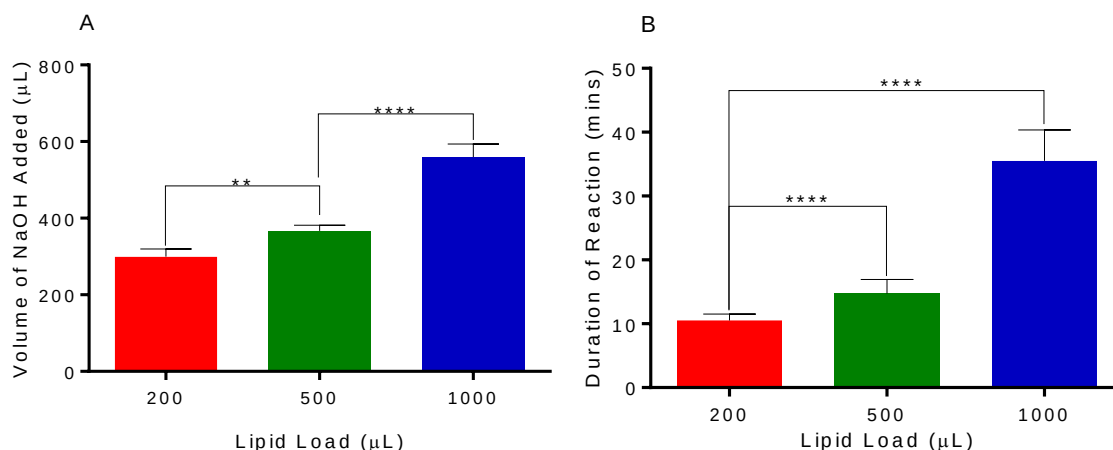


Figure 3-2. Volume of sodium hydroxide needed to maintain pH during lipolysis (A) and duration of reaction (B) for three lipid loads. Data presented as means $\pm$ SD, $n = 5$, ** denotes significance using one-way ANOVA with Tukey's correction, $p < 0.01$, **** = $p < 0.0001$.

Based on the low variability in titrant volume used and time required to finish digestion, these conditions were deemed fit for the purpose of assessing the intraluminal processing of THC in lipidic formulation.

3.2 Development and Validation of Analytical Assays to

Determine THC in Biological Matrices

The methods described in the previous sections all require the determination of THC in an accurate and precise manner. A number of different detection methods were attempted, including liquid chromatography with both ultra-violet and mass spectrometer detection, in a variety of biological and cell-free matrices. A large number of analytical assays for the determination of THC exist in the literature, each tailored for the purpose of the research group that developed it [177-181]. Many of the assays currently being used to determine the

concentration of THC in biological matrices use Liquid Chromatography coupled with Mass Spectroscopy or tandem Mass Spectroscopy (LC-MS or LC-MS/MS) [180, 182-185], which have lower limit of quantifications of 0.1 to 5 ng/mL. This section describes a number of different methods developed to accurately and reliably determine the concentration of THC in a number of biological matrices for application in lipolysis, chylomicron-drug association and pharmacokinetic studies.

3.2.2 Development and Validation of HPLC-UV Assay to determine THC in Lipolysis Media

3.2.2.1 Method Development

The original sample preparation method used ethanol as the more aqueous liquid in liquid-liquid extraction, since it is able to dissolve oils and micellar structures [186-188]. However, it was found that Tetrahydrofuran was also able to fulfil the same role, and does not require a special licence and allowance to use in the University of Nottingham.

3.2.2.2 Preparation of quality control samples

Independently prepared THC stock solutions of 10, 100, 500, 1000, 2000, 5000 and 40000 µg/mL dissolved in acetonitrile were used for preparation of quality control (QC) samples. Three levels of THC were chosen for each phase of lipolysis. These samples were then prepared for analysis as described in section 2.3.1.4.

3.2.2.3 Partial Method Validation

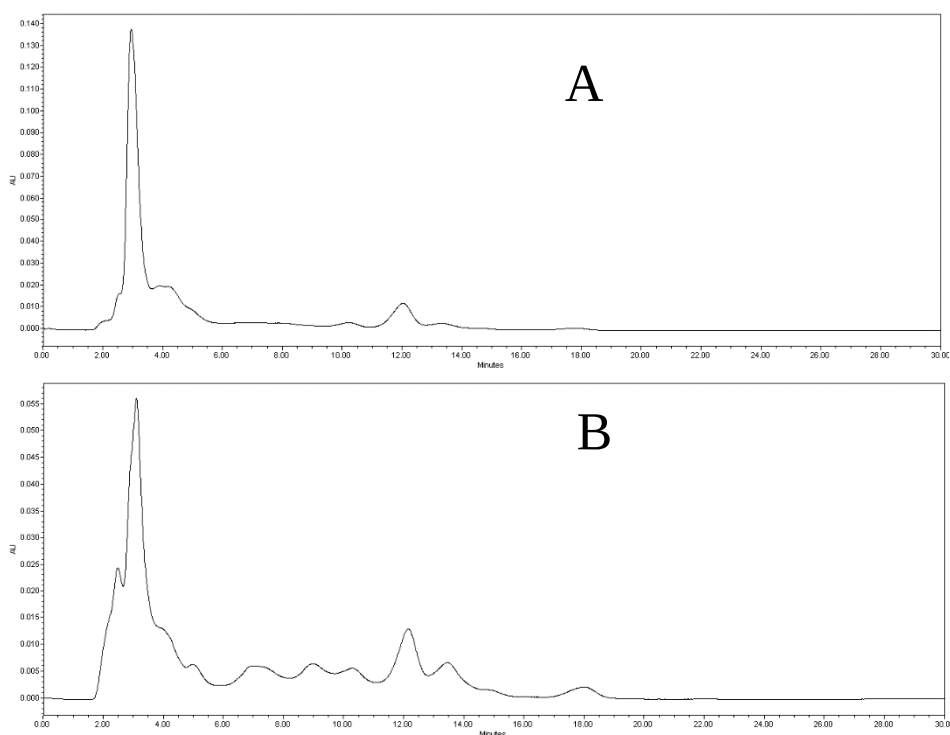
Since the nature of lipolysis yields three considerably different phases of liquid and the concentration range of THC found in them are also different, analytical assays to determine THC in each of these phases are different. The method was partially validated for specificity, sensitivity, linearity, accuracy and precision.

Specificity

The selectivity of the method for THC was studied by comparing the chromatographies of lipolysis samples of the digestion of 160 μL sesame oil without dissolved THC. Figure 3-3 shows a representative chromatography from the analysis of blank micellar, sediment and lipid phases using the assay described above. In all cases, the baseline peak minimally interfere with the elution of THC and IS at 220 nm, which eluted at 6.2 and 28 minutes respectively blank micellar, sediment and lipid phase samples.

Linearity

The linearity of the method was assessed using the calibration curves for quantification of lipolysis samples, and the correlation coefficient (r^2) values were calculated for each curve. The linearity of the assay was from LLOQ to 100, 200, and 4000 $\mu\text{g/mL}$ for micellar, sediment and lipid phases respectively. The linear regression parameters for each phase of lipolysis media is shown in Table 3-1. The calculated regression coefficients were all above 0.99 over the relevant concentration ranges for each of the phases, so the assays were deemed appropriate for the detection of THC.



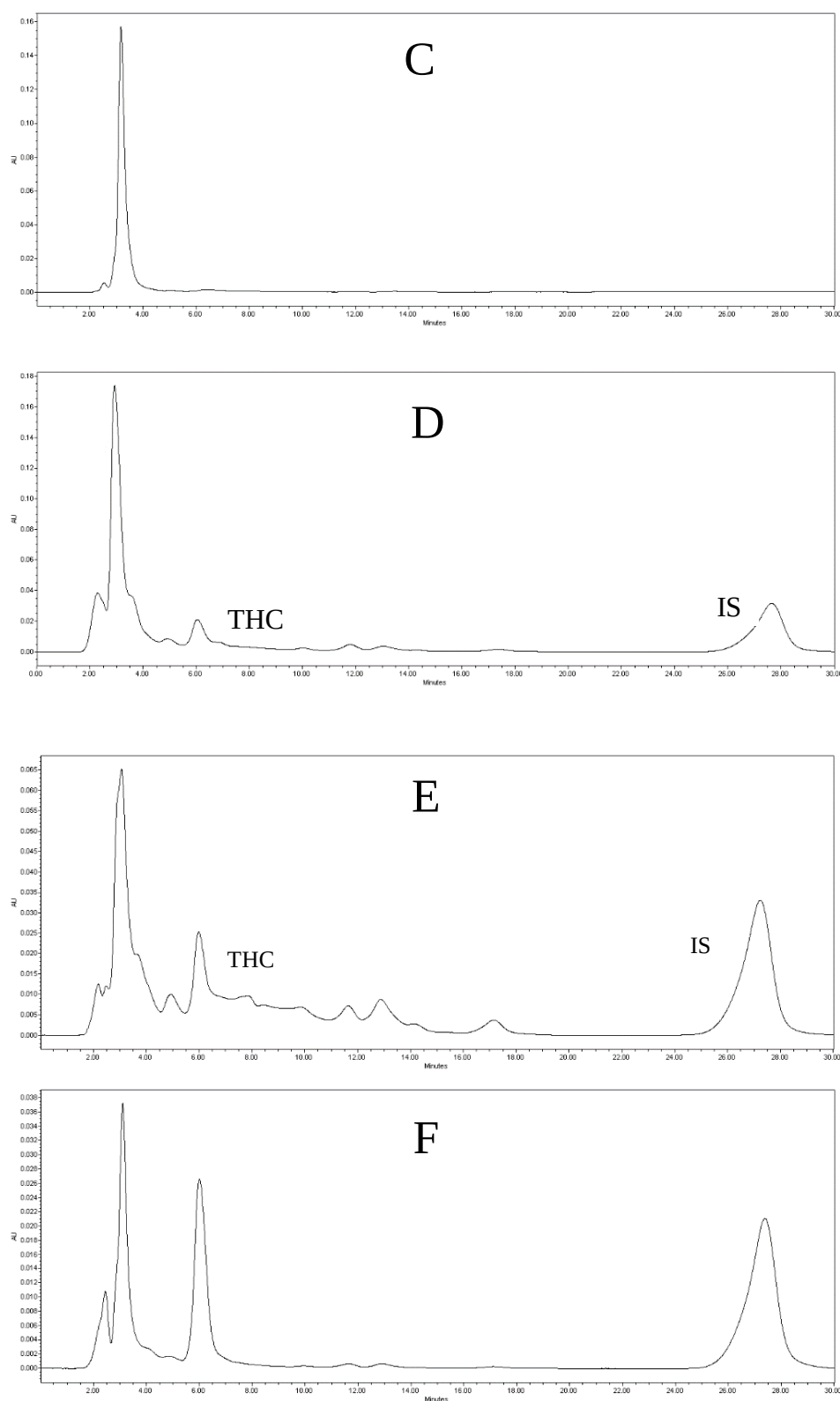


Figure 3-3. Representative chromatographies of HPLC-UV analysis of blank micellar (A), sediment (B) and lipid (C) phases with spiked with THC and IS (D, E and F for micellar, sediment and lipid phases respectively) detected at 220 nm. THC was found to elute at 6.2 minutes, and IS at 28 minutes.

Table 3-1. Linear regression parameters for the HPLC-UV method to determine THC in lipolysis media.

Phase	Equation	Regression Coefficient	Linear Range
Micellar	$y = 30.848x - 3.5443$	0.9987	1 – 100 µg/mL
Sediment	$y = 25.232x - 4.1163$	0.9930	1 – 200 µg/mL
Lipid	$y = 248.26x - 34.215$	0.9956	20 – 4000 µg/mL

Sensitivity

The lower limit of quantification (LLOQ) for THC was the lowest tested concentration of spiked sample that was considered precise and accurate (relative standard deviation and relative error within 20%) for intra-day analysis. The validated LLOQ for micellar and sediment phases were 1 µg/mL, and 20 µg/mL for lipid phase.

Accuracy and Precision

The inter- and intra-day accuracy and precision is shown in Table 3-2. The obtained values for the three quality control levels all fall within the acceptable range of 15%, so the assay is deemed sufficiently accurate and precise for the detection of THC in the three phases of lipolysis media.

Table 3-2. Intra- and Inter-day variability for the HPLC-UV method to determine THC in lipolysis media.

		THC (µg/mL)	Intra-day		Inter-day	
			Precision (RSD %)	Accuracy (RE %)	Precision (RSD %)	Accuracy (RE %)
Micellar Phase	Low	1	7.53	9.26	10.77	12.94
	Mid	10	1.48	3.87	5.50	9.25
	High	100	2.21	0.74	2.00	2.14
Sediment Phase	Low	1	11.30	6.97	12.07	10.73
	Mid	50	1.35	1.74	5.14	7.62
	High	200	1.66	2.82	3.57	3.16
Lipid Phase	Low	20	2.94	3.79	9.42	5.80
	Mid	500	1.23	7.04	3.35	1.72
	High	4000	0.27	3.69	0.93	1.52

As of the time of writing, this is the first time that the accuracy, precision and linearity of THC was assessed in three different phases of lipolysis media.

3.2.3 Development and Validation of HPLC-UV assay for determination of THC in rat plasma

In order to perform pharmacokinetic studies on THC, its concentration needs to be accurately and precisely determined in rat plasma. Analytical techniques therefore need to be used to do that, and there are a few published assays for the determination of THC. The early methods used radioactivity assays [121, 128, 189] but mostly used mass spectrometry as their method of detection [181, 183, 184, 190, 191]. The lower limits of quantification of these assays were of the range of 5 – 10 ng/mL [143, 192].

An LC-MS/MS method was initially attempted to determine THC in rat plasma and the lower limit of quantification (LLOQ) was assessed and compared with HPLC-UV. An Agilent 1100 series HPLC system linked to a Waters Micromass Quattro Ultra mass spectrometer was used, and a similar sample preparation protocol to lipolysis media was performed on plasma. The initial LC-MS injection showed the most prominent daughter ion had a mass/charge ratio of 241, and was subsequently selected as the ion for monitoring. Figure 3-4 overleaf shows the chromatography obtained from this run, detecting THC at 2 ng/mL. The chromatography shows that sufficient signal was obtained for THC at a concentration of 2 ng/mL, which was the lowest concentration tested and is in a similar sensitivity range to other LC-MS/MS assays that detect THC [181, 190]. Due to equipment availability and cost however, LC/MS/MS was not pursued further as a detection method for THC in rat plasma in this PhD. HPLC-UV was instead pursued as the more cost-effective option of detection, using a similar sample preparation process.

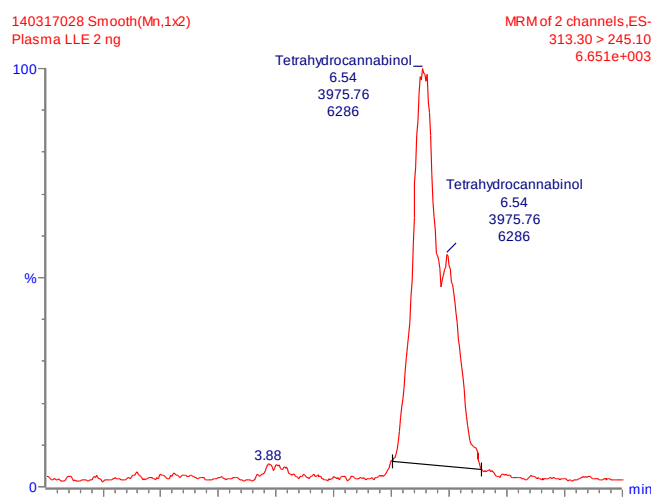


Figure 3-4. Chromatography of the daughter ion of THC obtained using LC-MS/MS.

HPLC-UV is a more cost-effective method of detection compared to LC-MS/MS. The assay described in section 2.3.3 was fully validated in accordance with FDA guidelines and was published in the Journal of Pharmaceutical and Biomedical Analysis [163, 193].

3.2.4 Method Development

Initially, the lipolysis HPLC-UV sample preparation method for THC (using THF and hexane) was attempted for plasma sample preparation, but the chromatography resulting from this method showed interference between background plasma and THC. THF was also not efficient in precipitating proteins, resulting in the blockage of a guard column. Different protein precipitating solvents were therefore attempted, and acetonitrile was found to be most effective [194]. Acetonitrile was also efficient in the extraction of lipophilic compounds such as THC [195-197]. Additional dilution of plasma with water resulted in a cleaner chromatography baseline, possibly because of improved retention of hydrophilic contaminants in water. Using hexane in the liquid-liquid extraction stage continued to be effective at separating THC from potential contaminants from plasma.

A number of different HPLC-UV columns were tested for their ability to separate THC from plasma background peaks, including a Phenomenex Luna C18 (2) 2.1 x 100 mm, 2 µm column,

an ACE Super C18 4.6 x 100 mm, 5 μ m column, and finally the ACE C18-PFP 4.6 x 150 mm, 3 μ m column, which gave the best separation of peaks for plasma.

Different IS were also tested for suitability to this assay, including dexamethasone, bifonazole, testosterone, benz[a]pyrene, cholecalciferol (vitamin D), α -tocopherol (vitamin E), probucol and DDT. The elution times for these compounds under the chromatographic conditions were found to be either too short so they eluted before THC (dexamethasone, bifonazole, testosterone, benz[a]pyrene), or too long that the total runtime was impractical (probucol, vitamin D, vitamin E). DDT was chosen as the most appropriate IS, but it initially overlapped with the eluting peak of THC in more hydrophobic mobile phase conditions. Reduction of the mobile phase composition to 62:38 acetonitrile:water (v/v) successfully separated the two peaks from each other, and from background plasma peaks.

It was possible to use a simple buffer-free mobile phase, which means this method would be easily compatible with LC/MS analyses. Column temperature and flow rate were set and optimised to give the final conditions described in this section.

3.2.5 Choice and preparation of standard solutions and quality control samples

Standard solutions of THC and IS (DDT) were prepared in acetonitrile at concentrations of 1.32 and 0.5 mg/mL respectively, and stored at -20 °C. Working standard solutions of 200 and 50 μ g/mL were prepared by appropriate dilution in acetonitrile and were also stored at -20 °C. Calibration curves and quality control samples were also prepared with CBD, the detection of which was also validated with this method, in collaboration with Atheer Zgair.

The working solution of THC was diluted in acetonitrile immediately before the preparation of calibration curves, to 0.15, 0.2, 0.3, 0.4, 1, 2, 4, 10, 20, 100 and 200 μ g/mL. These were spiked into plasma by adding 7.5 μ L (and also 7.5 μ L of CBD stock solution) into 135 μ L of rat plasma, to make up to 150 μ L total.

Independently, THC stock solutions of 0.2, 0.4, 2, and 20 µg/mL were used to prepare LLOQ, low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples, respectively. These samples were then prepared as described in section 2.3.3.3.

3.2.6 Assay Validation

Selectivity

The selectivity of the method was assessed by comparing the chromatograms of purchased blank rat plasma from different batches with THC at the LLOQ, and also with plasma samples obtained following intravenous (IV) administration of THC. Figure 3-5 overleaf shows blank chromatographies of rat plasma, where background peaks did not interfere with the detection of THC. This was also confirmed with plasma taken from rats in a subsequent intravenous pharmacokinetic study.

Accuracy and Precision

Accuracy and precision was expressed as RE and RSD respectively. Inter-day accuracy and precision was determined by analysing six replicates of rat plasma samples spiked with THC at concentrations of 20 (LQC), 100 (MQC) and 1000 ng/mL (HQC). RE and RSD values of inter- and intraday accuracy precision are shown in Table 3-3. All values obtained were within acceptable limits (RE within $\pm 15\%$ and $RSD \leq 15\%$ respectively).

Table 3-3. Intra- and Inter-day accuracy and precision values for detection of THC in rat plasma.

Level	Intra-day (n=6)		Inter-day (n=6)	
	RSD%	RE%	RSD%	RE%
LLOQ (10 ng/mL)	16.00	-15.87	-	-
LQC (20 ng/mL)	14.33	5.17	8.25	6.48
MQC (100 ng/mL)	4.97	3.13	4.62	3.23
HQC (1000 ng/mL)	3.54	1.81	3.25	-0.91

RSD, relative standard deviation, RE, relative error, LLOQ, lower limit of quantification, LQC, MQC, HQC, lower, medium and high quality controls respectively.

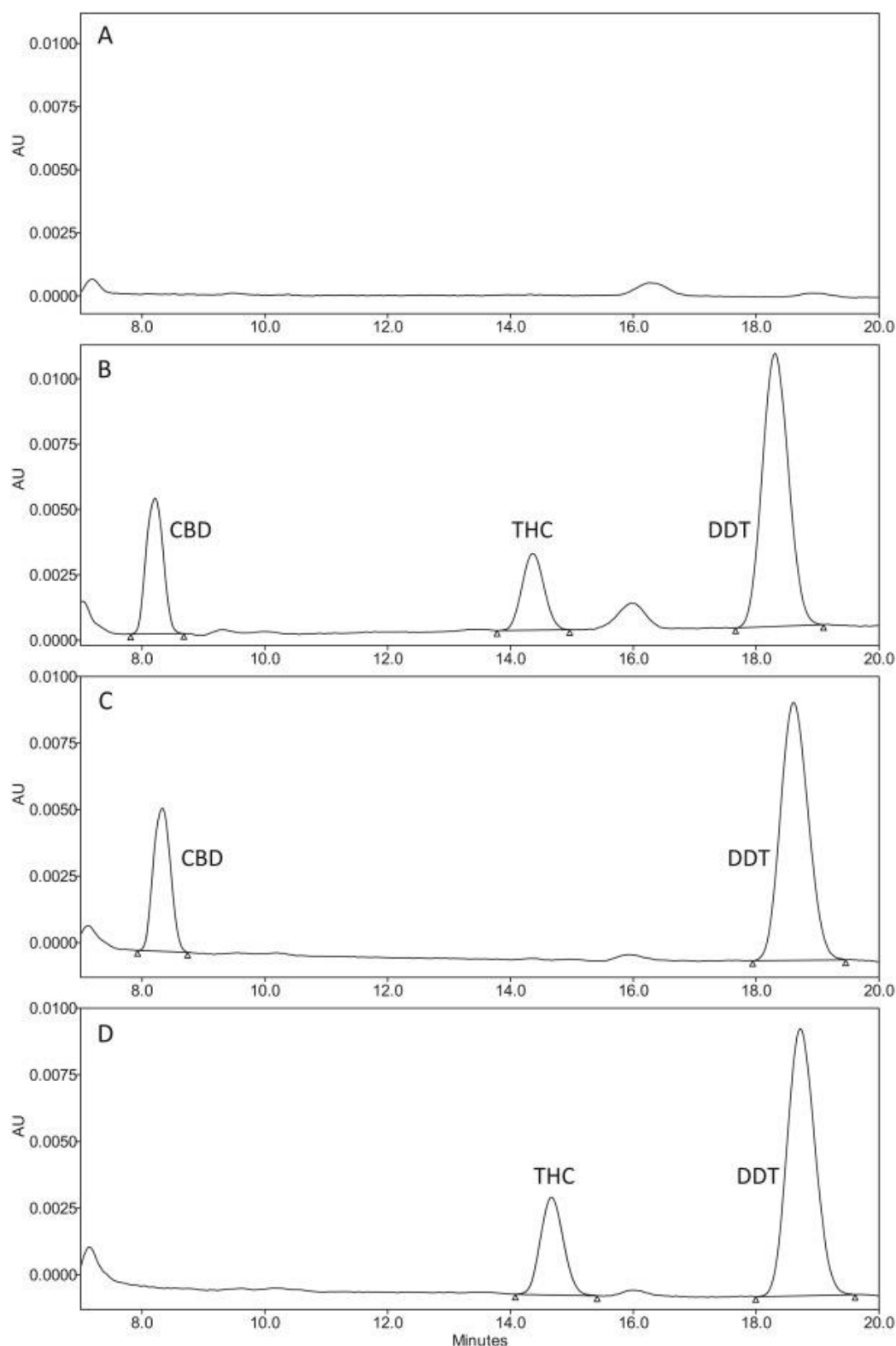


Figure 3-5. Representative chromatography of blank rat plasma (A), plasma spiked with cannabidiol (CBD) and Δ^9 -tetrahydrocannabinol (THC) at concentrations of 1,000 ng/mL in addition to DDT (B); plasma isolated from one rat given intravenous CBD in a pharmacokinetic study (C); and plasma isolated from one rat given intravenous THC (D), all absorbance was detected at $\lambda = 220$ nm.

Sensitivity

The validated LLOQ for THC was the lowest tested concentration of spiked plasma with sufficient accuracy and precision (RE and RSD below 20%) for intra-day analysis [163, 198, 199]. This concentration was 10 ng/mL, and the RE and RSD values are also shown in Table 3-3.

Recovery

To assess the extraction efficiency of the sample preparation process, the recovery of THC was determined by comparing the peak area of an extracted sample with stock solutions of the same concentration (using the LQC, MQC and HQC sample concentrations, n=5 for each level) [163]. The recovery of the IS was determined in a similar fashion, at the concentration used for sample preparation. The recovery of THC in plasma was $94.6 \pm 1.9\%$, $86.7 \pm 1.0\%$, and $93.1 \pm 1.3\%$, at the LQC, MQC and HQC concentrations, respectively. The recovery of IS was $74.6 \pm 0.7\%$. The recovery values reflect well on the high sensitivity of the assay.

Stability

The stability of THC in rat plasma was assessed under the following conditions: Freeze-thaw stability (3 cycles from $-80\text{ }^{\circ}\text{C}$ to room temperature), room temperature stability (6 hours), short-term stability (24 hours at $-80\text{ }^{\circ}\text{C}$), long term stability (30 and 60 days at $-80\text{ }^{\circ}\text{C}$) was performed at LQC, MQC and HQC (n=6 for each level). Also, autosampler stability of processed samples (16 hours at $4\text{ }^{\circ}\text{C}$) was performed at LQC, MQC and HQC (n=6 for each level). Samples were deemed stable if accuracy and precision values were within acceptable limits ($\text{RE} \pm 15\%$ and $\text{RSD} \leq 15\%$ respectively).

The stability of THC in different conditions at LQC, MQC and HQC are shown in Table 3-4. The values indicate that THC was stable in rat plasma after freeze-thaw cycles, room temperature, short-term storage and long-term storage. THC in processed samples was also

stable in the HPLC-UV autosampler. These results are consistent with previous stability results obtained for THC [129, 144] and also provides additional stability measures (room temperature, short- and long-term stability of THC in rat plasma were not previously studied), and prove that THC is stable in all the aforementioned conditions.

Linearity

The linearity of the assay was also confirmed over the concentration range of 10 – 10000 ng/mL based on 10 concentration levels with correlation coefficient (r^2) values ≥ 0.9996 in calibration curves.

Table 3-4. Stability values for detection of THC in rat plasma.

	Short Term (-80 °C, 24 h)		Long Term (-80 °C, 30 days)		Long Term (-80 °C, 60 days)	
Level	RSD%	RE%	RSD%	RE%	RSD%	RE%
LQC	14.9	-12.7	9.4	9.6	11.04	-5.98
MQC	6.8	-8.9	6.1	-12.2	9.25	11.6
HQC	2.1	-0.6	2.4	-12.3	3.26	-13
	Freeze-thaw (3 Cycles)		Room Temperature (25 °C, 6 h)		Autosampler (4 °C, 16 h)	
Level	RSD%	RE%	RSD%	RE%	RSD%	RE%
LQC	12.1	14.5	7.2	-14.5	9.9	-12.6
MQC	6.1	-11.1	6.2	-12.6	7.7	-1.8
HQC	3.9	-3.9	2.9	-5.9	1.5	1.0

h, hour, **RSD**, relative standard deviation, **RE**, relative error, **LLOQ**, lower limit of quantification, **LQC**, **MQC**, **HQC**, lower, medium and high quality controls respectively.

Compared to other published analytical assays, Valiveti and Stinchcomb [192] were able to produce a lower LLOQ than the one in this assay (5 ng/mL), but using mass spectrometry as the method of detection, and had a longer run time per sample (30 minutes). Therefore, this fully validated assay is able to detect THC with a similar sensitivity to other LC-MS methods, and can do so in a more time and cost-efficient way [144, 178, 183, 184, 190].

3.2.7 Application in Pharmacokinetic studies

This assay was used in three pharmacokinetic studies, one of which was published as a research paper, which I contributed more to the development and validation of the assay pertaining to THC [193]. The first application of this assay was a preliminary study where THC was administered intravenously. The obtained pharmacokinetic profile is shown in Figure 3-6 below. Since this was a demonstration of the use of the assay and a preliminary study, the most appropriate time-points for an intravenous pharmacokinetic study were not previously known; therefore only two rats were used.

This assay was also successfully used in further intravenous and oral pharmacokinetic studies described in Chapter 5.

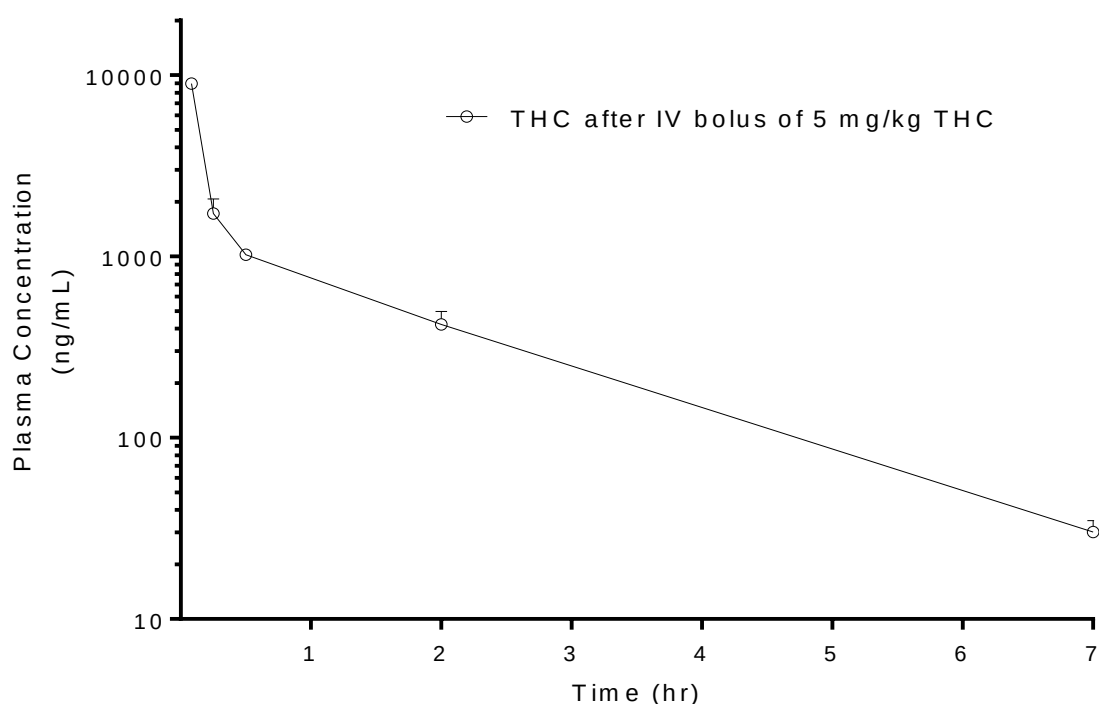


Figure 3-6. Plasma concentration-time profiles (means $\pm$ SEM) following single IV bolus administration (5 mg/kg) of Δ^9 -tetrahydrocannabinol (THC) to rats ($n = 2$).

3.3 Optimisation of the PBMC proliferation ELISA

Immunosuppressive effects of cannabinoids have been shown *in vitro* [135, 200], but observations in animals and humans have not been consistent with these findings [155, 201, 202]. There are a large variety of different methods that can be used to assess the immunosuppressive effect of a compound such as THC. These methods can be categorised into two large types: *in vitro* and *in vivo* assays. Previous *in vitro* studies on THC have used cell proliferation assays [112, 145], cell viability assays [119], cytokine assays [134, 146, 147], Polymerase Chain Reaction (PCR) [150, 200, 203] and microscope immunohistochemical analysis [119]. These assays can provide insights into how THC affects different cell processes, and at what concentrations these processes are affected. However, many of these assays are costly and require specialised apparatus not easily available.

In vivo studies to assess the immunosuppressive effect of THC have included testing the immune function of animals against viral infection by HSV2 [136, 148, 204], bacterial infection with *Listeria monocytogenes* or *Legionella pneumophila* [146, 148], or fungal infection with *Candida albicans* [151]. These studies monitored mortality, pathogen load and/or serum levels of cytokines after acute or chronic THC treatment.

Proliferation assays have been used to find the concentration at which THC significantly inhibits the mitogen response in lymphocytes. A common response to the activation of white blood cells is to proliferate in order to mount an effective immune response [205], and a proliferation assay is an essential first step in assessing the antiproliferative effect of a compound. Previous proliferation assay work has shown that THC is able to suppress proliferation of T cells derived from mouse spleen at 10 µg/mL [125], and also able to suppress proliferation of mouse thymus and lymph node cell cultures at 10 µg/mL [206]. Therefore, this was the initial maximal level used in this assay.

3.3.1 Development of Method

Since this method had not previously been performed for the analysis of PBMC at the University of Nottingham, and required optimisation to be effective. First, the initial density of cells to be seeded into 96-well plates was higher than the one stated in the manufacturer's instructions (the recommended cell number was $1 - 40 \times 10^4$ cells). A higher number of cells were required in order to give sufficient absorbance readings, used as an indicator of cell proliferation. This sensitivity issue was resolved when 50000 cells were seeded in each well. Second, a mitogen had to be chosen to stimulate proliferation. Two different mitogens were trialled to stimulate cell proliferation, PMA and Io used in concert, at 50 ng/mL and 1 mM respectively and PHA at 10 µg/mL. PMA and Io are often used in research in conjunction to stimulate the production of Interleukin 2 and 4 (IL-2 and IL-4), and subsequently the mitosis of T cells [205]. However, PMA and Io did not stimulate sufficient cell growth to produce consistent results in absorbance readings, and showed significant differences between negative control and DMSO vehicle treatment, as shown in Figure 3-7. DMSO has no previously published effects on cell proliferation at 0.1% v/v, and the reasons for these differences in proliferation could not be identified.

PHA was the second mitogen used to stimulate proliferation. PHA acts to stimulate the proliferation of T-lymphocytes, and used in previous studies to studying the role of THC in apoptosis [207, 208] and was also used in previous proliferation studies using THC [125, 206]. PHA produced more consistent experimental stimulation of PBMC stimulation, and did not show any difference in absorbance between negative and vehicle treatment (Figure 3-7), and thus was chosen as the mitogen for subsequent PBMC studies in both healthy and diseased states, which are discussed in detail in Chapter 6.

The effect of two other vehicles was also tested – the artificial lipid emulsion Intralipid® and isolated chylomicron emulsion, since their association was tested by another method in this PhD, and THC in the lymphatic system would likely be contained within chylomicrons. These different vehicles were also tested for toxicity on PBMC, as shown in Figure 3-8.

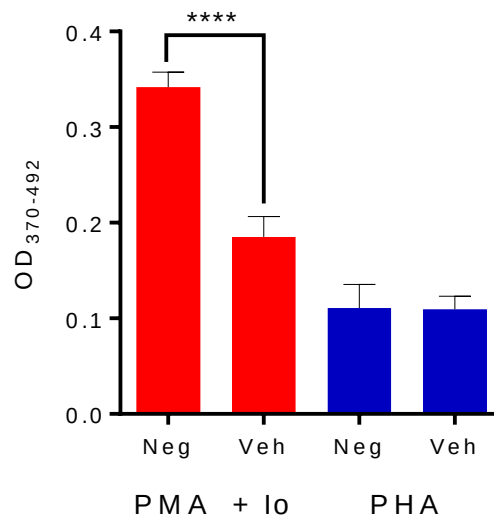


Figure 3-7. The immunosuppressive effect of DMSO Vehicle (0.1%) on the proliferation of mitogen-activated peripheral blood mononuclear cells (PBMC). PBMC from healthy human volunteers was exposed to DMSO for 30 minutes before addition of phorbol myristate acetate 50 ng/mL and ionomycin 1 μ M (PMA+Io, Red) or phytohaemagglutinin 10 μ g/mL (PHA). Cell proliferation was measured after two days using an ELISA. $n=4$, brackets indicate significance using one-way ANOVA, ****= $p<0.0001$.

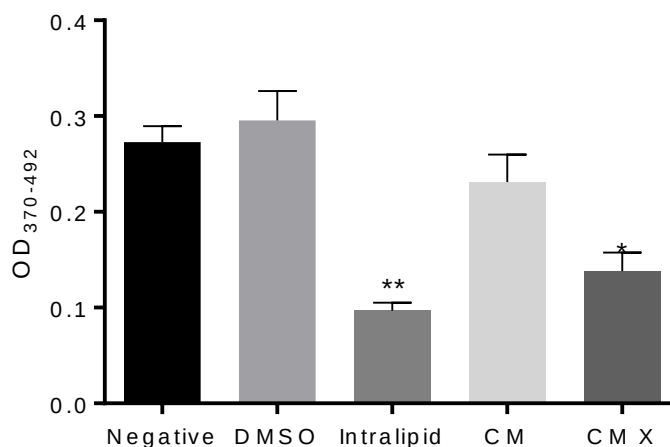


Figure 3-8. The effect of different vehicles on proliferation of peripheral blood mononuclear cells (PBMC). Chylomicrons were isolated from the blood of healthy volunteers and were diluted to a standard triglyceride concentration (1 mg/mL), and Intralipid was similarly diluted. The resulting emulsions were then added to PBMC by spiking in nutrient medium, and incubated for 30 minutes. CM X represents the use of one individual's chylomicrons on the PBMC of the other. DMSO was used at a final concentration of 0.1% v/v. PBMC were stimulated by addition of a mitogen (phytohaemagglutinin 10 μ g/mL) for two days. After this time the number of viable cells was assessed by ELISA, **= $p<0.01$, *= $p<0.05$ one-way ANOVA compared to negative control (no treatment but mitogen stimulated).

Intralipid® was significantly toxic to cells, most possibly due to the presence of glycerol, which has been previously shown to be cytotoxic in other cell types [209, 210]. An interesting observation from this experiment was that there was reduced proliferation when CM isolated from one individual was used on another individuals PBMC (shown as CM X on Figure 3-8). This could be the PBMC of that individual reacting to the foreign apolipoproteins contained within the CM, or other components separated by the process of CM isolation. As a result of this finding, subsequent PBMC studies using CM as a vehicle for THC were isolated from the same individual.

Chapter 4: Intraluminal processing of delta-9-tetrahydrocannabinol in lipidic formulation dispersed in simulated intestinal fluid

4.1 Introduction

The first step to study the intestinal lymphatic transport potential of THC is its processing in the intestinal lumen. The first barriers of intestinal absorption are solubilisation in the intestinal lumen and diffusion across the unstirred water layer. Since THC is a lipophilic cannabinoid and is soluble in oils, it belongs to the category of drugs with low solubility and high permeability, or the Biopharmaceutical Drug Class 2 as described by Amidon *et al* in his theoretical basis for the Biopharmaceutics Classification System (BCS) [211]. Many new drug candidates fall into BCS Class 2, and the *in vivo* absorption of drugs in this category are correlated to their intraluminal processing, in addition to metabolism and influx and efflux transporters [212]. Therefore, the development of drug delivery systems to improve the solubility of BCS Class 2 drugs, would also significantly improve their bioavailability [213]. A common drug delivery system used to do this are the lipid-based drug delivery systems (LBDDS).

LBDDS are largely categorised into four types – I, II, IIIA and IIIB, and IV [214]. Type I LBDDS consists of oils in the form of long- or medium-chain triglycerides (LCT or MCT). A typical Type 1 LBDDS is the one used in Marinol®, which contains sesame oil. A major advantage of this LBDDS type is that they are simple, biologically compatible, and easily inserted in capsules. However, the lipidic nature of Type I LBDDS makes it only suitable for highly lipophilic drugs. Type I LBDDS also require lipid digestion to form emulsions of drug in solution and the initial droplet size of these emulsions formed is large, in the range of 10 µm in diameter [215]. Pancreatic lipase/co-lipase acts at this oil/water interface to digest triglycerides into fatty acids and monoacylglycerols. Other types of LBDDS are more complex, contain hydrophilic cosolvents and form droplets of smaller sizes [216].

Classical *in vitro* techniques that assess drug dissolution such as the USP 1 Rotating Basket apparatus and the USP 2 Rotating Paddle apparatus are effective in comparing conventional oral dosage forms, but do not take into account lipid digestion and emulsification that the different types of LBDDS offer. This is particularly important for LBDDS that require digestion, which includes most Type I and some Type II and III formulations. Digestion models were therefore developed to mimic lipid emulsification and digestion and assess the fate of drugs in LBDDS. One such model is *in vitro* lipolysis. This model simulates the intestinal digestion of lipids, and the fate of a drug in this model is a good predictor of that drug's performance *in vivo* [217]. In this model, a lipid-based formulation is dispersed in a digestion medium mimicking intestinal fasted conditions, containing bile salts and phospholipids (shown in Chapter 2, Figure 2-1). Then, a lipase (usually pancreatic lipase) is added to initiate digestion. The breakdown of triglycerides by lipase releases fatty acids, which allows tracking of reaction progress by monitoring decreases in pH and titrating them using a base. Colloidal structures are also formed as a result of digestion, and lipophilic drugs are usually distributed to these structures before their absorption into the enterocyte *in vivo* [218].

After completion of lipolysis, the reaction mixture is separated by ultracentrifugation into three visually distinct phases – the upper lipid phase, the middle micellar aqueous phase, and lower sediment phase. Drug content is then determined in each of these phases, and represents the likely fate of the drug in the small intestine. Drug found in the micellar aqueous phase has associated with the colloidal structures formed as a result of lipid digestion, and is assumed to be readily available for absorption into the enterocyte, whereas drug found in the sediment or lipid phases are assumed to have no or delayed absorption [214].

Marinol® is contained in a 1 mL oil capsule containing sesame oil currently available in doses of 2.5, 5 and 10 mg. Marinol® contains sesame oil and no surfactants, which makes it a Type

I LBDDS and ideal for analysis using the lipolysis model. Despite the reported oral bioavailability of 10-20%, the fraction of THC available for absorption in the small intestine is currently unknown [122]. In order to assess the contribution of lymphatic transport to the absorption of THC, it is necessary to determine the fraction of THC available for absorption after lipid digestion under these *in vitro* experimental conditions, and which dose maximises the amount of THC available. To mimic the *in vivo* scenario, the doses that are assessed in the model will be the available doses stated above, in addition to two higher doses (20 and 40 mg/mL). The volume of oil used as formulation will be scaled down proportionally to the volume of the *in vitro* lipolysis reaction mixture.

4.2 Aims

The aim of this study is to:

1. Assess the intraluminal processing of THC in a Type I LBDDS using an *in vitro* lipolysis model, and determine the fraction of THC available for absorption after lipid digestion in the experimental conditions of the model.
2. Determine the dose of THC in LCT formulation that allows the highest fraction in the aqueous micellar phase while losing the least in the undigested lipid phase.

4.3 Materials and Methods

The materials and methods used for this work are described in Chapter 2, Section 2.1.1 and 2.3.1. The development and optimisation of the lipolysis model was outlined in Chapter 3, Section 3.1.

4.4 Results

4.4.1 Distribution of THC in lipolysis media

The distribution of THC in the three phases of lipolysis media in different doses is shown in Figure 4-1 overleaf. At 2.5 mg/mL, 59.5 ± 6.8 % of THC was found in the micellar phase. This decreased to 46.4 ± 6.2 % at 5 mg/mL and 48.6 ± 2.0 % at 10 mg/mL. When the dose was increased to 20 and 40 mg/mL, the fraction in the micellar phase decreased further. This decrease of THC in micellar phase was accompanied by a concomitant increase in the lipid phase, from 35.3 ± 6.2 % at the lowest to 62.9 ± 4.9 % at the highest dose. This suggests that THC transfers from being associated with the micelles in the micellar phase to re-associate with lipids in the lipid phase. The fraction of THC found in the sediment phase was similar across all doses, ranging from 5 to 10 %. The THC distribution in each of the three phases is shown in Figure 4-2 overleaf.

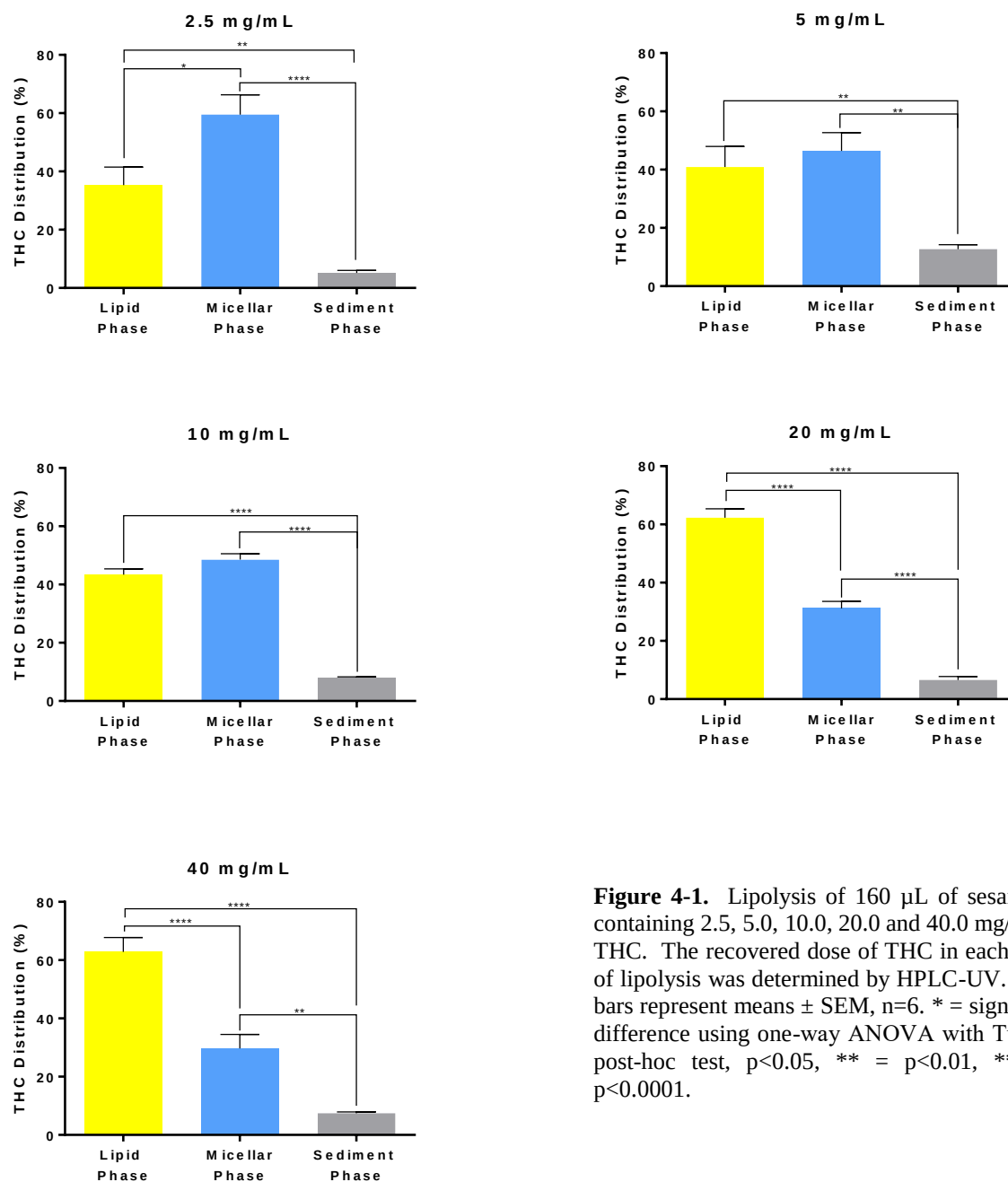


Figure 4-1. Lipolysis of 160 μ L of sesame oil containing 2.5, 5.0, 10.0, 20.0 and 40.0 mg/mL of THC. The recovered dose of THC in each phase of lipolysis was determined by HPLC-UV. Error bars represent means $\pm$ SEM, n=6. * = significant difference using one-way ANOVA with Tukey's post-hoc test, $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

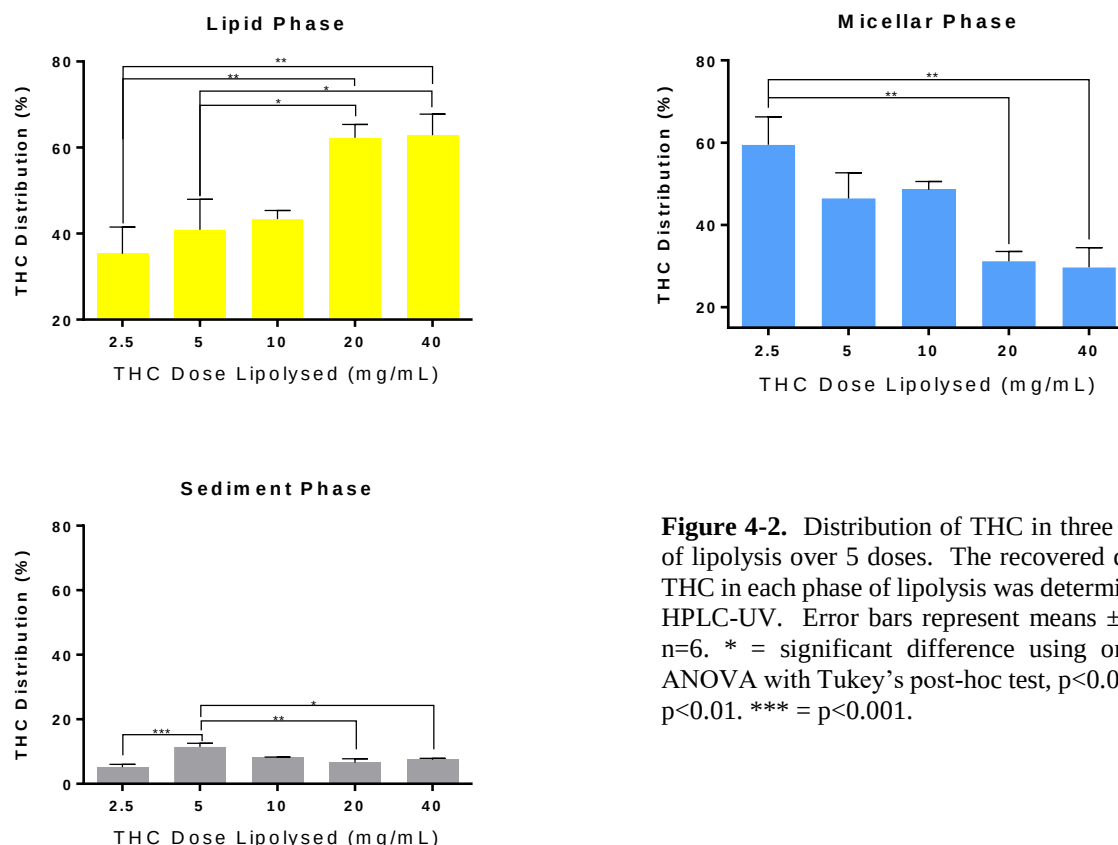


Figure 4-2. Distribution of THC in three phases of lipolysis over 5 doses. The recovered dose of THC in each phase of lipolysis was determined by HPLC-UV. Error bars represent means $\pm$ SEM, n=6. * = significant difference using one-way ANOVA with Tukey's post-hoc test, $p<0.05$, ** = $p<0.01$. *** = $p<0.001$.

4.5 Discussion

The distributions of THC at 5 and 10 mg/mL both show that 45 % of THC was found in the each of the micellar and lipid phases (totalling 90 % overall) and the remaining THC found in the sediment phase. Correspondingly, 20 and 40 mg/mL distributions both show 30 % in the micellar and 60 % in the lipid phase. It is important to reiterate that in the context of the *in vitro* lipolysis model, drug contained in the micellar phase is assumed to be readily available for absorption. As the name suggests, the micellar phase is where a variety of mixed micellar structures formed by the interaction of bile salts, triglycerides, liberated fatty acids and monoglycerides. Lipophilic drugs are also assumed to be associated within these micellar structures, making them available for diffusion across the unstirred water layer of the small intestine [32, 173, 176]. Unlike the digestion of MCT and SCT, lipolysis of LCT formulations usually generates an undigested lipid phase, because the pK_a of the fatty acids released are

higher than the set pH of titration. For sesame oil, these fatty acids are oleic acid and linoleic acid [219], which have pK_a values of 9.85 and 9.24 respectively [220]. As such, the lipid digestion of peanut oil (which is also majority oleic and linoleic acid) is typically only 12% complete at pH 6.8, compared to near-complete digestion of MCT formulations which typically do not have a lipid phase [161].

4.5.1 Comparison with other drugs

The intraluminal processing of THC when administered in an LCT formulation is similar to a number of other lipophilic drugs that have been analysed by *in vitro* lipolysis. A study by Yang *et al* assessed the fraction of Vitamin E, a drug that is more lipophilic than THC (a Log P value of 11.9) in LCT and MCT using an *in vitro* lipolysis model with added mouth and gastric digestion stages. Part of their study assessed the total amount of Vitamin E that was available to be absorbed after *in vitro* lipid digestion, and determined that 39% of total Vitamin E was available when LCT was used as the carrier oil, which is similar to the fraction found for THC [215].

A study by Dahan *et al* examined the intraluminal processing of two lipophilic drugs – dexamethasone and griseofulvin – by *in vitro* lipolysis, and performed an accompanying *in vivo* study to predict the bioavailability of these drugs based on their intestinal permeability. Dexamethasone had moderate aqueous solubility (100 $\mu\text{g/mL}$) whereas griseofulvin had much lower solubility (5 $\mu\text{g/mL}$). Both compounds have Log P values of 1.8, and are classed as BCS class 2 drugs. One gram of long, medium and short-chain triglyceride formulation containing 5 mg of each drug was used in the *in vitro* lipolysis and animal models. Using the *in vitro* lipolysis model, dexamethasone was highly solubilised in the micellar phase in all three formulations, and the highest fraction of griseofulvin was solubilised in the micellar phase when dissolved in medium-chain triglyceride formulation, then by long-chain and short-chain

triglycerides. The intraluminal processing of both drugs in the triglyceride formulations was positively correlated to the performance of those formulations *in vivo*, and the oral bioavailability of dexamethasone did not change significantly with formulation, whereas the same order of effectiveness (medium > long > short) was observed with griseofulvin. This suggests that the *in vitro* lipolysis model provided a good *in vitro-in vivo* correlation (IVIVC) between the performances of a formulation for BCS class 2 compounds, which could well be the case for THC [176].

Intestinal lymphatic transport may complicate the predictive power of the *in vitro* lipolysis model however. Another study by Dahan *et al* assessed the fraction of Vitamin D₃ and progesterone dissolved in peanut oil (a LCT), caprylic acid (a MCT) and triacetin (a SCT) in the micellar aqueous phase after *in vitro* lipolysis, and performed an accompanying *in vivo* oral bioavailability study using the same formulations. The experimental conditions of *in vitro* lipolysis in that study was almost identical to the ones performed and described in this chapter, with the exception of a different set pH for auto-titration (Dahan and colleagues used a set pH of 7.4), minimum rate of titrant addition and the volume of lipidic formulation used (1 gram of formulation was used). Vitamin D₃ has a similar lipophilicity of THC (Log P of 9.72), is poorly water soluble and is transported mainly in the lymphatic system [41], while progesterone has a lower lipophilicity (Log P of 4.04) but also has poor water solubility and significant pre-systemic metabolism in the wall the small intestine [221, 222]. The doses of Vitamin D₃ and progesterone were 5 mg/mL, which translate to 13 mM and 15.9 mM respectively and are similar (and almost identical in the case of progesterone considering it has the same molecular weight) in concentration to THC. Much like the study in 2005 by Dahan *et al*, the *in vitro* lipolysis portion of the study found MCT solubilised most of both drugs in the micellar phase, LCT was second best and SCT was third. In the *in vivo* study, the oral bioavailability of progesterone from the MCT formulation also was 4.1%, four-fold higher than

LCT (1.1%), and 11-fold higher than SCT formulation (0.33%), following the rank order obtained previously. However, the oral bioavailability of Vitamin D3 from LCT formulation was 40%, higher than MCT (30%) and SCT (20%). Upon repeating the animal study with co-administration of a lymphatic transport blocker cyclohexamide however, the MCT formulation out-performs the LCT. These results suggest that the *in vitro* lipolysis model may not be wholly predictive for drugs that benefit from intestinal lymphatic transport [176].

Based on these examples, mentioned, it would seem that the result obtained with THC is consistent with *in vitro* lipolysis drug assessment done previously, and the fraction of drug available for absorption in the micellar aqueous phase is similar to drugs that have similar lipophilicity. However, most studies mentioned only used one dose level of drug, and did not examine the effect of increasing drug dose on its distribution. The fraction available was also higher than the reported 10-20% bioavailability value previously stated for Marinol®, which suggest that other barriers to absorption are present that reduce bioavailability, such as hepatic metabolism and protein binding. Indeed, THC has considerable hepatic metabolism by cytochrome P450 enzymes and 95-99% protein binding when in plasma, which would provide substantial reductions in bioavailability [96, 121, 122].

One aim of this study was to determine the optimal dose of THC to use in subsequent *in vivo* work. Of the five doses studied here, 10 mg/mL is the optimal dose to use in subsequent work, since half of the total dose is solubilised in the micellar phase. At doses higher than 10 mg/mL, nearly 70% of the dose is lost in the lipid phase, for a modest increase in absolute amount of THC found in the micellar phase. This suggests that the micellar phase resulting from the mixture of digested lipids, phospholipids and bile salts is gradually becoming saturated.

4.5.2 Estimation of fraction available for absorption

In order to best estimate the fraction of THC available for absorption, the conditions by which lipolysis was conducted had to be as biologically relevant as possible. That meant controlling for factors such as lipid load, digestion buffer composition and pH conditions.

The volume of lipid used in this study was 160 μL . This is contrary to previous studies of the micellar solubilisation of a drug following *in vitro* lipolysis, which used lipid loads of 200, 500 and 1000 mg [160, 173]. Using 160 μL was to reflect one Marinol® capsule (which is assumed to be one 1 mL capsule containing sesame oil with dissolved THC). It is administered orally in the fasted state, taken with one glass of water. Assuming the approximate volume of liquid in the gastrointestinal tract is 250 mL [223], the proportional volume of sesame oil in the 40 mL lipolysis model reaction vessel would be:

$$\frac{1 \text{ mL Oil}}{250 \text{ mL Total Volume}} = \frac{x}{40 \text{ mL Reaction Vessel}} = 0.16 \text{ mL}$$

Previous *in vitro* lipolysis models have used a set pH of 7.4 [66, 173], which was set by Sek *et al* in 2002 to ensure fatty acid ionisation and efficient titration [175]. However, this did not fit the objectives set out for the current study for two reasons. First, the apparent ionisation constants of the major fatty acids in sesame oil are 9.85 (oleic acid) and 9.25 (linoleic acid), which means that the degree of ionisation at pH 7.4 would not have ensured additional ionisation of these fatty acids [220]. Second, a major objective of the current study is to simulate the conditions in the small intestine where lipid absorption takes place. The pH conditions of the first section of the small intestine in the rat have reported to be 6.8, making it a more appropriate set pH to use if subsequent animal studies will be done in rats, and the bioavailability of oral administration of THC is compared to the fraction of THC solubilised in the small intestine [71].

Using these proportions and calculations to perform the lipolysis experiment, approximately half of THC in a lipidic formulation is available for absorption at 2.5, 5 and 10 mg doses. Some of the drug delivery systems described in the introduction would likely be able to promote the formation of more mixed micelles in intestinal fluid, and improve the micellar phase content of THC. Types II, IIIA and IIIB LBDDS all contain triglycerides, but also have surfactants that would aid the formation of mixed micelles, providing more surface area for pancreatic lipase/colipase to work on to digest the formulation. In the context of the *in vitro* lipolysis model, this means that less THC would be in the lipid or sediment phases (if there was a lipid phase at all, there is also less triglycerides in a non-Type-I LBDDS) and a higher proportion solubilised in the aqueous phase [214]. Indeed, if medium-chain TG is used, a lipid phase might be completely absent since the fatty acids produced are soluble in aqueous media [160].

4.5.3 Limitations of the Study

In vitro lipolysis has its limitations – the major one being that the intestinal conditions being tested is the fasted intestinal state (in terms of bile salt and phospholipid osmolarity). Pancreatic juices are only secreted when stimulated by the presence of food in the duodenum, transitioning to the fed state. Different research groups have stated a number of different phospholipid and bile salt concentrations of the fed state of humans, ranging from 2-6 mM phospholipids and 5-15 mM bile salts [224]. This means there are 2-3 times more phospholipids and bile salts present in the intestinal lumen to emulsify food and lipids. This allows formation of more colloid and micellar structures for pancreatic lipase/colipase to bind. The intraluminal processing of a drug in this state may be different from its processing in the fasted state, usually increasing the capacity of the micellar phase for drugs to associate. However, the composition of phospholipids and bile salts in the fed intestinal state varies over time, and there are a number of different conditions used in the literature to simulate fed state intestinal fluid [225, 226]. There are many other potential lipolysis conditions to assess the

dissolution of THC in the fed state, but the only likely answer from this additional work is that more THC is found in the micellar phase due to increased solubilisation. It was therefore more relevant to perform lipolysis in the mimicked fasted state to allow more direct and robust comparisons with studies done on other lipophilic drugs, such as Vitamin D3 and dexamethasone described above.

A further complication is that there is not a uniformly accepted buffer composition for the performance of lipolysis, although some effort has been invested to attempt to standardise these experimental conditions for evaluation of LBDDS [227].

Another drawback of *in vitro* lipolysis is that the results from experiments are only indicative of a drug's dissolution in intestinal fluid, and only takes into account the intestinal portion of lipid digestion. Even though pancreatic lipase accounts for approximately 80% of the lipase activity in the body, some lipid digestion also takes place in the stomach [216]. *In vitro* lipolysis is also only simulating one step in the process of absorption, and at best only gives an approximation of dissolution, requiring the use of other studies to give permeability data.

4.6 Conclusions

The *in vitro* lipolysis model showed that up to 45 % of THC in a lipidic formulation is dissolved in mixed micelles under these experimental conditions. As the dose of THC increased, the fraction of drug found in the micellar phase transferred to the lipid phase, and the optimal concentration of THC in lipidic formulation assessed in this study was 10 mg/mL.

Chapter 5: Intestinal Lymphatic Transport, Pharmacokinetics and Biodistribution of delta-9-tetrahydrocannabinol.

5.1 Introduction

After assessing the intraluminal processing of THC in simulated intestinal fluid and the optimum dose for use in *in vivo* studies, the next step is to assess the lymphatic transport of THC. As mentioned previously, lipophilic drugs can access the lymphatic system by binding to lipid-rich lipoproteins called chylomicrons (CM) that move through the mesenteric lymphatic duct, joining general circulation at the external jugular vein.

Interactions of lipophilic drugs with other classes of lipoproteins have also been shown to affect their pharmacokinetics in the body [228]. Drugs such as ciclosporin, halofantrine and amphotericin B have been shown previously to bind to plasma lipoproteins, and this binding has changed their pharmacokinetics, tissue distribution and pharmacological effect [229-232]. In the case of amphotericin B, interaction with lipoproteins protected against the toxic effects of the drug on red blood cells, while maintaining its intended effect of targeting fungal infections [229]. In the case of halofantrine, binding to triglyceride-rich plasma lipoproteins increased its peak plasma concentration, which may have contributed to cardiac toxicity [231, 233]. THC is a drug that has well-documented immunosuppressive effects, and is highly bound to plasma proteins and fatty tissue due to its lipophilic nature [118, 234, 235].

If THC were to be transported via the intestinal lymphatic system, it would be associated with CM. After being synthesised in the enterocyte and entering the lacteal, CM drain through the lymphatic system to enter the mesenteric duct and thoracic duct, entering the general circulation at the right external subclavian vein. From there, CM interface with High Density Lipoproteins (HDL) to exchange apolipoprotein C2 and E for cholesterol [39]. Apolipoprotein C2 is a co-enzyme for lipoprotein lipase (LpL), an enzyme found on cell surfaces which is responsible for the liberation of fatty acids (and associated drugs like THC) from CM [45, 47].

Apolipoprotein E allows the binding of CM to specific receptors located on LDL and in the liver, allowing cholesterol exchange and eventual breakdown of CM [46, 48, 49, 236].

Research on the pharmacokinetics of THC in rats has been sparse. The available literature are from the 1980s or earlier and two recent studies conducted by Valiveti *et al* [184, 192]. Kreuz and Axelrod injected THC subcutaneously to rats for 1 to 26 days, and assessed the distribution in tissues with single and repeated administration. In this study the half-life of THC was 5 days, and was detected in the lung, liver and brain, but accumulated the most in fat, where it persisted two weeks after a single administration [237]. Levy *et al* studied the interaction between delta-1-THC and cannabidiol after intravenous administration to rats. Neither cannabinoid interfered with the elimination of the other, but pharmacokinetic parameters based on the plasma drug concentration-time profile were not calculated [238]. Alozie *et al* studied the penetration and distribution of THC, cannabinol and cannabidiol in the rat brain after intravenous administration, but also did not calculate derived pharmacokinetic parameters on the plasma drug concentration-time profiles obtained [189].

A study by Valiveti *et al* compared intranasal to intravenous administration of THC, and performed compartmental analysis to predict plasma concentrations for intravenous and intranasal dosing. The profile of the intravenous route fits a two-compartment model, and the intranasal route fits a one-compartment model with absorption [184]. Derived pharmacokinetic parameters for the other drug profiles in the studies mentioned above were very variable, most likely because of the low number of animals used.

To determine the contribution of intestinal lymphatic transport to the overall absorption of a drug, a concentration-time profile obtained after oral administration of a lipidic formulation of that drug (which activates intestinal lymphatic transport) could be compared with oral administration of a lipid-free formulation, which does not activate this transport. Previous

work has most commonly used rats with a cannulated jugular vein for repeated blood sampling and administration of intravenous drug to calculate absolute bioavailability [75, 239].

In vivo techniques looking to directly assess lymphatic transport involve complicated surgical procedures, and studies have been limited [240]. Historically, the first animal model describing the collection of lymph was by Bollman *et al* in 1948, where lymph could be collected from the liver, mesenteric duct or thoracic duct of a rat [241]. However, this procedure required that the rat be placed in specialised cages to prevent it turning. Roland Tasker expanded upon this technique in 1951, implanting an artificial cistern in the peritoneal cavity of the rat, so the animal is able to move freely [242]. More recently, an unconscious rat model was described by Porter *et al* [243], where three sites are cannulated in an anaesthetised rat – the carotid artery for blood sampling, the mesenteric lymph duct for intestinal lymph sampling, and duodenum for rehydration. Sometimes the jugular vein is also cannulated for ease of intravenous administration of compounds. An alternative conscious but restrained rat model is also used [68], and models in larger animals with higher lymph flow such as dogs [244], sheep [83], and pigs [56] have been established too.

The study by Gershkovich *et al* that assessed the lymphatic transport of two structurally related synthetic cannabinoids used the freely moving rat model. The work found that the lymphatic transport of dexamabinol, which had a moderate CM association (31.6%), contributed a small portion of its total absorbed dose. Conversely, the lymphatic transport of PRS-211,220, which had a higher CM association (66.1%), contributed over half of its total absorbed dose. CM association was a major determinant in extent for lymphatic transport for these two molecules [75]. THC has a similar chemical structure to these two cannabinoids, and could also have a similar association with CM to one of them.

However, in the context of this PhD, it was not essential to determine the absolute amount of drug transported via the intestinal lymphatics, so there was no need for *in vivo* surgical cannulation models. An alternative non-surgical method to assess the intestinal lymphatic transport of a drug *in vivo* is to assess the distribution of THC in lymphatic organs after oral administration of the drug in a lipidic formulation (such as Marinol®). This is because dietary lipids in the formulation activate intestinal lymph flow, which is usually low in the fasted state [245]. Assessing the concentration of THC in the MLN at times which intestinal lymphatic transport is activated would confirm this phenomenon. The ideal time to assess drug concentration in the MLN would be surrounding the time of peak plasma concentration after oral administration ($T_{\max}$), since that would be when THC is being transported through the intestinal lymphatics at the highest levels.

5.2 Aims

1. To predict the association of THC to CM using the *in silico* model, and its experimental association with natural CM isolated from rats and humans.
2. To assess the effect that lipids have on the oral bioavailability of THC by administration in lipid-free or lipidic formulations.
3. To confirm lymphatic transport of THC by assessing its concentration in MLN after oral administration of lipid-free or lipidic formulations at peak plasma times.

5.3 Materials and Methods

The methods used here are described and discussed in more detail in Chapter 2, Section 2.2.1, 2.2.2, 2.3.2, 2.3.3, 2.4.1, and 2.5.1.

5.4 Results

5.4.1 *In silico* chylomicron association of THC

Table 5-1 shows the calculation of the *in silico* CM association for THC. The final predicted association value is 36.64%. The high Log D_{7.4} value of THC contributed most positively to its predicted value (since Log D_{7.4} is an indicator the hydrophobicity of a molecule at pH 7.4), while the number of hydrogen bond acceptors contributed the most negatively (an indicator of the hydrophilicity of a molecule).

Table 5-1. Calculation of *in silico* association of delta-9-tetrahydrocannabinol to chylomicrons using a combination of its physicochemical properties.

Physicochemical Property	THC	Regression Coefficient	Calculation
Log D 7.4	7.25	0.299879	2.1741
Log P	7.68		
Log P - Log D	0.43	-0.238127	-0.1024
PSA (Å ²)	29	-0.00855215	-0.2480
H-Acceptors	2	-0.184359	-0.3687
FRB	4	0.0805226	0.3221
Density (g/cm ³)	1	1.45337	1.4534
Molar Volume (cm ³)	309.7	0.00545912	1.6907
H-Donors	1	0.0823094	0.0823
Constant			-5.2414
Association with CM (Y)			-0.2379
10^{^Y}			0.5782
% Association with CM			36.64%

5.4.2 HPLC Method Development and Validation

The development and partial validation of the HPLC-UV method for the determination of THC in Intralipid®, rat and human CM emulsions are described in Chapter 2. The method was partially validated for selectivity, sensitivity, linearity, accuracy and precision under the FDA guidelines for bioanalytical assays [163].

The HPLC-UV assay for determination of THC in rat plasma was developed and fully validated for accuracy, precision, recovery, stability, sensitivity and selectivity in accordance with FDA guidelines on Bioanalytical Assays [163]. This is described in Chapter 3, Section 3.2.3.

5.4.3 Association of THC with Intralipid and CM

The association of THC to Intralipid® is shown in Figure 5-1. Out of the three emulsions, THC had the highest association with Intralipid®, $76.6 \pm 7.4\%$. The association of THC with Rat and Human CM were $72.5 \pm 8.0\%$ and $59.9 \pm 6.8\%$ respectively. There were no significant differences between these three experimental association values (one-way ANOVA, $p=0.1036$).

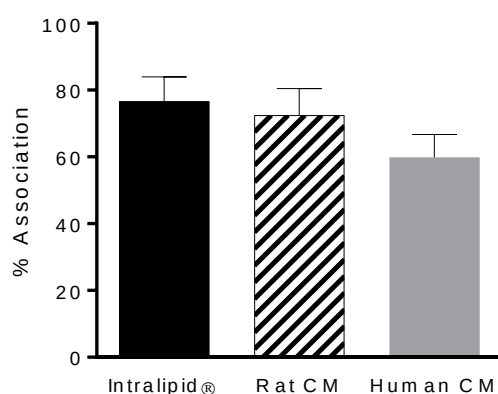


Figure 5-1. Association of THC with Intralipid®, Rat and Human Chylomicrons (CM). Stock solutions of THC was added to a triglyceride-standardised emulsion and incubated at 37 °C. CM were isolated by density gradient ultracentrifugation and THC associated in the emulsion was determined by HPLC-UV $n=6$, bars represents means $\pm$ SD.

5.4.4 Oral bioavailability of THC following administration in lipid-free and lipidic formulation

5.4.4.1 Non-compartmental approach

The plasma THC concentrations were quantified after IV and oral administration in lipid-free and lipidic formulations. The mean plasma concentrations obtained after these treatments are shown in Figure 5-2. The pharmacokinetic parameters derived from this data after non-compartmental analysis are summarized in Table 5-2. The oral bioavailability of THC was increased three-fold when given in lipidic formulation (18.5%) compared to lipid-free

formulation (5.73%). The $T_{\max}$ for the lipidic formulation was 4 hours, and the $T_{\max}$ for lipid-free formulation was 3 hours.

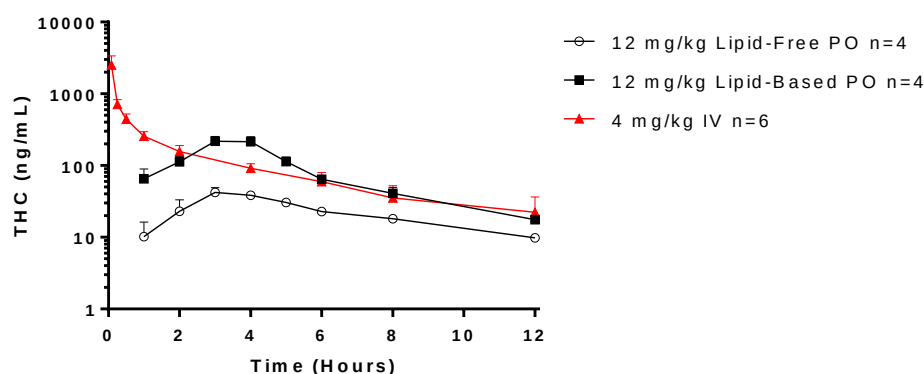


Figure 5-2. Plasma THC concentration-time profile semi-logarithmic plot (means $\pm$ SEM) following intravenous bolus (IV, $n=6$) and oral administration in lipid-free and lipidic (PO, $n=4$).

Table 5-2. Pharmacokinetic parameters using non-compartmental analysis of THC derived from IV bolus and oral administration in lipid-free and lipidic formulation. Values represent means $\pm$ SEM, $n=6$ for IV administration, and $n=4$ for both oral administration groups.

	$AUC_{inf}/Dose$ ([hr.ng/mL]/[mg/kg])	V_d (mL/kg)	CL (mL/h/kg)	$t_{1/2}$ (h)	
IV	477 ± 101	9367 ± 2558	2714 ± 750	3.32 ± 1.39	
	$AUC_{inf}/Dose$ ([hr.ng/mL]/[mg/kg])	$T_{\max}$ (h)	$C_{\max}$ (ng/mL)	$t_{1/2}$ (h)	F (%)
Lipid-Free	27.3 ± 3.3	3	44 ± 11	5.11 ± 0.77	5.73 ± 0.70
Lipidic	88.4 ± 6.0	4	232 ± 70	3.32 ± 0.22	18.5 ± 1.3

5.4.4.2 Compartmental Approaches

Intravenous Profiles

On visual inspection, the plasma THC concentration-time profile appeared to exhibit a biexponential decline that suggests two first-order processes – distribution and elimination. Therefore, a two-compartment model was used to model the pharmacokinetics of THC. A one-compartment model was also used to compare the fit of the two models. To better fit the data to this biexponential model, a biexponential weighting factor was applied all plasma data ($1/y^2$ weighting) so that all points have equal importance [246].

For each model, individual fittings of data were obtained, and a predicted plasma drug-concentration profile was generated using the derived pharmacokinetic parameters. Figure 5-3 shows the comparison between one- and two-compartment models to fit the observed IV concentration profile of THC. In the one-compartment model, only four out of six animals had profiles that fit the model, and all animals fit the two-compartment model, as demonstrated by the better fit between the observed points and predicted line.

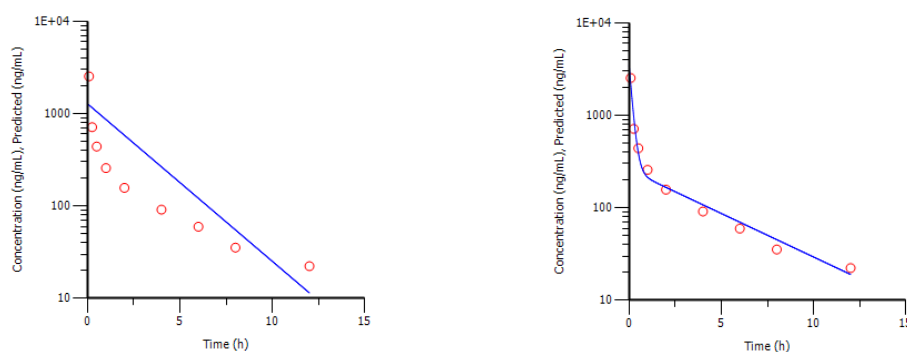


Figure 5-3. Semi-logarithmic plot of plasma drug concentration-time course after IV administration of THC with observed values denoted with solid circles, and the predicted one-compartment (Left) and two-compartment (Right) models in solid lines.

This improved fit of the two-compartment model is demonstrated further by comparing the observed concentration values at each time-point with the predicted value generated by the model, shown in Figure 5-4.

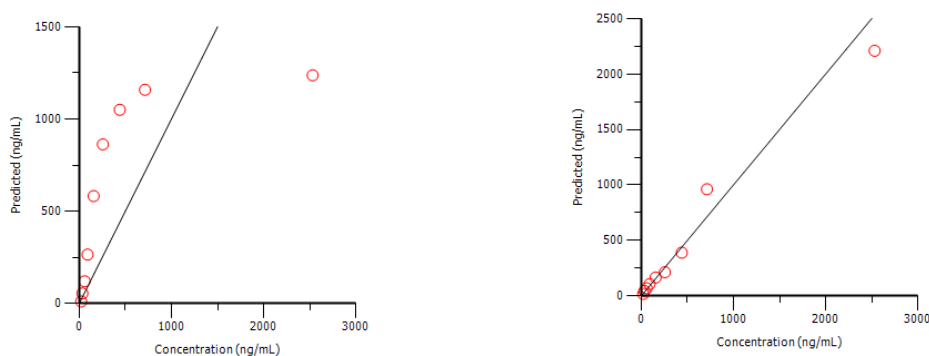


Figure 5-4. Comparison of observed plasma drug concentration values after IV administration with those predicted using one-compartment (Left) and two-compartment (Right) modelling denoted by solid circles. Solid line denotes the identity line.

These trends based on the graphical results are confirmed by comparing the AIC values for all profiles. The mean AIC value for a one-compartmental model was 111.4 (n=4), and the mean AIC value for a two-compartmental model was 80.0 (n=6). Since the two-compartmental model had a lower average value and a higher number of profiles that fit, the IV pharmacokinetic profile of THC is best described by a two-compartment model with first-order elimination.

The derived pharmacokinetic parameters for this model are summarised in Table 5-3. The dose-normalised AUC and CL using the two-compartmental model was similar to that obtained via the non-compartmental approach. The ratio of distribution constants between compartment 1 and 2 was high (3.76), which suggests that THC distributes to a peripheral compartment over the central one quickly.

Table 5-3. Estimated and derived pharmacokinetic parameters of THC obtained using the two-compartmental modelling approach based on drug concentration-time profile after IV administration.

Parameter (Unit)	Mean $\pm$ SEM
V_1 (mL/kg)	1475 $\pm$ 459
CL (mL/kg/hr)	2840 $\pm$ 825
k_{10} (1/hr)	2.39 $\pm$ 0.60
k_{12} (1/hr)	5.14 $\pm$ 0.73
k_{21} (1/hr)	1.23 $\pm$ 0.37
AUC/D ([hr.ng/mL]/[mg/kg])	463 $\pm$ 99
α -Half-life (hr)	0.0897 $\pm$ 0.0138
β -Half-life (hr)	3.47 $\pm$ 1.46
V_{ss} (mL/kg)	5507 $\pm$ 2462

V_1 , Volume of Distribution of first compartment; CL, Clearance; k_{10} , Elimination rate constant; k_{12} , Distribution constant between compartment 1 and 2; k_{21} , Distribution constant between compartment 2 and 1; V_{ss} , apparent volume of distribution at steady state; AUC/D, Dose-normalised areas under the curve.

Oral Profiles

For the drug concentration-time profile after oral administration in lipid-free and lipidic formulations, a similar method of fitting to one- and two-compartment models was performed. To be consistent with the intravenous model fitting, 1/y² weighting was also applied to this data. The predicted profile for THC to fit the mean oral pharmacokinetic profile in rats given

lipid-free formulation is shown in Figure 5-5, and the predicted profile for THC to fit the mean oral pharmacokinetic profile in rats given lipidic formulation is shown in Figure 5-6. The profiles of all four animals receiving THC orally in both formulations fit a one-compartment model, but only one animal from each group fit the two-compartmental model. For the reasons stated above, it was not possible to generate a reliable predicted plasma concentration-time profile using two-compartment modelling, and the pharmacokinetic profile of THC after oral administration was best described as a one-compartment model with first order absorption and first order elimination. The derived pharmacokinetic parameters for this model are summarised in Table 5-4. The predicted AUC, $T_{\max}$ and $C_{\max}$ using this model were similar to that obtained via the non-compartmental approach.

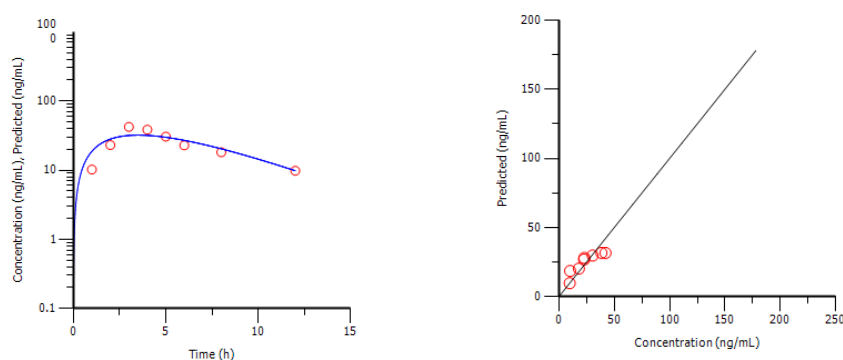


Figure 5-5. (Left) Semi-logarithmic plot of plasma drug concentration-time course after oral administration of THC in lipid-free formulation, with mean observed values in open circles, and predicted profile as a solid line. (Right) Comparison of observed and predicted plasma concentration values using a one-compartment modelling (denoted by solid circles), with the solid line of identity.

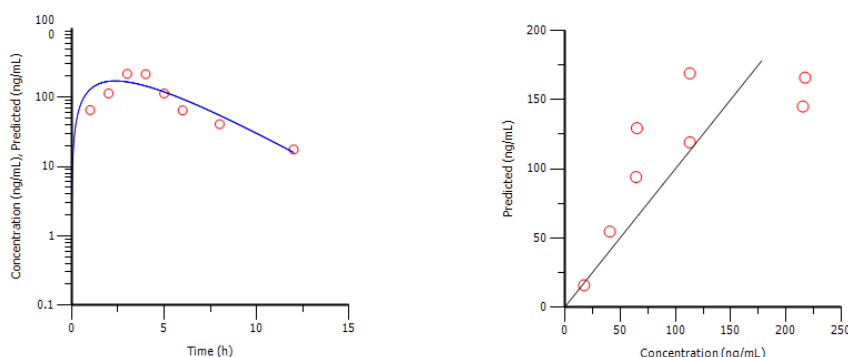


Figure 5-6. (Left) Semi-logarithmic plot of plasma drug concentration-time course after oral administration of THC in lipidic formulation, with mean observed values in open circles, and predicted profile as a solid line. (Right) Comparison of observed and predicted plasma concentration values using a one-compartment modelling (denoted by solid circles), with the solid line of identity.

Table 5-4. Estimated and derived pharmacokinetic parameters of THC after oral administration in lipid-free and lipidic formulations obtained with the one-compartment modelling approach.

Parameter (Unit)	Lipid-Free	Lipidic
k₀₁ (1/hr)	1.01 ± 0.25	0.386 ± 0.036
k₁₀ (1/hr)	0.181 ± 0.024	0.382 ± 0.035
AUC (hr*ng/mL)	339 ± 22	1189 ± 143
T_{max} (hr)	2.65 ± 0.67	2.67 ± 0.25
C_{max} (ng/mL)	45.4 ± 10.6	163 ± 9

V_d, Volume of Distribution; **k₀₁** Absorption rate constant; **k₁₀**, Elimination rate constant; **AUC**, area under the curve; **CL**, clearance; **T_{max}**, time of peak plasma concentration; **C_{max}**, peak plasma concentration.

5.4.5 Biodistribution of THC in the Mesenteric Lymph Nodes (MLN)

MLN were successfully found nestled near the end of the ileum and beginning of the caecum.

The brain, liver and spleen were also successfully found and homogenized. The

biodistribution of THC in the MLN is shown in Figure 5-8. The oral T_{max} for THC in lipid-free formulation from the pharmacokinetic study was 3 hours, and therefore one hour prior to T_{max} was 2 hours. Likewise, oral T_{max} for THC in lipidic formulation was 4 hours. At one hour before T_{max}, the concentration of THC in MLN of rats receiving a lipidic formulation of the drug was 27-fold higher than the MLN of rats receiving a lipid-free formulation, with a mean concentration of 8660 ± 3132 ng/g in the lipidic group, and 313.6 ± 150 ng/g in the lipid-free group. At T_{max}, this concentration differential between MLN of rats receiving lipid-free and lipidic formulation reduces to 14-fold with the concentration of THC in lipidic MLN being similar to an hour earlier (8260 ± 2788 ng/g), and the concentration of THC in the lipid-free MLN increased to 587.1 ± 324.4 ng/g. At 12 hours, this difference between concentrations in the MLN becomes statistically insignificant, while the mean concentration of THC in lipid-free MLN was 134.6 ± 22.6 ng/g and THC in lipidic MLN was 2599 ± 332 ng/g. This suggests that the lipidic formulation no longer has any lymphatic targeting effect 12 hours after oral administration.

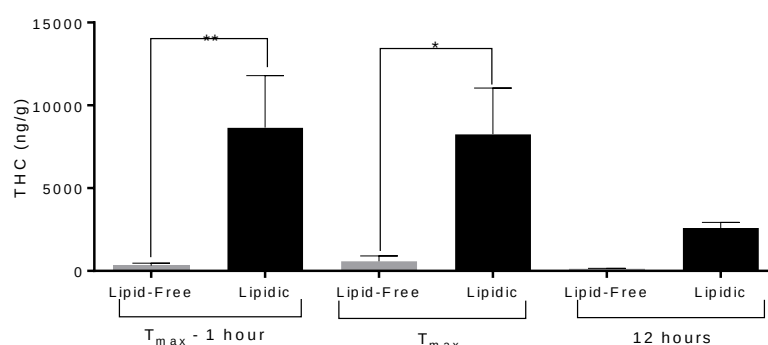


Figure 5-8. Concentration of THC in mesenteric lymph node (MLN) homogenate after oral administration of lipid-free or lipidic formulation, at one hour before the formulation's T_{max} (as determined by a previous oral pharmacokinetic study), T_{max} and end of experiment (12 hours). Data presented as means $\pm$ SEM, ** denotes significance using one-way ANOVA, with Fishers Least Significant Difference test, $p < 0.01$, * = $p < 0.05$.

5.4.6 Distribution of THC in other tissues

The distribution of THC in the spleen, brain and liver is shown in Figure 5-9. THC was found in the brain, spleen and liver of the lipidic group, but was not detected in the brain and liver of the lipid-free groups at either sampling time. The concentrations of THC found in all tissues were higher at T_{max} compared to one hour earlier. THC was found in the highest concentrations in the spleen at T_{max} , with an average concentration of 1230 ± 21 and 1253 ± 4.2 ng/g for lipid-free and lipidic rat spleens respectively. One hour prior to T_{max} , the concentration of THC found in the liver of rats in the lipidic formulation group was higher than that found in the spleen, whereas this trend is reversed at T_{max} .

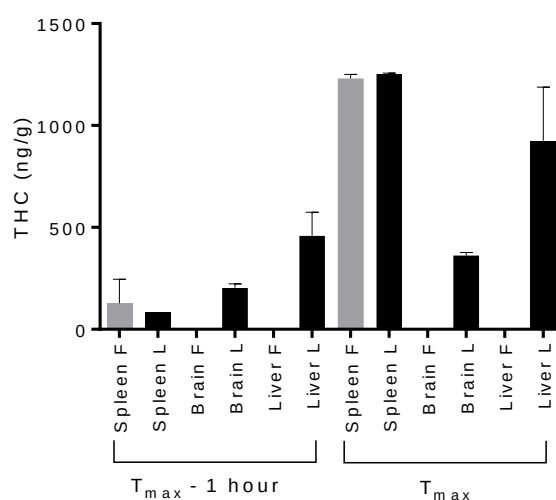


Figure 5-9. Concentration of THC in rat spleen, brain and liver homogenate after oral administration of lipid-free (F) or lipidic (L) formulation, at one hour before the formulation's T_{max} (as determined by a previous oral pharmacokinetic study) and T_{max} . Data presented as means $\pm$ SEM, $n=3$.

5.5 Discussion

5.5.1 *In silico* prediction of Chylomicron Association

Compared to the experimental CM association values obtained, it would seem that the model has under-predicted the association of THC. One reason for this could be that in the molecules used for the development of the model, there are many compounds that have a Log P - Log $D_{7.4}$ value of zero or a value of two or higher (so either non-ionising or highly ionised molecules) but not as many compounds in-between like THC which has a value of 0.43. Since the most important physicochemical properties determined in this model were Log $D_{7.4}$ and Log P - Log $D_{7.4}$, it could be possible that the predictive power of this model is higher for molecules that have Log P - Log $D_{7.4}$ values closer to zero or higher than two. Since this method of estimating the association of a drug to CM does not require any experimental setup, it is a useful tool for preliminary screening of drug candidates for their lymphatic transport potential.

5.5.2 Experimental Association of THC with Intralipid® and Chylomicron Emulsions

Isolation of chylomicrons

Sufficient amounts of CM were obtained using the gavage method described. The isolation of human CM however, was not previously done and needed optimisation. In this process, different volumes of blood were taken, and different waiting periods post-meal were attempted to find the optimum time to collect CM from blood. Based on the work of a previous study, a starting time of two hours was attempted [40], but some volunteers produced more CM at four hours instead of two. Meals containing high levels of fat were inspired and modified from a study on high-fat meal induced post-prandial inflammation, which provided the fat content of many types of meals [247].

Association values of THC with emulsions

Intralipid® is an artificial lipid emulsion containing soy bean oil, egg phospholipids and glycerin and most commonly used to provide intravenous nutrition for those unable to have an oral diet [248]. The emulsion has many of the same lipidic components to CM, and Gershkovich *et al* showed that the association of a drug to the emulsion isolated at a similar density to CM was similar to the drug's association with CM. This finding provides an additional method of assessing the lymphatic transport potential of a drug without the use of animals [70]. The similarity between association values to Intralipid® and CM was also found in this study. The experimental association of THC with rat chylomicrons was also high and similar to the association of other lipophilic drugs such as halofantrine, vitamin D and DDT to CM. There was no statistical difference between the three association values ($p=0.1036$, one-way ANOVA), which supports the view that the three emulsions are similar. Previous work has also shown a correlation between the associations of a drug to CM to the fraction of dose absorbed into intestinal lymphatics [1]. Figure 5-10 shows a reproduction of a graph from [1], showing this correlation. Although the quantitative cumulative intestinal lymphatic transport work has not yet been performed for THC, the red dot denotes where it would fit in the trend, which suggests that over 20% of the administered dose of THC could be absorbed via the intestinal lymphatics.

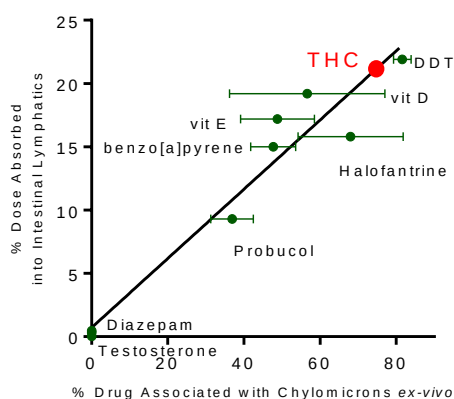


Figure 5-10. The correlation between a drug's association with rat chylomicrons and the fraction of dose absorbed into intestinal lymphatics, with the chylomicron association of THC indicated in red. Figure reproduced from [1].

Another study looked at the intestinal lymphatic transport of two structurally related synthetic cannabinoids, dexanabinol and PRS-211,220. The compounds were lipophilic, having Log P and Log $D_{7.4}$ values of 8.2 and 8.5 respectively but had very different CM association values (dexanabinol has 31.6% association and PRS-211,220 has 66.1%). These drugs were administered intragastrically in lipid-free and lipid-based formulations, and a drug concentration-time profile was obtained for plasma and lymph. When these cannabinoids were orally administered to rats in a lipid-based formulation, the compound with higher CM association (PRS-211,220) was found to be transported much more via the intestinal lymphatics (9.7% of the administered dose compared to 1.6% of the administered dose of dexanabinol). Considering that THC has similar physicochemical properties to both of these cannabinoids, and an even higher experimental CM association value than PRS-211,220, it is highly likely that it is transported via the intestinal lymphatics if administered in conditions that activate intestinal lymphatic transport.

5.5.3 Pharmacokinetic studies

To our knowledge, this is the first time that the lymphatic transport potential of THC has been studied in the rat, the first time a compartmental approach was used to analyse the oral pharmacokinetic profiles and the first time tissue distribution of THC has been examined in the rat after oral administration.

The absolute bioavailability of THC orally administered in a lipid-free formulation was poor (5.73%), and is similar to that reported in the literature [99, 122]. However, bioavailability was improved over three-fold when the formulation was changed to a lipidic one (to 18.5%). This is likely due to the presence of oils, which lead to the activation of intestinal lymphatic absorption. Activation of intestinal lymphatic transport has previously been shown to be able to improve the bioavailability of a drug, especially if those drugs are lipophilic [68, 75, 249-

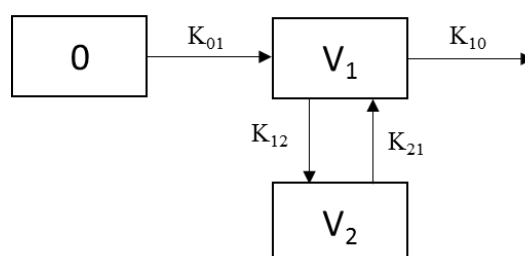
252]. Comparing this effect to the previous study in synthetic cannabinoids dexamabinol and PRS-211,220, the increase in bioavailability from using a lipidic formulation was similar to the latter [75]. PRS-211,220 has similar physicochemical properties to THC, a relatively high association with chylomicrons (66%), has a low oral bioavailability when administered in a lipid-free formulation and this bioavailability also improved threefold when a lipidic formulation was used. The study also revealed that intestinal lymphatic transport contributed to 60% of this bioavailable dose, which suggests that approximately 20% of the total administered dose was transported via the intestinal lymphatics. This number corresponds well to the correlation between the association of a drug with CM and the dose absorbed via the intestinal lymphatics *in vivo*, strongly suggesting that THC is absorbed in a similar way to PRS-211,220 [1].

Some studies have conducted pharmacokinetic analysis of THC using non-compartmental or compartmental approaches. Wall *et al* conducted a study that found that the oral bioavailability of THC dissolved in 0.5 mL of sesame oil was between 10 and 20%, but it was unclear whether the participants in this study were fasted or non-fasted otherwise during the experiment. The authors of this study found that the intravenous drug concentration-time profile yielded a biexponential curve that fit a two-compartment model, and also calculated a similarly high V_d of 10.2 L/kg [122]. Chiang and Barnett also proposed an open two-compartment model to describe the relation to the subjective high effect of THC from smoking one marijuana cigarette, with the effect compartment being directly linked to the central compartment [126]. The most recent pharmacokinetic study on THC by Valiveti *et al* also found that the intravenous concentration-time profile also fit a two-compartment model, with similar α - and β -half-lives and V_{ss} (the values obtained by Valiveti *et al* were 0.0967 ± 0.0116 h, 6.93 ± 2.08 h, and 3220 ± 1894 mL/kg respectively). After correcting for dose, the observed AUC values obtained in that study were also similar (331 ± 119 compared to 463 ± 99 in this study) [184].

Compared to other oral pharmacokinetic studies on THC, the oral bioavailability of THC in a lipid-free formulation obtained here is most similar to that reported in humans by Ohlsson *et al* [99]. That study found that the oral bioavailability of THC given to men in one chocolate cookie was $6 \pm 3 \%$. Other studies conducted oral studies without an accompanying intravenous study [130, 143, 253] or studied the smoking route of administration, with or without accompanying intravenous studies to calculate absolute bioavailability [180, 254-259].

By contrast, the concentration-time profile for the oral administration of THC in lipid-free or lipidic formulation only fit a one-compartment model. This could be due to the high volume of distribution of THC, and the high intercompartmental clearance in favour of the peripheral compartment. This means that THC very quickly distributes to the periphery when it arrives in the central compartment, which many lipophilic and fat soluble drugs do [260]. It is also likely that the low concentrations obtained after oral administration of THC affected the ability of the software package to reliably fit the data.

The activation of dietary fat absorption by lipidic formulation also tripled the exposure of THC, delayed $T_{\max}$, and increased half-life. Since the intravenous administration of a drug does not include an extravascular input, the pharmacokinetic model for THC can be best described by the following schematic, a two-compartment model with first-order extravascular input and first-order elimination:



These results are similar to halofantrine and DDT, drugs that have been proven to be transported via the intestinal lymphatics. Halofantrine also has a high Log P value and similar

affinity to CM to THC (8.5 and $68 \pm 13.8\%$ respectively) [1]. The oral bioavailability of halofantrine also tripled if administered after a fatty meal [261, 262], and subsequent work confirmed that this improvement in bioavailability was due to intestinal lymphatic transport [263]. DDT had a lower Log P but higher CM association value to THC (6.79 and $81.6 \pm 2.3\%$ respectively), and has been found to be transported via the intestinal lymphatics [69, 264].

The lipophilicity of THC suggests that it is very permeable and would easily penetrate tissues. This would explain the rapid distribution phase after intravenous administration [265]. THC has also been shown to accumulate in less-vascularised tissues and fat. It is very likely that fatty tissue would constitute the second compartment in the two-compartment model described here [237, 266].

5.5.4 Biodistribution studies

Previous work indicates that 88% of THC in the blood is distributed to the plasma, and the remaining 12% distributed to red blood cells [234]. 95-99% THC has also been shown to bind to plasma proteins, which include lipoproteins such as CM, as corroborated by the association studies shown above [267].

Overall, the amount of THC found in the organs of rats given a lipidic formulation was higher than the organs of rats given a lipid-free formulation. In particular, this was the most pronounced in the MLN. At the two times sampled, the concentration of THC in the MLN was extremely high compared to the plasma concentration (compared in Figure 5-11). In the lipid-free group, the concentration of THC in the MLN was over 10 times higher than the plasma, and over 30 times higher than plasma in the lipidic group. This suggests that THC localises selectively to the intestinal lymphatics, regardless of the formulation and oral administration of THC in a lipidic formulation increases the magnitude of this localisation. The peak

concentration of THC in the MLN was higher than the levels seen to cause suppressive effects *in vitro* [114, 139, 149, 151, 268, 269].

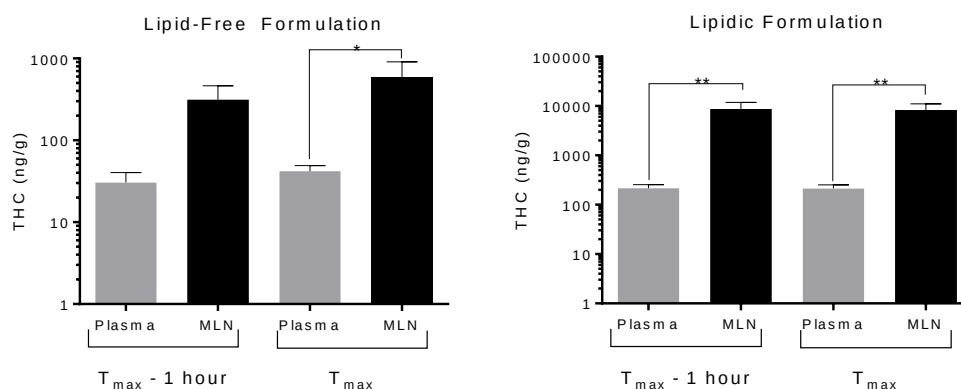


Figure 5-11. Re-representation of data obtained in this study, comparing the concentration of THC one hour before T_{max} and T_{max} in plasma and mesenteric lymph nodes (MLN). Data presented as means $\pm$ SEM, $n=3$ for MLN, $n=4$ for plasma. * denotes significance using the student's t-test, $p<0.05$, **= $p<0.01$.

The implication that being in the fed state triples the exposure of THC to the body and the lymphatic system in particular is very important for patients that ingest THC after a fatty meal. Marinol® is currently used to manage loss of appetite associated with AIDS and nausea and vomiting associated with cancer chemotherapy [155], and both conditions involve immunosuppression. AIDS patients have a low CD4+ T cell count, which reduces the body's defence to infection, and the majority of chemotherapy treatments also drastically reduce the number of white blood cells in the body [270, 271]. The presence of another immunosuppressive drug such as THC transported via the lymphatic system, where a large portion of lymphocytes reside in the body may cause even more targeted and pronounced immunosuppression, which may further compromise the immune systems of the patients that take the medication. This local immunosuppression in the gut could have a systemic immunosuppressive effect. The MLN are an integral part of the gut-associated lymphoid tissues, which prevents invasion of pathogenic bacteria from the intestine into the body [272].

The amount of THC found in other organs in this biodistribution study was much lower. In the lipid-free group, THC was only found in the spleen, but THC was found in all the organs

analysed in the lipidic group. It is interesting to note that THC was found in the spleen at both groups and time points examined, with a concentration proportional to the concentration of THC found in the plasma at those times. A possible reason that THC was found in the spleen could be because the spleen utilises the triglycerides contained within lipoproteins including CM [49], and the THC associated with CM persists in the tissue.

The natural function of CM is to transport dietary lipids from the intestine to adipose, cardiac and skeletal muscle tissue. When they are synthesised in the enterocyte, they contain the apolipoprotein B-48, and after transport through the intestinal lymphatics, CM enter general circulation where they interact with High Density Lipoproteins (HDL), which donates apolipoprotein C2, an important cofactor for Lipoprotein Lipase (LpL), the enzyme primarily responsible for converting triglycerides in CM to free fatty acids [45, 273]. These fatty acids can then be transported into the cell or tissue that LpL is on. LpL is also present in a number of organs in the body, including the liver and brain and macrophages [47, 48, 51]. After losing the majority of their contained triglycerides, CM reduce in size (from up to 1000 nm to 50-100 nm in diameter), lose apolipoprotein C2 (which is returned to HDL) and are ultimately taken up by Low Density Lipoprotein (LDL) receptors in the liver to be disassembled [48]. The distribution of LpL in the brain and liver and overall increase in systemic exposure to THC would explain why THC was found in the liver at the times assessed at higher concentrations than the brain, assuming THC is co-localised with the triglycerides in CM.

Conversely, THC was not detected in the brain or liver in the lipid-free group, and this was probably due the lower systemic exposure to the drug. The absence of lipids would mean that intestinal lymphatic transport is not activated. However, THC might still be able to associate with Very Low Density Lipoprotein (VLDL) produced in low amounts from enterocytes in the fasted state [274].

5.5.5 Limitations of study

Due to practicalities of animal research, only four rats were allocated to each oral treatment group. This low number of animals made the fitting of the data to compartmental models less reliable. The biodistribution study done in this study at oral $T_{\max}$, one hour prior to oral $T_{\max}$ and at the end of experiment was a good alternative to prove that THC was transported in the intestinal lymphatic system after oral administration, but this procedure was terminal, and it was not possible to monitor the concentration in the lymphatic system of single animals.

5.6 Conclusions

The high Log P and Log $D_{7.4}$ values of THC suggest that it has a high association with CM, but the *in silico* predicted association value was lower than expected when compared to other lipophilic drugs. This under-prediction was confirmed when experimental association studies showed that THC has an extremely high CM association similar to other lipophilic drugs that are transported by the intestinal lymphatics, implying that intestinal lymphatic transport is a major pathway in the oral absorption of THC.

Oral administration of THC in a lipidic formulation improved absolute bioavailability threefold compared to a lipid-free formulation. Coupled with THC's high degree of association with CM, this strongly suggests that intestinal lymphatic transport is a major contributor to this improvement. Biodistribution studies showed extremely high concentrations in the MLN at $T_{\max}$ and one hour before $T_{\max}$, disproportionate to the distribution in other organs at those times. The concentrations of THC achieved in the MLN were 27-fold higher than those in plasma, and previously shown to be immunosuppressive in *in vitro* studies. This could mean that the current use of THC by immunocompromised patients exposes their mesenteric immune systems to very high local concentrations of THC.

Chapter 6. The Antiproliferative Effect of Delta-9-Tetrahydrocannabinol.

6.1 Introduction

Following on from the previous pharmacokinetic and biodistribution studies in the rat, it was found that oral administration of THC in conditions that facilitate intestinal lymphatic transport (in a lipidic formulation) improved absolute oral bioavailability by three-fold. It was also found that 100-fold higher levels of THC were found in the MLN compared to organs in the general circulation (such as the spleen or liver), with concentrations around 8 µg/g. This value is similar to the concentration ranges used in the aforementioned studies showing immunosuppressive effects *in vitro* [125, 131, 136]. THC travels associated with chylomicrons (CM) while in the lymphatic system since it is lipophilic and has extremely high experimental affinity to CM (72.5%).

THC needs to bind to cannabinoid receptors in order to exert its effect, which means it also needs to dissociate from CM by the action of LpL. LpL is secreted and expressed by macrophages [43, 51, 275, 276]. Previous studies have shown that LpL is expressed by macrophages resident in the MLN, and is active there [50]. LpL is also present on other populations of white blood cells, such as natural killer cells [277], and B cell lymphocytes [52]. In particular, the study by Calder *et al* shows that lymphocytes isolated from the cervical lymph node and thoracic duct have LpL activity, and are able to breakdown triacylglycerol emulsions into fatty acids and glycerol [278].

There are many clinical trials that study the effects of THC, cannabis-based medicines, and synthetic cannabinoids. These trials have focused on the analgesic and antiemetic properties of THC, and have generally shown that THC performs better than placebo to treat cancer pain and neuropathic pain (reviewed in [279]). However, not many studies have examined the response of immune cells to THC. Marinol®, a commercial formulation containing THC, has been approved to be used in the treatment of nausea associated with cancer chemotherapy.

Chemotherapy is one of the main types of cancer treatment, and uses drugs to kill cancer cells. Cancer cells divide and grow rapidly, and many chemotherapy drugs prevent cell division by a variety of mechanisms. Since immune cells are among the most rapidly dividing cell types in the body, they are commonly also affected by chemotherapy drugs. This commonly causes some form of immunosuppression [280]. While chemotherapy drugs are in effect, white blood cell, red blood cell and platelet counts can decrease dramatically.

The addition of another potentially immunosuppressive drug like THC may create one of two possible scenarios. First, THC may have an additive effect and further suppress the immune system, immunocompromising subjects that take it. This may in turn render THC unsafe for these individuals. Second, the pathways that chemotherapy drugs trigger apoptosis may already be saturated, and the addition of THC would not result in more suppression. In contrast with the first scenario, this may suggest that THC is safe for these individuals. Even though THC had little or no effect in HIV subjects as shown by Bredt *et al* [155], a similar study has not been done in subjects receiving or recovering from chemotherapy. It would be highly beneficial to assess any potential difference in reaction to THC in this regard, to confirm the safety of THC being prescribed to cancer patients. According to the World Drug Report 2015, there are 181.8 million global users of cannabis, with the highest number residing in the United States. The changes in legislation in some states have drawn attention to cannabis use, and the prevalence of cannabis use in the general population is steadily increasing [90].

As previously discussed in Chapter 3, Section 3.3, there are many methods to assess the cellular effects of a potentially immunosuppressive drug, and many have been used to study THC [125, 131, 147, 206]. Among the *in vivo* and *in vitro* methods available, an appropriate preliminary method for the assessment of the suppression of proliferation is the colorimetric BrdU proliferation Enzyme-Linked Immunosorbent Assay (ELISA). The method is simple, effective

and cost efficient compared to other cell-culture methods available to assess the antiproliferative effect of THC.

It is therefore plausible that THC is able to dissociate from CM while travelling through the lymphatics (lymph nodes in particular), and possibly exert an antiproliferative effect.

6.2 Aims

- To determine the concentration of THC that is antiproliferative in PBMC isolated from healthy individuals, and patients receiving chemotherapy.
- To determine whether THC associated with chylomicron emulsion is also able to exert an antiproliferative effect on PBMC.
- To determine the bio-relevance of this threshold antiproliferative concentration by comparing to the concentrations obtained from previous pharmacokinetic and biodistribution studies.

6.3 Materials and Methods

The development and optimisation of PBMC and CM isolation, THC association with CM emulsion and HPLC analysis was described in Chapter 2 and Chapter 3.

6.4 Results

6.4.1 Proliferation of PBMC isolated from healthy individuals after exposure to THC

6.4.1.1 THC dissolved in 0.1% DMSO vehicle

THC had a dose-dependent suppressive effect on the proliferation of PBMC isolated from healthy individuals, shown in Figure 6-1. At 10 and 20 µg/mL THC suppressed the proliferation of PBMC, and this suppression was statistically significant ($p < 0.0001$, one-way

ANOVA). Cells exposed to 10 and 20 $\mu\text{g/mL}$ of THC had similar levels of proliferation to unstimulated cells. For better comparison between stimulated and unstimulated cells, the absorbance values were normalised to cells stimulated with DMSO vehicle. Subsequent analysis of unstimulated cells exposed to THC (PBMC isolated from patients recovering from chemotherapy and treatment of PBMC isolated from healthy individuals with THC associated with CM emulsion) showed no change in proliferation compared to unstimulated vehicle treatment and were not displayed.

Since THC had no effect at 0.01 and 0.1 $\mu\text{g/mL}$, these concentrations were not used in subsequent proliferation studies using THC associated with CM emulsion, and studies using PBMC isolated from cancer patients.

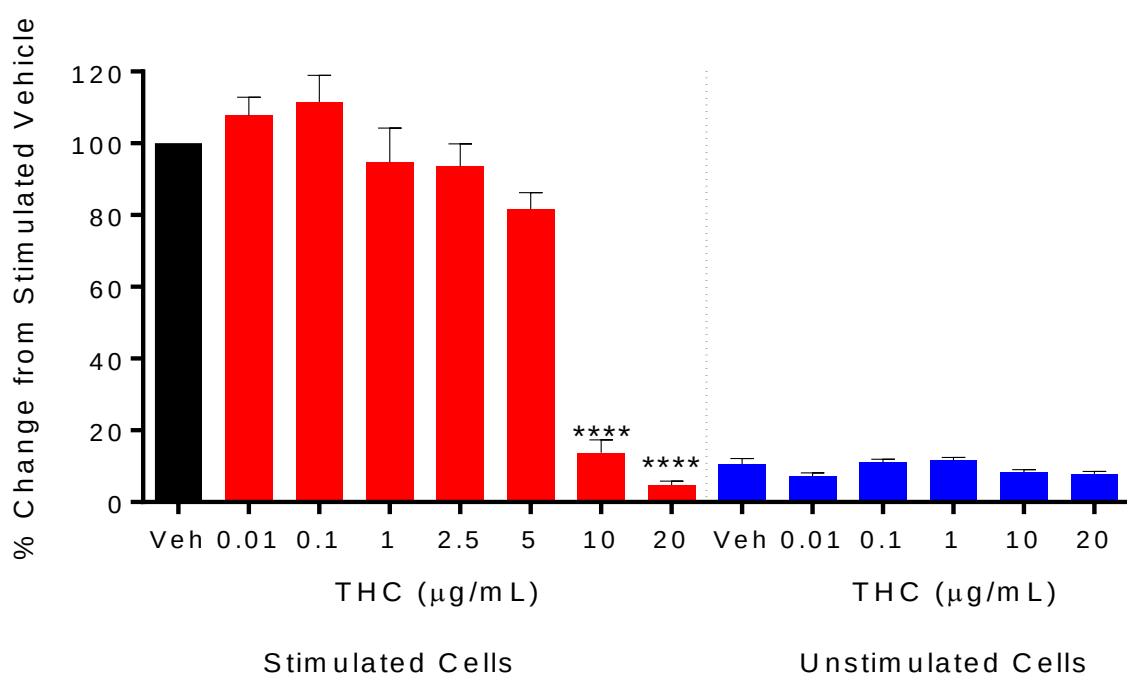


Figure 6-1. (Left) Proliferation of PBMC isolated from healthy male volunteers after exposure to increasing concentrations of THC and a mitogen (PHA, 10 $\mu\text{g/mL}$). (Right) Proliferation of PBMC isolated from healthy male volunteers after exposure to increasing concentrations of THC, without mitogenic stimulation. **** denotes significance by one-way ANOVA with Dunnett's correction compared to vehicle treatment, $p < 0.001$, $n = 9$ for stimulated cells, $n = 6$ for unstimulated cells.

6.4.1.2 THC associated with Chylomicron emulsion

Figure 6-2 shows the suppressive effect of THC exposure to PBMC isolated from healthy individuals in 1% CM emulsion. THC suppressed proliferation in a dose-dependent manner, similar to 0.1% DMSO treatment. This suppression was statistically significant at 10, 15 and 20 $\mu\text{g/mL}$ ($p < 0.01$, $p < 0.01$ and $p < 0.0001$ using one-way ANOVA with Dunnett's correction respectively) but the suppression elicited was less pronounced than the suppression by THC dissolved in DMSO (values compared in Table 6-1).

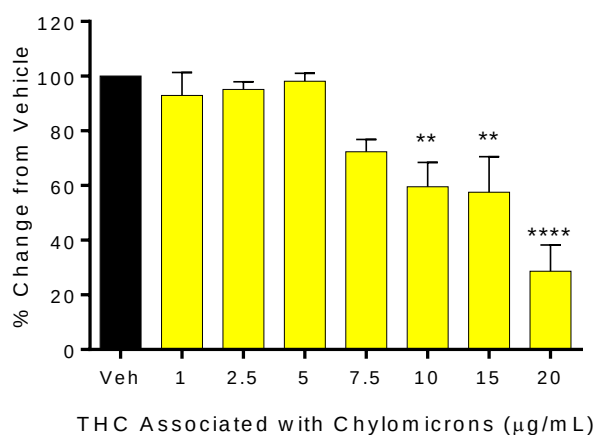


Figure 6-2. Proliferation of PBMC isolated from healthy male volunteers after exposure to increasing concentrations of THC in 1% CM emulsion vehicle. ** denotes significance by one-way ANOVA with Dunnett's correction compared to vehicle treatment, $p < 0.01$, ****= $p < 0.0001$, $n=4$.

6.4.2 Proliferation of PBMC isolated from cancer patients after exposure to THC

The treatment and blood count data of the cancer patients who donated blood samples are shown in Table 6-2. Six out of the seven patients that donated blood for PBMC isolation were receiving chemotherapy for the treatment of Non-Seminomatous Germ Cell Tumours (NSGCT); one patient had mixed seminomatous and non-seminomatous germ cell tumours. All patients were males aged 21 to 59 years old. There was variability in the patient's white blood cell (WBC) and platelet count (Plts), but with similar quantities of lymphocytes. Sufficient amounts of PBMC were isolated from blood samples to conduct the cell proliferation assay (approximately 5 million PBMC were required to seed the 96-well plate for THC treatment). One patient (ID 2931) had both very high white blood cell and minimally raised lactate dehydrogenase (LDH) levels, which suggested that some cells were damaged by the

time of analysis. This was reflected in the result of that patient, where their cells responded differently to mitogenic stimulation, leading to large variability in absorbance readings. For this reason, the results obtained from this patient were excluded from the amalgamated results.

Two of the individuals were receiving their first cycle of chemotherapy, three individuals were receiving their second cycle, and two individuals were receiving their third cycle. All the treatment regimens included the use of bleomycin, etoposide and cisplatin (BEP), with the addition of carboplatin and vincristine (CBOP) for individuals with a poor prognosis (PP). The two patients in their first cycle of treatment also had significantly higher WBC and Absolute Neutrophil Counts (ANC). Conversely, patients in their second and third cycle of chemotherapy treatment had lower WBC and ANC, with only patient 1445 having ANC above $1 \times 10^9/l$. Haemaglobin (Hb) levels however stayed more or less constant between the patients irrespective of treatment regimen, chemotherapy cycle or prognosis. It is possible that PBMC isolated from the blood of patients in their third cycle of treatment would have a different distribution of lymphocytes and that they would respond differently to mitogenic stimulation, but these differences, if present, were not noticeable under these experimental conditions.

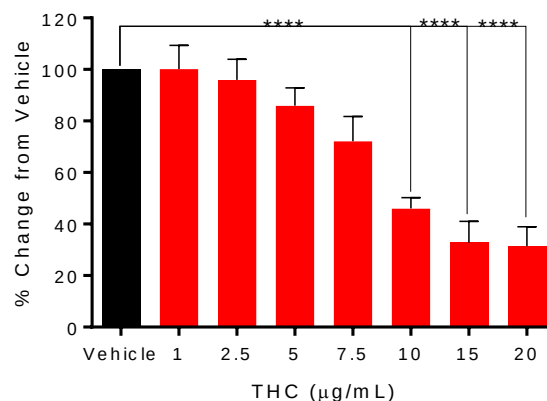


Figure 6-3. Proliferation of PBMC isolated from male patients recovering from chemotherapy to treat non-seminomatous germ cell tumours after exposure to increasing concentrations of THC. **** denotes significance by one-way ANOVA with Dunnett's correction compared to vehicle treatment, $p < 0.0001$, $n = 5$.

Figure 6-3 shows the antiproliferative effect of THC in DMSO vehicle when exposed to PBMC isolated from patients recovering from chemotherapy. THC suppressed proliferation in a dose-dependent manner, similar to the exposure of PBMC to THC in CM vehicle. This suppression was statistically significant at 10, 15 and 20 $\mu\text{g/mL}$ ($p < 0.0001$ using one-way ANOVA with Dunnett's correction). The degree of suppression at 10, 15 and 20 $\mu\text{g/mL}$ was similar to the degree of suppression of THC in CM vehicle when exposed to PBMC isolated from healthy individuals (Table 6-1).

Table 6-1. % Change in absorbance from vehicle control by colorimetric ELISA after exposure of THC to PBMC at 10 and 20 $\mu\text{g/mL}$.

PBMC Source	Vehicle	% Change in Absorbance from Vehicle Control	
		At 10 $\mu\text{g/mL}$ THC	At 20 $\mu\text{g/mL}$ THC
Healthy Individuals	DMSO	13.8 $\pm$ 3.6 %	4.79 $\pm$ 1.10 %
Healthy Individuals	CM	59.6 $\pm$ 8.9 % ^{***}	28.6 $\pm$ 9.6 % ^{**}
Cancer Patients	DMSO	46.0 $\pm$ 4.3 % ^{***}	31.3 $\pm$ 7.6 % ^{**}

^{***} Statistically significant difference to PBMC from healthy individuals treated with DMSO by one-way ANOVA, $p < 0.0001$

^{**} Statistically significant difference to PBMC from healthy individuals treated with DMSO by one-way ANOVA, $p < 0.001$

Table 6-2. Cancer Patient Treatment Blood Count Information for Cell Studies.

ID	Age	Gender	Collection Date	Tumour site and stage	Treatment	WBC 10 ⁹ /l	ANC 10 ⁹ /l	Ly 10 ⁹ /l	Plts 10 ⁹ /l	Hb g/l	Evidence for haemolysis
2748	35	M	24/11/15	GP met NSGCT	Cycle 2 BEP	1.1	0.1	0.8	105	125	No
2930	59	M	01/12/15	GP met NSGCT	Cycle 3 EP	2.2	0.5	1.4	151	120	No
2678	21	M	07/12/15	IP met NSGCT	Cycle 3 BEP	2	0.5	1.2	104	94	No
2931	21	M	14/12/15	PP met NSGCT	Week 10 CBOP BEP	6.4	3.8	1.7	286	102	Possibly minor as LDH raised minimally
1395	36	M	26/04/16	GP met NSGCT	Cycle 2 BEP	2.9	0.4	1.2	416	108	No
1445	43	M	26/04/16	GP met mixed GCT	Cycle 2 BEP	4.3	1.5	2.2	338	115	No
1573	29	M	03/05/16	PP met NSGCT	Week 7 CBOP BEP	6.1	3.6	1.7	375	100	No

WBC White Blood Cell Count

ANC Absolute Neutrophil Count

Ly Lymphocyte

Plts Platelets

Hb Haemoglobin

PP Poor Prognosis

GP Good Prognosis

IP Intermediate Prognosis

met Metastasized

NSGCT Non-seminomatous Germ Cell Tumour

CBOP/BEP Carboplatin/Bleomycin/Etoposide/Vincristine/Cisplatin

BEP Bleomycin/Etoposide/Cisplatin

LDH Lactate Dehydrogenase

6.5 Discussion

This study is the first showing a dose-dependent suppression of proliferation elicited by THC exposure to human PBMC isolated from healthy individuals and patients recovering from chemotherapy. This study also assessed whether this suppression was affected by the use of a biologically relevant vehicle (chylomicron emulsion).

6.5.1 Comparison of the proliferation of PBMC from healthy individuals and cancer patients after exposure to THC

Under these experimental conditions, the PBMC of patients recovering from chemotherapy to treat NSGCT responded in a similar manner to those from healthy volunteers. However, the degree of this suppression at statistically significant exposure levels (10 and 20 $\mu\text{g/mL}$) was more pronounced in healthy volunteers.

All proliferation profiles show a dose-dependent suppression of PBMC proliferation in response to THC, but the PBMC of patients in the third cycle of chemotherapy seemed to have a much more pronounced suppression of proliferation at 15 and 20 $\mu\text{g/mL}$ THC, reducing cell numbers to 10 and 7% of vehicle treatment respectively compared to 35-50% reductions seen in the other four patients. This could be related to the cycle of chemotherapy that the patient is receiving. The repeated and prolonged administration of chemotherapy drugs reduced the number of total white blood cells, as shown by the higher WBC in first cycle and lower WBC in second and third cycle patients (despite similar lymphocyte numbers). It is possible that the distribution of the different subpopulations of WBC contained within the isolated PBMC was different in the patients receiving their first cycle of chemotherapy, possibly having a higher proportion of macrophages and NK cells, which are less receptive to THC. However, this trend was only observed in this small subset of patients and warrants further study on a larger scale.

The chemotherapy drugs that these patients were receiving varied slightly based on their prognosis, but all included the use of cisplatin and etoposide. Cisplatin is a platinum-containing alkylating agent that also prevents cell division by binding to DNA, causing them to be unable to be replicating and ultimately triggering apoptosis as well [281-283]. Once it is transported into the cell, cisplatin binds to DNA, preventing its replication and cell growth [284]. Previous work by Vancurova and colleagues showed that cisplatin also has a dose-dependent antiproliferative effect on PBMC after PHA stimulation, suggesting that it work synergistically with THC if co-exposed under these experimental conditions [285].

Etoposide is a plant-derived anticancer drug, and prevents cell division by inhibiting DNA topoisomerase II, an enzyme responsible for unwinding DNA during its replication. This inhibition prevents DNA strands from re-ligating, breaking the DNA strand and causing cell cycle arrest and apoptosis [81, 164, 286]. Inhibition of DNA topoisomerase II by etoposide triggers apoptosis by activation of the p38 mitogen-activated protein kinase signalling pathway, which in turn plays an integral role in the activation of the caspase-dependent apoptotic pathway [287, 288]. Interestingly, THC has also been shown to be involved in activating the caspase-dependent apoptotic pathway by regulating Bcl-2 and caspase activity [133]. Previous cell work by Mühl and colleagues showed a dose-dependent increase in DNA fragmentation and release of chemokines associated with apoptosis in PBMC [289]. It is likely that this apoptotic effect of etoposide would synergise with the antiproliferative effect of THC, but since proliferation was not assessed as part of that study, etoposide's potential interaction with THC in this context is only speculative.

In this study THC dissolved in DMSO had a dose-dependent antiproliferative effect when exposed to PBMC isolated from both healthy individuals and cancer patients. While the concentration at which this antiproliferative effect is statistically significant is the same in both

sources of PBMC (10 µg/mL), the proliferation of PBMC from healthy individuals were more suppressed at that concentration compared to PBMC from cancer patients (Table 6-1). This suggests that PBMC from cancer patients were less sensitive to THC, either because of differences in lymphocyte cannabinoid receptor expression, or differences in subpopulations of PBMC that were not suppressed by THC and stimulated by PHA. This also suggests that those PBMC were still suppressed by a certain extent by chemotherapy drugs. However, since THC was still able to suppress proliferation in these PBMC at a similar threshold concentration, it may mean that THC is able to cause additional suppression to PBMC isolated from subjects receiving chemotherapy, potentially suppressing their immune systems further if they are continually taking Marinol®. Possible evidence for this is that the lymphocyte counts of patients were on the lower range of normal (from 0.7 to 1.2 million cells/mL). Unfortunately, since the study requires a large number of PBMC in order to successfully conduct (approximately 5 million cells are required per blood sample), it would not be possible to isolate sufficient PBMC closer to the nadir of chemotherapy. While this method of proliferation assessment was appropriate for the preliminary nature of this study, alternative assessments of proliferation, apoptosis or cytokine expression may be more suitable for subsequent work (some of which are described in Chapter 7).

Relating the current work to that previously performed, the concentrations of THC that suppressed proliferation in under these experimental conditions also had other effects on immune cells. Klein *et al* found that THC suppressed proliferative responses of T cells to mitogens at concentrations of 8 µg/mL and above (one mitogen used was also PHA, [125]). Zhu *et al* showed that at concentrations of 5 and 10 µg/mL, THC triggered apoptosis in T cells and macrophages through suppression of the anti-apoptotic protein Bcl-2 [133]. At 10 µg/mL, THC was also found to suppress the cytolytic activity of mature cytotoxic T cells [131].

6.5.2 Comparison of THC associated with chylomicron emulsion with THC dissolved in DMSO

Figure 6-2 shows the effect of THC in a CM emulsion. It would seem that THC associated in CM vehicle reduces its antiproliferative effect on PBMC under these experimental conditions. While the concentration at which this antiproliferative effect is statistically significant was the same in both vehicles (10 µg/mL), the proliferation of PBMC from the exposure to THC in DMSO vehicle was more suppressed at that concentration compared to PBMC exposed to THC in CM vehicle (Table 6-2). However, since THC is still able to exert an effect on PBMC at the same this still suggests that THC is able to dissociate from CM to enter cells. It is possible that THC is drawn out of CM by the action of LpL or a concentration gradient with the surrounding medium. Since THC has high binding affinity with serum albumin, the bovine serum albumin present in fetal bovine serum could also have drawn out THC from CM [234].

The process of isolating CM and PBMC was technically challenging and often required separate blood sampling to isolate CM and PBMC to be successful. The production of human CM depends on the individuals gastric emptying after a meal, age, insulin resistance and a number of other extraneous factors [44]. In healthy volunteers, this delay varied from 2-4 hours after a fatty meal. Due to the variability of cancer subjects' age and availability, it was not possible to reliably isolate CM from their blood sample, and therefore the CM portion of the PBMC study was not performed on cancer patients. During the development and optimisation of this cell proliferation assay, it was found that treating an individual's PBMC with CM isolated from another individual had a mild but statistically significant antiproliferative effect, thereby necessitating the treatment of the PBMC of an individual with THC associated with their CM (shown in Chapter 2).

Relating the data obtained here to the THC biodistribution study described in Chapter 5, the concentrations of THC found in the MLN one hour before plasma $T_{\max}$ and at $T_{\max}$ are high enough to potentially be antiproliferative to lymphocytes there (a mean concentration of 8.6 and 8.3 $\mu\text{g/g}$ respectively), but not high in other organs such as the spleen, brain or liver to be immunosuppressive (where the maximum was 1.2 $\mu\text{g/mL}$, found in the spleen at $T_{\max}$). This suggests that THC orally administered under conditions that facilitate intestinal lymphatic transport creates a high local concentration in the MLN after absorption, and could exert a local antiproliferative effect which could have knock-on effects for the body. For example, the MLN act as an important first line of defence from pathogenic and commensal intestinal bacteria penetrating the systemic immune system [272].

6.5.3 Limitations of the study

Due to the preliminary and exploratory nature of this study, it was only possible to assess proliferation of PBMC as a whole, so the populations of different types of WBC within PBMC isolated from each individual is not known. Therefore, the antiproliferative effect of THC on individual white blood cell populations such as B- and T-cells also could not be examined. The potential apoptotic effect of THC was also not assessed, only the effect on proliferation in response to mitogenic stimulation.

There were a number of limitations in the method for the isolation of PBMC, there was no lysis step to ensure no red blood cell contamination in the final cell suspension. Likewise, the CM vehicle used was generated from a separate blood sample from the same individual after ingestion of a high-fat meal, and it is unknown whether there are other soluble components of that sample that persisted through the isolation process and is also exposed to PBMC. However, there were multiple wash steps in the PBMC and CM isolation protocol, and on visual inspection of PBMC during cell counting and after seeding into 96-well plates no

contaminating red blood cells were found. It was also unknown how much of THC spiked to cell culture medium in either DMSO or CM vehicle was exposed to PBMC, nor the relative levels of LpL expressed on the surfaces of PBMC to interface with CM.

As part of the assay, it was necessary to normalise the absorbance values obtained to the absorbance of vehicle-treated cells. While this enabled more consistent comparisons of the effect of THC between different plate readings, it did not allow comparisons of absolute absorbance values, which may be indicative of the overall proliferation of an individual's PBMC.

Due to variability in blood sampling times for cancer patients, it was not possible to reliably isolate both CM and PBMC from a single blood sample, although each patient was advised to eat a high-fat meal prior to sampling. CM are produced some time after ingestion of a high fat meal, but the exact time varied based on a large number of factors, most notably age and insulin resistance [44]. As a result, insufficient CM were isolated from blood samples and it was not possible to assess the antiproliferative effect of THC associated with CM on PBMC isolated from patients recovering from chemotherapy.

Regarding the levels of THC used in this study, it is unlikely that the PBMC of patients taking Marinol® would be exposed to these high levels of THC in blood, even though the indications for Marinol® can be up to 20 mg/day, and heavy marijuana smokers can use up to 20 mg/kg/day [145]. The highest concentration of THC found in rat plasma after oral administration of THC in a lipidic formulation to rats was 232 ± 70 , which is nearly two orders of magnitude lower than the concentrations used in this study. However, the concentrations used in this study were similar to those found in the mesenteric lymph node homogenate after oral administration of THC in a lipidic formulation (8.6 µg/g), which suggests that high levels

of THC could be present in the intestinal lymphatics and potentially affect the lymphocytes residing within.

6.6 Conclusions

This study shows for the first time that THC suppresses proliferation in PBMC isolated from patients recovering from chemotherapy to treat non-seminomatous germ cell tumours in a dose-dependent manner. PBMC isolated from these patients were less sensitive to THC than PBMC isolated from healthy individuals, and THC suppressed PBMC isolated from patients recovering from chemotherapy to a lesser degree compared to PBMC from healthy individuals.

This work also shows that THC associated with CM emulsion is also able to have a dose-dependent antiproliferative effect on PBMC, suggesting that THC is able to dissociate from CM and enter cells, although further study is required to elucidate the exact mechanism that this occurs. However, THC associated with CM emulsion was less suppressive to PBMC than THC dissolved in DMSO at the same concentrations.

Chapter 7: General Conclusions and Future Work

7.1 General Conclusions

The results presented in this work demonstrates that delta-9-tetrahydrocannabinol (THC) is transported via the intestinal lymphatic system, especially after oral administration in the presence of dietary fats. It also showed that THC has a dose-dependent antiproliferative effect on peripheral blood mononuclear cells (PBMC) *in vitro*, that THC is also able to exert this effect while associated with chylomicron (CM) emulsion.

7.2 Implications

In vitro lipolysis studies showed that using commercially available dose strengths of 2.5, 5 and 10 mg, up to half of the administered dose of THC in a lipidic formulation is solubilised in mixed micelles. As concentration of THC in lipidic formulation increased, THC increasingly partitioned to the undigested lipid phase. Compared to other lipophilic drugs subjected to similar studies, THC is solubilised in the aqueous micellar phase in a similar proportion to other drugs that were assayed using the same method [160, 176, 217]. The solubilised fraction of THC under these experimental conditions was also higher than the reported oral availability of THC in Marinol®, which is between 6-20% [265], which suggests that there are other factors that reduce the absorption of THC.

CM association studies showed the strong intestinal lymphatic transport potential of THC. With an association value of 72% in the experimental conditions, THC has a similar or higher CM association compared to other lipophilic drugs that have been shown to be transported via the intestinal lymphatics such as halofantrine, Vitamin D₃, and Vitamin E [244, 290]. Using the linear regression between association with chylomicrons and % of dose absorbed via intestinal lymphatics, potentially up to 20% of an administered dose of THC would be absorbed through the lymphatic system [1].

The oral pharmacokinetic study confirmed that the absorption of THC is facilitated by activation of intestinal lymphatic transport, since there was a threefold increase in absolute oral bioavailability when THC was administered in a lipidic formulation compared to a lipid-free formulation. This increase in bioavailability is similar to that observed by Caliph *et al* when halofantrine was administered in a lipidic formulation compared to a lipid-free formulation [251] and by Gershkovich *et al* with a synthetic cannabinoid PRS,211-220, which had an absolute oral bioavailability of $13 \pm 3\%$ in lipid-free formulation, and $35 \pm 6\%$ in lipidic formulation [75]. Therefore, patients taking Marinol® may increase their exposure to THC by up to three-fold if intestinal lymphatic transport is activated.

Intestinal lymphatic transport was further confirmed with biodistribution studies of THC after oral administration showing that the concentration of THC in mesenteric lymph node (MLN) homogenate was 30-fold higher than plasma at the time of maximal plasma concentration. The mean concentration of THC in lymph nodes at oral $T_{\max}$ was $8.6 \mu\text{g/mL}$, which was nearly 10-fold higher than the concentration found in other internal organs. This was more pronounced one hour before oral $T_{\max}$, confirming that THC enters the lacteal before entering general circulation. Therefore, intestinal lymphatic transport of THC could create high local concentrations there, at levels previously shown to be immunosuppressive *in vitro*. This could also change how Marinol® is prescribed by physicians, reduce variation in efficacy and reduce the dose prescribed.

Pharmacokinetic modelling showed that the intravenous concentration-time profile of THC fits a two-compartment model, with a high distribution coefficient from the first to the second compartment, most likely fatty tissue. This is in agreement to previous work that performed pharmacokinetic analysis using a compartmental approach [184, 265]. For the first time, the

oral pharmacokinetic profile of THC was obtained and shown to fit a one-compartment pharmacokinetic model.

Finally, the *in vitro* study on the antiproliferative effect of THC on peripheral blood mononuclear cells (PBMC) showed that there is a dose-dependent reduction in proliferation. At concentrations 10 µg/mL and above the capacity for PBMC to proliferate in response to a mitogen is significantly suppressed. Since THC is a lipophilic molecule, it has poor solubility in aqueous solutions such as cell culture media and generally requires dissolution in a lipophilic or organic vehicle. Previous work has used propylene glycol [238], ethanol [202, 204, 291] or DMSO as vehicle for solubilising THC [206, 292]. Propylene glycol is very viscous, and is difficult to accurately measure. Ethanol is highly volatile, and may cause significant concentration change in long-term storage and use. DMSO has neither of these disadvantages, so was therefore deemed the most appropriate solvent to use for cell work. Even though DMSO has been shown to have its own cytotoxic effects on cells the concentration at which this effect is seen is above 10%, much higher than the 0.1% used in this work [293].

The main vehicle of drug transport in the intestinal lymphatic system after absorption of dietary fats is CM, generated by enterocytes [236, 273]. Cells require lipoprotein lipase (LpL) present on its cell surface in order to lipolyse CM and access its contents [43, 45, 47]. Therefore, it was important and relevant to assess whether THC is able to dissociate from CM and act as it would dissolved in another vehicle such as DMSO. The results show that this is indeed the case, as THC was able to suppress proliferation in a dose-dependent manner, albeit to a lesser extent compared to THC dissolved in DMSO. LpL has been found to be secreted by many different macrophages [43, 294], Natural Killer cells [277], and B cell lymphocytes [52]. Work by Lichtenstein *et al* also shows that macrophages specifically resident in the MLN express

and secrete LpL, suggesting that drugs associated with CM such as THC are able to dissociate in lymph nodes [50].

Another observation from this cell study was the reduced antiproliferative effect of THC on PBMC from cancer patients compared to PBMC from healthy individuals. This suggests that the PBMC from patients were still affected by the chemotherapy drugs, one of which has been previously shown to be antiproliferative to PBMC [285].

A conclusion in this work that links all the aims together is the biodistribution study, which showed that the concentrations found in the MLN after oral administration of THC in a lipidic formulation was also potentially antiproliferative – the average concentration found at oral $T_{\max}$ and one hour prior was 8.6 $\mu\text{g/g}$, near the 10 $\mu\text{g/mL}$ that was the significantly antiproliferative concentration level in the PBMC study. This suggests that these antiproliferative effects seen *in vitro* could translate *in vivo*. The concentration of THC in MLN also corresponds to immunosuppressive effects of THC studied *in vitro*, such as reducing the proliferation of splenocytes [125, 136, 295], inhibiting adenylate cyclase [291], inducing apoptosis in macrophages [133], and suppressing cytotoxic T cell function [140].

7.3 Recommendations for Future Work

Whilst the research undertaken for this thesis has addressed some areas that were previously lacking, it also indicates where further work would be beneficial. In particular, there are a number of further studies that could be done in order to prove the extent of intestinal lymphatic transport and elucidate the antiproliferative effect of THC in patients with immunocompromising conditions.

7.3.1 Measurement of intestinal lymphatic transport of THC following oral administration in lipid-free and lipidic formulation

The work done over the course of this PhD measured the concentration of THC in the lymph nodes draining the intestine, but did not directly measure the extent of its intestinal lymphatic transport. If possible, a most convincing study to conduct would be similar to the one by Gershkovich *et al* on two structurally similar synthetic cannabinoids, dexanabinol and PRS-211,220 [75]. A few experimental elements of this study were also done on THC – the calculation of physicochemical parameters, the experimental association with CM, and the intravenous and oral pharmacokinetic studies, with the oral study comparing a lipid-free formulation with a lipidic one.

To assess intestinal lymphatic transport, a freely moving lymph cannulated rat model was used based on previous methods which allow sampling of lymph but also normal lymph flow during the recovery period post-surgery [241, 296]. The right jugular vein and stomach were also cannulated. This surgery would allow intragastric administration of formulations, and blood and lymph collection to obtain drug-time profiles. The concentration of THC in the lymphatic system would also be obtained, which would reflect on over what period that THC is above an immunosuppressive level dictated by PBMC studies. The cumulative lymphatic transport of THC can also be determined, as well as that lymphatic transport's contribution to overall oral bioavailability. With regard to the cell work done in this PhD, these studies would also highlight the window by which the concentration of THC in the lymphatic system is above the significant antiproliferative concentration for PBMC (10 µg/mL). In the figure examining the plasma and lymph concentrations, lymph levels of PRS-211,220 remained above 10 µg/mL until 8 hours after administration [75]. If THC were transported to a similar extent by the intestinal lymphatic system, it would likely follow a similar lymph concentration profile and exert an immunosuppressive effect over that time.

7.3.2 *In vivo* assessment of immunosuppression after oral administration of THC

In the pharmacokinetic and biodistribution studies performed as part of the research for this thesis, THC was confirmed as being transported via the intestinal lymphatics. As mentioned previously, the concentration of THC found in the MLN was high, and corresponded to immunosuppressive effects previously seen *in vitro*. In order to assess the extent of immunosuppression *in vivo*, the immune functions of rats given THC in lipidic formulation can be assessed. The recommended dosing for Marinol® varies according to the desired effect, and is reversed when treatment is stopped. To be used as an antiemetic (the most common prescription for Marinol®), most patients respond to 5 mg capsules three to four times daily, depending on chemotherapy cycle. In order to replicate this regimen, a similar dosing could be done in animal studies, and the chronic effect of THC on the immune system while receiving this dosing regimen could be observed. If THC persists in the lymphatic system after oral administration, its immunosuppressive effect might also persist.

The previous studies assessing the immunosuppression brought on by THC *in vivo* did not administer it orally. The study which found that THC reduces mouse resistance to *Legionella pneumophila* administered the drug by intravenous injection [146]. Another study by Blumstein *et al* which found that THC reduced mice resistance to secondary *Candida albicans* infection administered the drug by intraperitoneal injection [151]. Specter *et al* also administered drug by intraperitoneal injection in their study which found that THC augmented retroviral induced immunosuppression in mice [292]. LeCapitaine *et al* chronically administered THC by intramuscular injection in their study on Rhesus Macaques [152]. One study in which THC was given orally was the one by Buchweitz *et al* which found that THC modulates airway response of mice to influenza infection, once every day for five days, and influenza infected on the third day [149].

Massi *et al* studied many of the functional and biochemical parameters of the immunosuppressive effects of acute and repeated doses of THC (one dose, and 7 or 14 days of daily dosing). The group assessed splenocyte proliferation by aseptically removing spleens from mice receiving THC and harvesting cells from them. The group also assessed white blood cell and lymphocyte counts, natural killer cell activity, and expression of interleukin 2, 4 and interferon gamma [268]. The study showed many different aspects of immunosuppression after repeated dosing, such as reduced splenocyte proliferation, suppressed NK activity, reduced expression of interferon gamma, and reduced numbers of T lymphocytes with a mirrored increase in B lymphocytes. However, THC was administered subcutaneously in this study, and it would be very interesting to assess if oral administration in this case and activation of intestinal lymphatic transport would produce a higher or more sustained level of immunosuppression, considering the alternative route of absorption.

Other aspects of immune function can be assayed – blood samples can be taken and tested for haematological parameters such as red blood cell, white blood cell and lymphocyte counts, haemoglobin levels and platelet counts. In addition, the immune organs can be collected at the end of experiments. The spleen, thymus and MLN can be weighed and/or lymphocytes isolated from them and studied for cytokine expression.

7.3.3 Immunohistochemistry work to further elucidate the anti-proliferative effect of THC

The cell work done in this PhD has looked at the reduction of proliferation elicited by THC in PBMC. There are many different types of PBMC isolated from blood, including lymphocytes (B-, T- and Natural Killer cells) and monocytes (macrophages and dendritic cells). THC has been shown to have differing effects on each of these cell types, most notably and well-characterised to T-cells [131, 134, 141]. Since the PBMC isolation protocol lacks cell-type-

specific staining or further separation, it was not possible to specifically tell which cell populations were being affected by THC. Since different types of PBMC have very different functions and THC affecting one cell-type over others may have a knock-on effect on immune function of that individual. It was also not possible to assess the expression or change of cytokine expression in response to THC. THC has been shown to change cytokine expression in a myriad of ways, reviewed in [297, 298]. Therefore, the larger story of the immunosuppression by THC is likely to be much more complex than only reducing proliferation in a dose-dependent manner.

One way to do this would be to use fluorescence immunohistochemistry paired with flow cytometry of isolated PBMC. Different cell-types can be stained for their specific cell-surface proteins – helper T cells, which regulates the activity of other immune cells by releasing cytokines, express the surface protein CD4; cytotoxic T cells, which are responsible for killing cancer or damaged cells, express the surface CD8 [200, 299]; B cells express the cell surface markers CD19 and CD20 [300, 301]; Natural Killer cells express Neural Cell Adhesion Molecule, or CD56 [302] and macrophages express cell surface markers such as CD14 and CD33 [294, 303]. All these markers can be stained for by antibodies, and the numbers of cells expressing these markers can be monitored under the same experimental setup (pre-treatment with THC, then stimulation with mitogen). This way, the proliferation of the different PBMC can be monitored more specifically, and which populations are more affected by THC. This experiment would give a more detailed breakdown of THC's effect, and whether this effect is mirrored in cancer patients receiving or recovering from chemotherapy.

By the same or similar method, intracellular molecules can also be assessed by flow cytometry. Since previous work has shown that THC activates different aspects of cannabinoid signalling, apoptosis in particular, and a number of cytokines are secreted which can also be stained for

by antibodies. For example, a commonly expressed cytokine after THC treatment is Interferon-gamma (IFN- γ) and Interleukin 2 (IL-2), both of which are critically involved in the immune response [112, 134, 141, 304]. Again, the regulation of cytokine secretion by THC can be monitored in the experimental setup to assess whether this regulation is still present in cancer patients receiving or recovering from chemotherapy.

There seemed to be some interaction between THC and the cancer chemotherapy drugs used on cancer patients sampled in this work. The time at which their PBMC are sampled was during the recovery period after their chemotherapy, when these PBMC populations have mostly been replenished, and the effects of chemotherapy drugs such as etoposide and cisplatin (which were given to all patients in the study) were subsiding. More specifically, both etoposide and THC have been shown to promote apoptosis by cell cycle arrest at the G2/M phase [117, 305, 306]. Both compounds also have effects on the Mitogen-Activated-Protein Kinase (MAPK) signalling pathway. For THC, Borner *et al* found that cannabinoids are able to activate MAPK in Jurkat cells (an immortalised T-lymphocyte cell line) [112], and Kobayashi *et al* showed that activation of cannabinoid receptors also activates MAPK in a dose-dependent manner [307]. Tsai *et al* showed that inhibition of the MAPK pathway in a lung cancer cell line decreased their sensitivity to etoposide, suggesting that etoposide exerts its cytotoxic effects via MAPK [308]. Cisplatin and THC have been shown to be involved in the caspase-dependent apoptotic pathway, which also suggests there would be overlap in their effect [133, 309]. As previously mentioned, Vancurova and colleagues also showed that cisplatin had a dose-dependent antiproliferative effect on PBMC after PHA stimulation, suggesting that it work synergistically with THC if co-exposed [285]. It would therefore be plausible that if THC were given while cisplatin or etoposide were still in effect, THC may cause additional suppression of immune cells, potentially further compromising patients taking it.

One possible way to assess this compounding effect of THC on etoposide and/or cisplatin would be to attempt to repeat the proliferation assay in PBMC from cancer patients receiving chemotherapy, at a time nearer the time of drug administration so that these drugs are very much still in effect. After the preliminary results obtained in cancer patients, a randomised placebo-controlled trial could be conducted in the same patient population. In a clinical setting, one of the effects of immunosuppression is increased chance of infection, as shown by some work conducted in animals to monitor the *in vivo* effect of THC [146, 149, 151]. Therefore, incidence of infection could be monitored in patients receiving chemotherapy, and also taking Marinol® to treat nausea. If THC compounded the immunosuppression brought on by chemotherapy, patients taking Marinol® would be more susceptible to infection.

7.3.4 Translate the experiments done to other cannabinoids

Even though THC is the most abundant and psychoactive phytocannabinoid, there are many other cannabinoids that are present in strains of cannabis. Of note, there are the metabolites of THC – which include cannabinol (CBN), which is structurally related to THC but is less abundant in the cannabis plant [310]. It is also a partial agonist of CB1 and CB2 receptors, but has lower affinity for both of them compared to THC [311]. Other phytocannabinoids include cannabigerol, cannabivarin and cannabichromene [103, 312].

While this work has been ongoing, my colleague Atheer Zgair has been doing related work on another major cannabinoid, cannabidiol (CBD). CBD is a non-psychoactive phytocannabinoid, and much of its mode of action has been linked to antagonism of the cannabinoid receptors rather than activation [265, 313]. CBD has also been shown to be antipsychotic, and reverses some of the psychoactive effects of THC [314-316]. CBD is currently part of two pharmaceutical preparations, Sativex®, an oromucosal spray approved for treatment of spasticity due to multiple sclerosis [101, 317], and Epidiolex, an investigational

drug for treatment of Dravet and Lennox-Gastaut syndromes, both types of epilepsy in infants [318]. Much of the same studies have been conducted (*in vitro* lipolysis, CM association, pharmacokinetic studies, cell work), and it is also likely that CBD is transported in the intestinal lymphatics.

In addition to phytocannabinoids, synthetic cannabinoids have also been developed to mimic their effects, possibly without psychoactive side effects, binding to cannabinoid receptors at all, or are selective for CB1 or CB2. Nabilone is one of those cannabinoids, and was approved in 1985 as an analogue to THC (Figure 7-2 shows the chemical structures of both cannabinoids). It also acts as an agonist to CB1 and CB2 with no preference for either receptor [319]. It was proven to be an effective antiemetic in patients receiving cancer chemotherapy [154, 320]. Much like Marinol®, it is also approved for the treatment of nausea and vomiting associated with chemotherapy, and has been used in many clinical trials as a sleep aid, analgesic and pain associated with multiple sclerosis [321, 322].

Dexanabinol or HU-211 is an enantiomer of 7-hydroxy- Δ^6 -tetrahydrocannabinol, which was discovered in 1989 as a non-psychotropic blocker of *N*-methyl-D-aspartate receptors (NMDA) with no cannabinoid receptor agonism [323]. It is currently being studied for the treatment of brain injury and stroke [324], and has a similar pharmacokinetic profile to THC [102]. Previous studies have shown that Dexanabinol has been transported in the intestinal lymphatics when administered in a lipidic formulation, but did not contribute significantly to its overall absorption [75]. WIN 55,212-2 is a specific agonist for the CB1 receptor, which produces similar effects similar to THC even though it has a different molecular structure (Figure 7-2 D) [325]. It is a potent analgesic in a rat model of neuropathic pain [326]. JWH-133 is a specific agonist for the CB2 receptor which has also been shown to exert immunosuppressive effects on T cells via CB2 receptor activation [327].

These synthetic cannabinoids were discovered nearly 20 years ago, but have recently resurfaced in the last ten years - Nabilone was approved by the FDA in 1985, but marketing for the drug began in 2006; Dexanabinol was discovered in 1989 and is currently in Phase 1 trials; JWH-133 was described in 2000 and is currently in a pre-trial phase [328]; and WIN 55,212-2 has only been used in a cell culture and non-human animal models [184, 326].

Considering the structural similarities of all these alternative cannabinoids to THC, they also likely have similar physicochemical properties, particularly lipophilicity. It is likely that these compounds have moderate to high association of cannabinoids, and have high intestinal lymphatic transport potential. In particular, the increased specificity for either cannabinoid receptor of JWH-133 or WIN 55,212-2 can be particularly important if they are transported in the intestinal lymphatics to the same extent as THC, and act upon immune cells there.

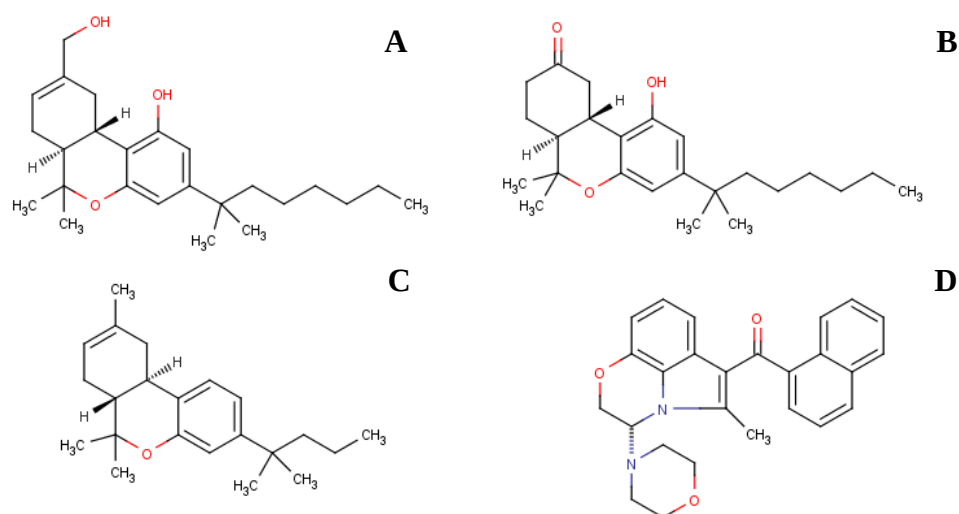


Figure 7-2. Chemical structure of synthetic cannabinoids, Dexanabinol (A), Nabilone (B), JWH-133 (C), and WIN 55,212-2 (D)

The predominant cannabinoid receptor present in the intestinal lymphatics is CB2, therefore JWH-133 and Nabilone which have high binding affinities for the receptor may have a greater local immunosuppressive effect than WIN 55,212-2 and THC, who have lower binding affinities for CB2 [107, 311].

In summary, the additional studies listed above, I feel, would expand upon the conclusions already drawn, and would provide more insight into the safety and efficacy of medicinal cannabinoids.

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