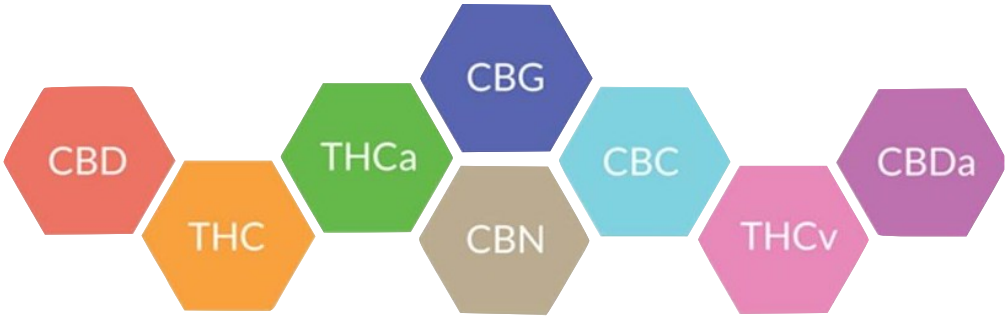
School of Science & Technology

Postgraduate Program of Studies:

Chemical and Biomolecular Analysis

Postgraduate Dissertation

«Mechanism of action of cannabinoids in human body and methods of determination of cannabinoids in human urine»



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Patras, Greece, June, 2024

«Mechanism of action of cannabinoids in human body and methods of determination of cannabinoids in human urine»

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To my family that supports me every day

And to Jason.

Abstract

This thesis deals with a hot topic of recent years, cannabinoids. Cannabinoids, having a history of many years of use, have invaded our lives and are now being consumed at a rapid pace. This sometimes leads to extremes in their use, sometimes leading to their becoming addictive illegal substances. However, despite their legal prohibition in most countries of the world, it has been found that cannabinoids can function not only as addictive substances but also as an aid in the treatment or even suppression of serious diseases such as cancer and epilepsy.

In addition to the above, the metabolic pathways and the pathways of action of the no-fibroids in the human body, their synthesis and degradation and the way in which they interact with some systems of the body, such as the Central Nervous System, are analysed.

In this context, some of the most important diseases or conditions for which the use of cannabinoids is a therapeutic tool and the legislation that prevails in Europe and Greece are discussed. Finally, the aim of this thesis is to mention the main analytical methods through which the use of cannabinoids in a population of individuals can be determined through the analysis of urine samples.

Keywords

Cannabinoids, THC, CBD, Endoccanabinoid System, cancer, urine

Περίληψη

Η παρούσα διπλωματική εργασία πραγματεύεται ένα φλέγον ζήτημα των τελευταίων ετών, τα κανναβινοειδή. Τα κανναβινοειδή έχοντας ιστορία πολλών ετών χρήσης, έχουν εισβάλλει στη ζωή μας και καταναλώνονται με γοργούς ρυθμούς πλέον. Το γεγονός αυτό οδηγεί άλλοτε σε ακρότητες ως προς την χρήση τους, καταλήγοντας να αποτελούν εξαρτησιογόνες παράνομες ουσίες. Ωστόσο, παρά τη νομική απαγόρευση τους στις περισσότερες χώρες του κόσμου έχει διαπιστωθεί ότι τα κανναβινοειδή μπορεί να λειτουργήσουν όχι μόνο ως εξαρτησιογόνες ουσίες αλλά και ως βοήθημα στην αντιμετώπιση ή και καταστολή σοβαρών ασθενειών, όπως ο καρκίνος και η επιληψία.

Παράλληλα με το παραπάνω, αναλύονται οι μεταβολικές οδοί και τα μονοπάτια δράσης των κανένα ινοειδών στον ανθρώπινο οργανισμό, σύνθεσης και αποσύνθεσής τους αλλά και ο τρόπος με τον οποίο αλληλεπιδρούν με κάποια συστήματα του οργανισμού, όπως το Κεντρικό νευρικό Σύστημα.

Στο πλαίσιο αυτό, γίνεται λόγος για κάποιες από τις σημαντικότερες ασθένειες ή καταστάσεις για τισ οποίες η χρήση των κανναβινοειδών αποτελεί θεραπευτικό μέσο καθώς και η νομοθεσία που επικρατεί στην Ευρώπη αλλά και στην Ελλάδα. Στόχος τέλος της παρούσας διπλωματικής είναι ανά αναφερθούν οι βασικότερες μέθοδοι ανάλυσης, μέσω των οποίων μπορεί να διαπιστωθεί σε έναν πληθυσμό ατόμων εάν γίνεται χρήση κανναβινοειδών, μέσα από την ανάλυση δειγμάτων ούρων.

Λέξεις – Κλειδιά

Κανναβινοειδή, THC, CBD, Ενδοκανναβινοειδές Σύστημα, Καρκίνος, Ουρία

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List of Abbreviations & Acronyms

CBD: Cannabidiolie

CBDA: Cannabidiolic acid

CBGA: Cannabigerolic acid

CBN: Cannabidiol

THC: Tetrahydrocannabidiol

THCA: Tetrahydrocannabinolic acid

MS: Mass spectrometry

GC: Gas Cgromatography

LC: Liquid Chromatography

FID: Flame Ionization Detector

TLC: Thin Layer Chromatography

HPLC: High- Perfomance Liquid Chromatography

FAAH: Fatty Acid Amide Hydrolase

CNS: Central Nervous System

PNS: Peripheral Nervous System

MAGL: Monoacylglycerol Lipase

AG: 2-Arachidonylglycerol

AEA: Anandamide

cAMP: Cyclic Adenosine Monophosphate

DAG: 1,2-Diacylglycerol

ECS: Endocannabinoid System

CHAPTER 1: CANNABINOIDS AND THEIR CATEGORIES

1.1 Cannabinoids

Hemp was first used in 3000 BC, for the purpose of textile production, because of its durable fibre. The plant was present in many places around the world and today three species of cannabis are known: Cannabis sativa, Cannabis indica and Cannabis ruderalis.

Ancient medical texts from India and China mention that its medicinal use gradually spread to the Arab world through Persia in the tenth century. Later, Cannabis sativa was introduced to the continent of Europe and at the same time attracted the scientific interest of America.

The cannabis plant was used in various activities, including religious ceremonies, but it also had many medicinal actions similar to those attributed to it today.

Hemp was incorporated into the British first into the British Pharmacopoeia and then into that of the United States. Extracts of the plant were used as sedatives, hypnotics and more.

In recent years, there has been a growing interest in the medicinal benefits of cannabis. California was the first state to legalize the use of botanical cannabis for medical purposes with the supervision of physicians through the Compassionate Use Act in 1996.

Cannabinoids are essential for controlling synaptic transmission in the Central Nervous System (CNS) through the activation of CB1 and CB2 receptors, although each receptor has its own specific mechanism. Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are the primary endocannabinoids present in mammals. In 1992, Anandamide was identified as the first endocannabinoid by researchers. It is a neurotransmitter produced from the non-oxidative metabolism of arachidonic acid, a type of fatty acid. Anandamide is derived through various processes from N-arachidono-phosphatidylethanolamine and is metabolized by the enzyme FAAH into ethanolamine and arachidonic acid. Anandamide impacts both the CNS and the PNS by activating CB1 and CB2 receptors. It exists in small amounts in the body and is only there temporarily because of FAAH (1). 2-AG was found in the year 1995. 2-AG acts as the natural ligand for the CB2 receptor. It consists of arachidonic acid and glycerol. Unlike anandamide, 2-AG is plentiful in the CNS and relies on calcium. MAGL and FAAH separate it through hydrolysis. In summary, it acts as a CB1 receptor agonist (2).

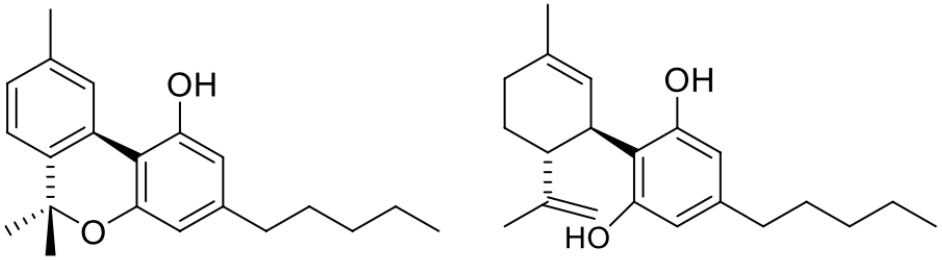

Figure 2: Representation of the natural components of cannabis (cannabinol on the left and cannabidiol on the right)

Cannabinoids are the terpenoid substances found in cannabis and its derivatives, with (-)D9-THC serving as the primary psychoactiveelement. This substance is an optically active compound, can dissolve in fats, and does not dissolve at all in water (3,4).

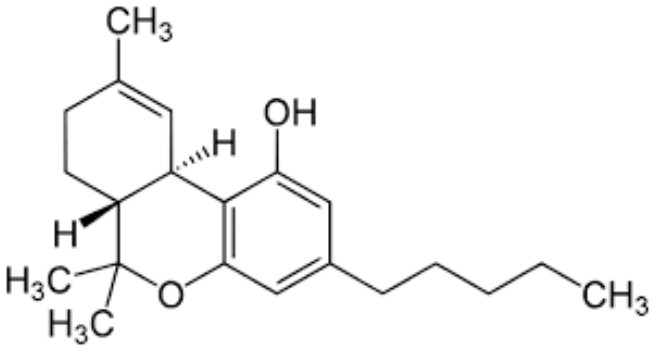

Figure 1: Visualization of (-)Δ^9-THC

Substances like cannabidiol (CBD) and cannabinol have been extracted from hemp. CBD does not have psychoactive properties, however, it does hinder the impact of THC on the nervous system.

As of 1986, more than 300 cannabinoid analogs had been created for the purpose of researching their activity related to structure (5). Because of the complex stereostructure of (-)Δ9-THC, there was an emergence of a requirement for a receptor that engages with cannabinoids (6). The cloning of CB1, the first cannabinoid receptor, took place in 1988. The identification of CB2, the second cannabinoid receptor, in 1993, showed that it is mainly found in immune cells. Finding the CB1 receptor in the brain prompted the understanding that there are likely endogenous compounds that bind to it, given that cannabinoids are not indigenous to the brain. In 1990, the first discovery of an internal cannabinoid compound was made, which had a structure akin to arachidonic acid ethanolamide. The cannabinoid compound was given the name anandamide because it derives from the Sanskrit word ananda, which signifies inner joy (7).

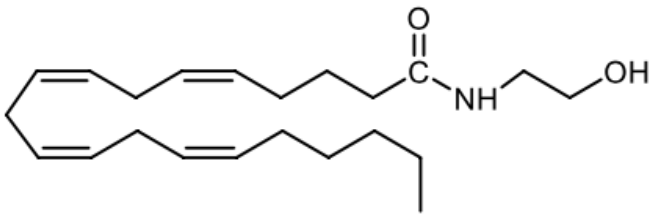

Figure 3: Anandamide

Extensive research on Anandamide has confirmed that it meets three key criteria as an endogenous cannabinoid molecule: production dependent on activity, activation of cannabinoid receptors, and biological inactivation.An increase in intracellular calcium (which leads to an increase in the activity of acyltransferase, the enzyme that produces the

precursor molecule N-arachidonoylphosphatidylethanolamine, NARE) or activation of specific neurotransmitter receptors leads to an increase in the synthesis of anandamide from phospholipid precursor molecules located in the cell membrane. The enzymes that biosynthesize anandamide have not yet been fully elucidated. N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) was considered the key biosynthetic enzyme (8), which hydrolyzes N-acylphosphatidylethanolamine to anandamide and phosphatidic acid. Recent data also indicate that α/β hydrolase acts as a biosynthetic enzyme (9), along with phosphatases like PTPN22, which target phosphoanandamide (10). FAAH is the enzyme responsible for breaking down anhydrase. This process is followed by this mechanism. Once Anandamide enters the cell, it is broken down into arachidonic acid and ethanolamine by the FAAH enzyme.

The anandamide transporter has the ability to operate in two different pathways. This indicates that it has the ability to remove anandamide from the cells (11,12). Immunohistochemical examinations were conducted on the aforementioned enzyme (FAAH), revealing its significant presence in cortical pyramidal neurons, hippocampal pyramidal cells, and Purkinje cells in the cerebellum. Similar results were observed in the human brain, with increased staining in the neocortex and medulla, thalamus, and minor variations in other areas (13).

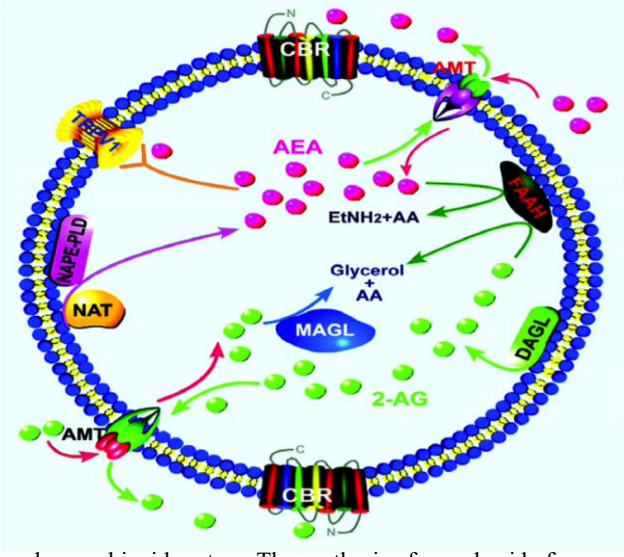

Figure 4: The endocannabinoid system. The synthesis of anandamide from membrane N-arachidonoylphosphatidylethanolamines is catalysed by NAPE-PLD, which releases anandamide and phosphatidic acid. Anandamide (AEA) is transported into or out of the cell via the specialized transporter AMT and, once taken up into the cell, is hydrolyzed by FAAH to ethanolamine and arachidonic acid. The main targets of anandamide are CB1 and CB2 receptors (extracellular binding site), as well as type 1 vanilloid receptors (intracellular binding site). 2-AG is also released from membrane lipids and the biosynthetic enzyme is DAGL, while it is hydrolysed by FAAH or MGL (mainly) and this leads to the release of glycerol and arachidonic acid. Transport of 2-AG is either via AMT or another transporter and the target for this is CBRs, but not TRPV1

1.1.1 THC- CBD

It is reported that cannabis causes both behavioral and physiological effects. The first ones include lack of concentration and the feelings of euphoria and relaxation, while the other ones include dry mouth and throat, increased appetite and high diastolic blood pressure (14). Apart from these effects, cannabis

THC is the main addictive substance that causes a number of temporary and dose-dependent effects, acting as an agonist at CB1 receptors (15) and causing its immediate effects via the type 1 endocannabinoid receptor (CB1R) (16). Symptoms of the reported effects are depression, impaired reflexes and increased cravings by the cannabinoid receptors CB1 and CB2. Once people consume cannabis, THC connects to cannabinoid receptors in the brain and central nervous system, resulting in a variety of influences such as euphoria, relaxation, altered time perceptin and increased appetite, commonly referred to as "high". In addition to its recreational use, THC has potential medicinal potential. It is being researched for its abilities to provide symptom relief in diseases such as chronic pain, chemotherapy-associated nausea and nausea, and muscle spasticity in diseases such as multiple sclerosis. D9- THC is characterized by a 21-carbonyl tricyclic structure without nitrogen and two chiral centers in trans-arrangement (17), and it is a volatile thick oil with high fat solubility, low water solubility, and a pKa of 10. In cannabis, D9-THC is found as a combination of monocarboxylic acids that can be readily and effectively decarboxylated when heated (17). Exposure to air, heat, or light can cause degradation of the substance, which readily bonds to glass and plastic (18). Two theories explain the in vivo activity of Δ9-THC. The initial suggestion is that Δ9-THC, when discharged as glucuronide, functions by engaging with cellular and organic membranes in the brain, leading to a breakdown of membranes (19,20). The second one supports that Δ9- THC interacts with specific cannabinoid inceptors (21,22). Given that Δ9-THC acts on a various intercellular targets (ovoid receptors, the prostaglandin metabolic pathway etc), it is currently challenging to establish a single mechanism of action (23). Furthermore, cannabinoids inhibit macromolecular metabolism in a dosage- dependent way and have a variety of impacts on enzyme systems, hormone release and neurotransmitters (24,25). The evidence for multiple and widespread in vivo effects provides strong confirmation of the hypothesis of non- specific interaction for THC.

Studies have conclude that THC supplementation in animals refused anxiety in low doses but increased anxiety at higher doses (26).

THC's psychoactive effects are decreased by the CB1R antagonist rimantabant, suggesting that these effects are mediated through the activation of CB1R receptors bound to G proteins, which lower cAMP levels by blocking adenylate cyclase (27). Because endogenous cannabinoid receptors are neural and temporal specialized against THC, endogenous THC disturbs precisely calibrated endogenous cannabinoid receptor transduction networks. THC competes with endogenous agents that have a higher receptor affinity than THC in conditions of low CB1R density. Additionally, THC allosterically modulates opioid receptors, potentially offering further indirect mechanisms for modifying dopamine transmission. Moreover, THC possesses psychoactive metabolites that have a preference for CB1R, which complicates the examination of receptor binding research (28,29,30,31).

THC acts by binding to the CB1 receptors in the brain, as a partial agonist, and to the CB2 receptors in the immune system, as an agonist. The receptors that are activated are the ones that cause the psychoactive effects. These are signalled by a decrease in the cAMP messenger as anenyl guanylate cyclase is inhibited (32,33). Its lipid composition leads the THC molecule to bind to receptors and tissues in addition to its typical affinity for major cannabinoid receptors (34). This suggests that its pharmacological action is characterised by complexity since it acts through adverse effects and drug-drug interactions. Because of its partial agonistic action, THC exhibits chemical competition with other cannabinoids that act as full agonists or antagonists. Competition at the receptor level is based on chemical affinity, whereas competition at the effect level is based on the ability of molecules to act agonistically or antagonistically on cannabinoid receptors to activate or deactivate them. Finally, the presence of the phenolic molecule in THC, which may provide some protection against excitatory toxicity induced in neurons, may account for the fact that this compound exerts a partial antioxidant effect. Being aware of the fact that Δ9-THC is lipid-dependent, it has therefore been proposed a number of the cannabinoid influences have to do with the specific disorder of cell membrane that stimulates or inhibits membrane-associated enzymes and alters channels (35). THC builds up in adipose tissue and the spleen and can work as a site of chronic stasis (36). Nevertheless, due to its strong lipophilicity it breaks down the blood-brain barrier and can be found in high concentrations in the brain (37). Smoking D9-THC rapidly crosses the brain and is found in blood plasma within minutes of first inhalation (38).

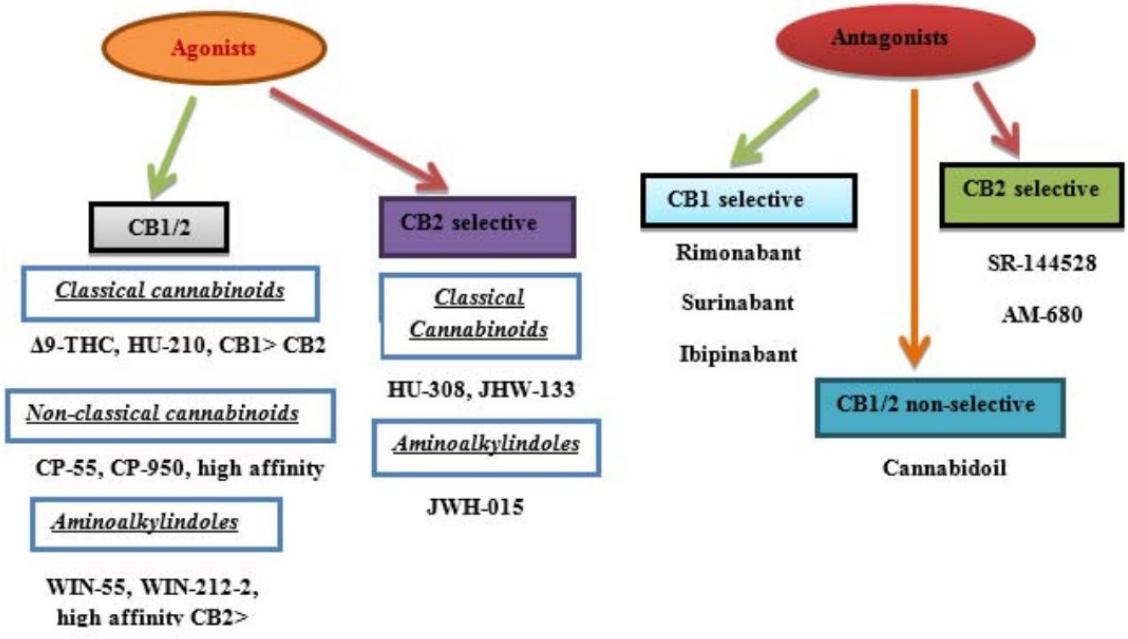

Figure 5: Agonists and antagonists of cannabinoids receptors.

The abbreviation CBD represents cannabidiol. It is a compound found in cannabis plants that does not have psychoactive effects, present in both marijuana and hemp. CBD does not cause a feeling of being "high" like THC does, nor does it change one's level of awareness. CBD interacts with the body's endocannabinoid system, which plays a role in controlling different physiological processes like mood, hunger, and sleep (39,40). CBD has become popular due to its possible healing advantages. Some research and personal stories indicate that CBD might possess properties that help reduce inflammation, relieve pain, reduce anxiety, and protect neurons. It is being investigated as a possible remedy for conditions like chronic pain, anxiety disorders, epilepsy, and more. It should be noted that CBD can come from either marijuana or hemp. CBD derived from hemp has minimal THC levels (0.3% or lower) and is lawful in numerous locations, whereas CBD derived from marijuana may contain elevated THC levels and is subject to stricter rules. Besides these combinations, there are other compounds present in cannabis plants such as cannabinol (CBN), cannabichromene (CBC), tetrahydrocannabivarin (THCV), Cannabidiolic acid (CBDA), Cannabigerolic acid (CBGA), etc (41).

CBD shows low affinity to CB1 and CB2 receptors. CBD blocks the breakdown and absorption of endocannabinoids and influences cannabinoid receptors (42). A recent evaluation of the mind-altering qualities of CBD suggests that CBD could potentially counteract the hallucinogenic effects of THC (43).

1.2 Types of cannabinoids

There are over 66 active terpenophenols called cannabinoids derived from 2-substituted 5amyl resorcinol that are present in cannabis and its products. The compounds are naturally derived from the Cannabis flower and belong to the C21 group of organic compounds, each offering distinct advantages. Three types of Cannabis plants include Cannabis ruderalis, Cannabis indica, and Cannabis sativa. More than 500 compounds are found in these species, with 150 of them being phytocannabinoids. The main cannabinoids are (-)-trans-delta-9-tetrahydrocannabinol (Δ9-THC) and (-)-cannabidiol (CBD) (4,44).

From a biological perspective, cannabinoids are chemical substances that engage with cannabinoid receptors. These compounds can be divided into cannabimimetics, which exhibit cannabinoid activity and primarily activate CB1 receptors, and antagonists that interact with cannabinoid receptors without inducing cannabinoid effects, but rather block these receptors for other substances (45).

1. **Phytocannabinoids:** Phytocannabinoids, also referred to as pCB, are natural cannabinoids found in the cannabis plant. They are produced in the glandular trichomes of the plant and have multiple roles, such as protecting against pests and environmental stress. Some instances are Tetrahydrocannabinol (THC): The primary psychoactive compound in cannabis, responsible for the euphoric "high" sensation, Cannabidiol (CBD): Non-psychoactive cannabinoid known for its potential therapeutic effects, such as anti-inflammatory, analgesic, and anxiolytic properties and Cannabinol (CBN): Formed through the degradation of THC, CBN has mild psychoactive effects and is often associated with aged or oxidized cannabis. Plant cannabinoids show great diversity. This is a result of non-enzymatic changes triggered by elements like light and oxygen in the atmosphere. Different phytocannabinoids exhibit different affinities for CB1 and CB2 receptors, and some phytocannabinoids have been discovered to interact

with molecular targets beyond the endocannabinoid system. The last-mentioned ones engage with other G protein-coupled receptors.

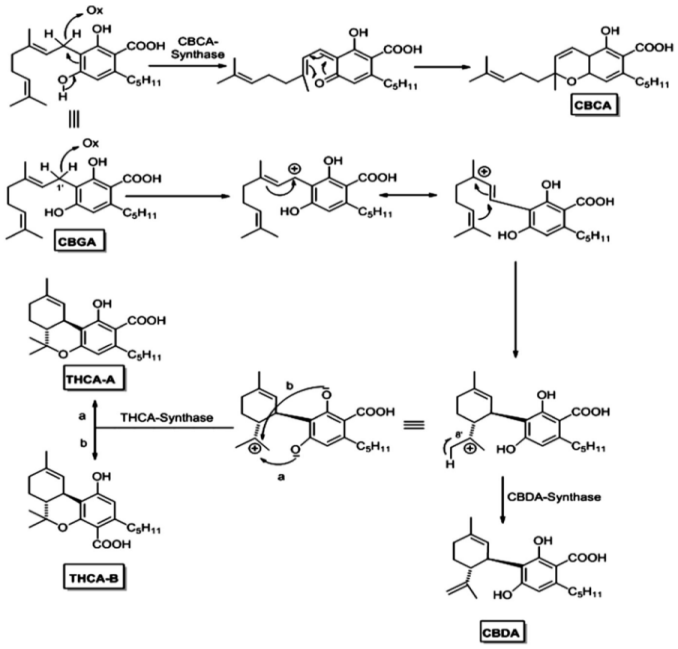

Figure 6: The biosynthetic origin of the main plant cannabinoids.

2. **Endocannabinoids:** Endocannabinoids are naturally present in humans or genetically in a living organism and depending on the needs of the organism they are produced from lipid precursors in the cell membranes. However, unlike neurotransmitters, endocannabinoids are not stored in vesicles. These molecules undergo rapid cleavage by enzymes to stop signalling. They act as signaling agents and affect important functions such as pain, appetite, memory and others. Anandamide (AEA), is called the "bliss molecule" since it regulates brain function and is involved in pain perception. 2-AG is another endocannabinoid that plays a role in neurotransmitter release and synapse plasticity. From Figure 6, it can be seen that endocannabinoids are small lipid messengers involved in several signaling pathways. They are released "on demand" in an activity-dependent manner through the cleavage of membrane phospholipids. They are then released rapidly without being stored in vesicles. To date, two cannabinoid receptors (CB1 and CB2) have been identified. The CB1 receptor is expressed in the CNS and is present on presynaptic terminals and regulates the activity of channels and allows or prevents the release of neurotransmitters. However, the evidence also strongly suggests that there is also a postosynaptic lane for CB1 receptors. CB2 receptors are less expressed than CB1. In fact, they are predominantly localized outside the CNS. More recently, data have shown that CB2 receptors are expressed by microglia during inflammatory processes as well as in brainstem neurons. However, the activity of CB2 receptors in the CNS is still unclear. However only two have been studied to date: Arachidonoyl ethanolamide (AEA-anandamide) and 2-arachidonoylglycerol (2-AG) are the main endocannabinoids and are synthesized and metabolized by separate pathways. AEA is primarily produced through the hydrolysis of a membrane phospholipid precursor known as N-arachidonoylphosphatidylethanolamine (NAPE) by the enzyme

phospholipase D (PLD), which is facilitated by the enzyme N-acyltransferase (NAT) in a calcium-dependent fashion. NAT causes the arachidonic acid group to move from phosphatidylcholine's SN-1 position to phosphatidylethanolamine. 2-AG can be generated through various mechanisms, with one of them being the hydrolysis of 2-AG by PLC, which is a phospholipase that acts on phosphatidylinositol (PI) to create diacylglycerol (DAG). After that, the process of converting DAG back into 2-AG is catalyzed. DAGL is the enzyme responsible for this process. AEA and 2-AG can diffuse passively through neurons' phospholipid bilayer. Various hydrolases internally deactivate these cannabinoids within cells. FAAH breaks down AEA by hydrolyzing it. On the other hand, monoacylglycerol lipase (MAGL) is strongly linked to nerve endings and facilitates the breakdown of 2-AG. Inhibitors of both FAAH and MAGL do not facilitate the breakdown of endocannabinoids. This means they work as indirect agonists and can be used as potential pharmaceutical and therapeutic agents (46).

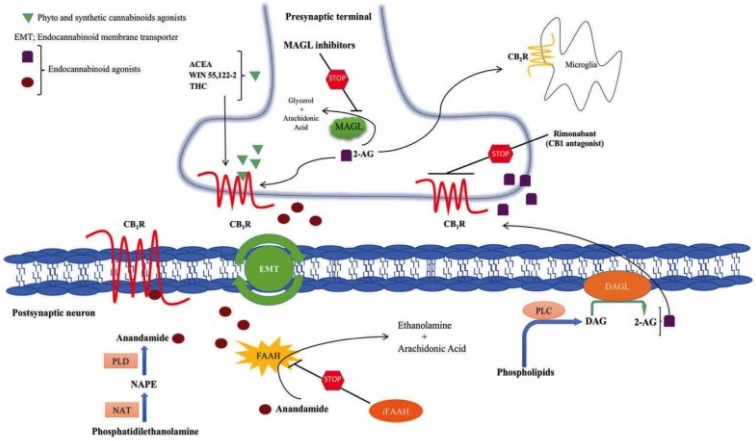

Figure 7: The endocannabinoids are small lipid messengers involved in several signaling processes.

3. **Synthetic Cannabinoids:** Synthetic cannabinoids are man-made substances created in labs to imitate the properties of cannabinoids found in nature. Frequently utilized in research environments or for medicinal purposes. Synthetic cannabinoids consist of a variety of chemical structures. Various categories of compounds such as aminoalkylindoles, cyclohexylphenols, naphthoylindoles, and others are considered as common structural classes. JWH-018 is an instance of a synthetic cannabinoid that functions as a strong agonist of the cannabinoid receptors. It was frequently discovered in illegal mixtures of synthetic cannabinoids called "Spice" or “K2" and CP-55,940, another synthetic cannabinoid employed in research for studying the endocannabinoid system and possible therapeutic uses.

1.2.1. SYNTHETIC CANNABINOIDS

The structure of the cannabinoid system enables it to interact with a variety of compounds, making it a more vulnerable target for synthetic drugs than other systems. Synthetic Cannabinoids are the largest group in terms of the quantity of different substances observed by the EU Early Warning System, with 190 substances documented from 2008 to 2018 and

approximately 280 substances recorded globally by UNODC (47,48). Many new analogues have been identified in pharmaceutical formulations, introducing new Synthetic Cannabinoids with names likely selected by those involved in marketing the products. The synthetic compound XLR-11 is named after the first liquid fuel rocket in the USA, possibly indicating the vendor's intentions for consumers (49). Synthetic cannabinoids are mainly lipophilic compounds and non-polar. They consist of 22- 26 carbon atoms and this makes them highly volatile when smoked. A common element of these compounds is a side chain of 4-9 saturated carbon atoms (50). Many of these compounds are not structurally related to common cannabinoids (D9THC etc.)

In order to systematise the chemical structures of synthetic cannabinoids, a model of their different structural types was presented by EMCDDA. The general structure of the model consists of 4 basic building elements, which are as follows:

α. The core and its substituents

β. The link

c. The ring and its substituents; and

δ. The tail

This allows for the identification of the chemical makeup of the cannabinoid without needing to use the lengthy chemical name (51). Yet, there are cannabinoids that deviate from the typical structure shown in the diagram. The synthetic cannabinoids family is extensive and consists of many substances from various chemical groups and subgroups. The classical cannabinoids, hybrid cannabinoids, aminoalkylindoles, eicosanoids, and miscellaneous cannabinoids are the main categories.

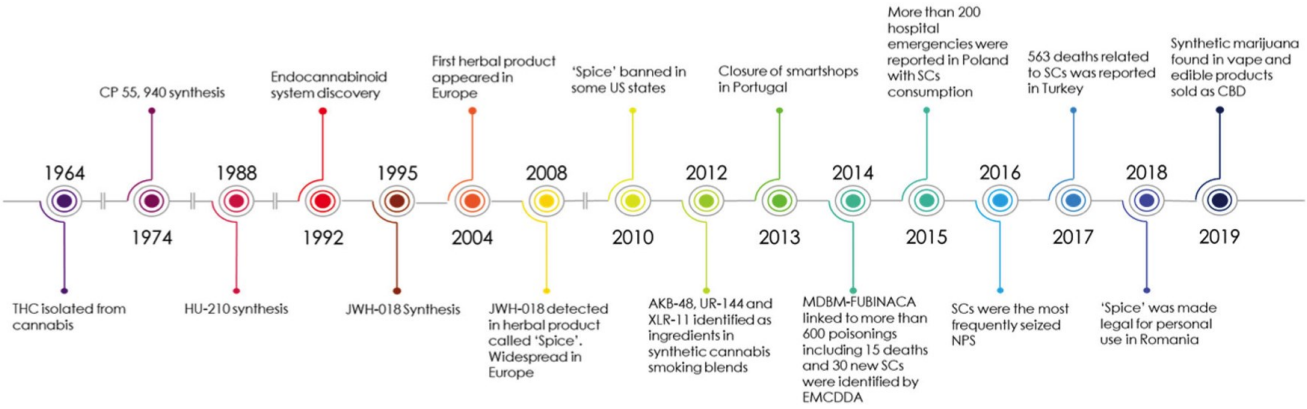

Figure 8: Timeline of the main events related to synthetic cannabinoids.

- **CLASSICAL CANNABINOIDS**

Traditional cannabinoids dissolve easily in lipids, alcohols, and non-polar organic solvents, but they do not dissolve in water. The primary psychoactive element of cannabis and cannabinoids falls into this classification. Cannabinoids, specifically classical ones like

nabilone and dronabinol found in cannabis, are derived from a tricyclic dibenzopyran ring structure.

Many studies on classic cannabinoids have concentrated on modifications to the C3 side chain. While most analogs have saturated linear or branched alkyl chains, certain C3 side chains contain unsaturated alkyl chains, heteroatoms, and functional groups such as esters, carboxylic acids, ethers, nitriles, and heterocycles, which affect CB1 and CB2 potency and selectivity differently.

- **NON- CLASSICAL CANNABINOIDS**

They are in fact a subclass of classical cannabinoids and differ from them in terms of the pyrrole ring (B), because in non-classical cannabinoids this is absent, but they include AC-dicyclic and ACD-tricyclic compounds (52). Non-classical cannabinoids include bicyclic and tricyclic structures, including CP47, 497. CP compounds are similar to the D9-THC structure because they have the alkyl chain attached to the central phenol portion of the compound. This is also related to the interaction of these compounds with cannabinoid receptors (53). The compound CP55,940 is a prominent example in this class because it has a strong affinity for both CB1 and CB2 receptors, precise stereospecificity, and powerful pain-relieving effects.

- **HYBRID CANNABINOIDS**

Hybrid cannabinoids combine characteristics of classical and non-classical cannabinoids. AM-4030, for example, belongs to this category and possesses the dibenzopyran ring common to classical cannabinoids and an aliphatic hydroxyl group common to the CP family of non-classical cannabinoids.

- **AMINOALKYLINDOLES**

They are a new generation of cannabinoids that came to replace the non-classical cannabinoids. They are characterized by low lipophilicity compared to the first two classes and their analgesic properties are related to their interaction with cannabinoid receptors. They show different affinity for the CB1 and CB2 receptors, depending on the compound in each case.

Aminoalkylindoles do not resemble D9-THC. However, they do possess cannabimimetic properties and are the synthetic cannabinoids most commonly found in mixtures, perhaps because they are more easily synthesised (54). This category is further divided into other classes such as naphthoylindoles (e.g. JWH-015), phenylacetylindoles (e.g. JWH-250), benzoylindoles (e.g. AM-694) etc.

- **EICOSANOIDS**

  Eicosanoids are another class of synthetic cannabinoids and are analogues of endocannabinoids such as anandamide.

Classic classification system	New classification system	Street names of SCs	Adverse effects of SCs
Classical cannabinoids Nonclassical cannabinoids Hybrid cannabinoids Aminoalkylindoles Eicosanoids Others	Phytocannabinoids Endocannabinoids Synthetic cannabinoids	K2, Spice (Gold, Silver, Diamond), Smoke, Black Mamba, Bombay Blue, Fake Weed, Genie, Joker, Kronic, Yucatan Fire, Chill X, Algerian Blend, and others	Tachycardia, agitation, nausea, anxiety, drowsiness, hallucinations, delusions, confusion, psychosis, dizziness, cardiovascular, cerebrovascular, neurological and renal problems

Table 1: Cannabinoids: classification, street names and adverse effects of SCs.

1.3 ENDOCANNABINOID SYSTEM (ECS)

The ESS is made up of endocannabinoids AEA and 2-AG, receptors CB1 and CB2, and enzymes FAAH and MAGL that process endocannabinoids (55). More than 100 diverse cannabinoids can be identified in marijuana. THC and CBD, the most famous ones, regulate the human endocannabinoid system. The endocannabinoid system consists of at least two cannabinoid receptors (referred to as CB1 and CB2) that are involved in different brain functions (56,57). In addition to receptors, the ECS consists of endogenous cannabinoids (endocannabinoids) and the enzymes that create and degrade these endocannabinoids. Endocannabinoids and their receptors can be found in the blood, brain, organs, and immune system, with each tissue having unique functions for the cannabinoid system. However, the final goal stays consistent and mutual: to maintain homeostasis. The blood and brain contain cannabinoid receptors that regulate hunger and pain, among other conditions. Two receptors for cannabinoids, CB1 and CB2, have been identified in the brain.

Both receptors belong to the G-protein coupled receptor family and consist of 7 transmembrane domains. Their activation inhibits the functioning of adenylate cyclase.

Endocannabinoids are natural lipids that activate receptors, affecting behavior similarly to the psychoactive elements in cannabis, particularly D-9-THC. The initial endocannabinoids that were discovered and most well-known are an amide and 2-arachidonyl glycerol (2-AG). The edocannabinoid precursors are found in lipid membranes. Typical neurotransmitters are produced beforehand and kept in synaptic vesicles, whereas endocannabinoids are quickly released through one or two enzymatic processes before being released into the extracellular space. The inherent effectiveness of internal cannabinoids fluctuates. 2-AG is a very potent activator for both CB1 and CB2 receptors. Nevertheless, amide has low efficacy as an agonist at the CB1 receptor and even lower efficacy at the CB2 receptor (58,59). When the receptor expression is weak or the receptors are bounded weakly to signal transduction pathways, andamide compete with the effects of more effective agonists (60). There are some others endogenous compounds such as vidrodamine and 2-arachidonyl glycerol ether

(61) that widen the range of endoccanabinoids, however their biology is not understood as the biology of andamide and 2-AG.

The endogenous cannabinoid system is tonically active, i.e. it consistently releases endogenous cannabinoids. In a specific region of the grey matter that regulates pain, elevated levels of endogenous cannabinoids have been found. It is necessary to emphasize that breast milk contains cannabinoids chemically and two reasons. It stimulates infants' appetite so that they feed properly and it helps to relax the infant so that he or she remains calm. This suggests that cannabinoids play an important role from the earliest stages of human existence (62).

1.3.1 ENZYMES OF THE ENDOCANNABINOID SYSTEM

The endocannabinoid system is made up of enzymes like fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). They are responsible for regulating the appropriate utilization of endocannabinoids (63). Fatty acid amide hydrolase belongs to the serine hydrolase enzyme family. It is the primary enzyme responsible for catalyzing the function of anandamide. Research indicates that blocking FAAH function may potentially serve as a treatment for anxiety disorders. MAGL also falls under the serine hydrolase family. It is the main enzyme that breaks down 2-AG by hydrolyzing it. It aids in the decomposition of triglycerides in fat cells together with lipase via hydrolysis. Studies on mice have shown that significant deactivation of MAGL results in an increase in 2-AG levels (64,65).

CHAPTER 2:USE OF CANNABINOIDS FOR THERAPEUTIC PURPOSE AND AS ADDICTIVE SUBSTANCE

Cannabinoid consumption brings about a number of changes in the way our body functions and these changes range from mild effects to very significant changes in the way the user perceives reality. The consumption of cannabinoids has been associated with the practice of smoking. When smoking a powdered mixture of dried cannabis leaves and flowers in the form of a cigarette, about 20% of the THC is absorbed. Cannabinoids are employed for either medicinal purposes or for recreational use that can lead to addiction. THC, cannabidiol, synthetic cannabinoids, and their analogues all demonstrate a powerful pain-relieving effect in both preclinical and clinical trials. For instance, THC affects pathophysiological processes by activating CB1 and CB2 receptors. Nevertheless, its practicality in clinical settings is restricted by negative effects on the central nervous system, which act through CB1 receptors in the brain. Cannabidiol, another key phytocannabinoid, shows minimal affinity for CB1 and CB2 receptors. In contrast to THC, it does not result in psychoactive effects, yet it provides numerous beneficial pharmacological properties, including anti-anxiety, anti-epileptic, antibacterial, anti-inflammatory, anti-cancer, and anti-diabetic effects. There are multiple ways to introduce cannabis into the body, such as smoking, vaping, consuming through the mouth or throat mucous membrane, or using it as a cream or lotion topically.

The study of the biological action of cannabinoids combined with the discovery of selective receptor antagonists and inhibitors of the mechanism that terminates the action of endocannabinoids, has provided a platform to stimulate biological targets of the endocannabinoid system.

The endogenous cannabinoid system is a cytothoracic communication mechanism and has as its main component endogenous cannabinoids, receptors and the endocannabinoid signal deactivation system. It has a regulatory role and is localized and acts mainly in the brain (66).

Cannabinoid analogues that act antagonistically to CB1 can be used for diseases such as in people at low risk of developing depression. CB1 antagonists can be used to treat muscular dystrophies based on the differential activity of this receptor in muscle cells. The utilization of positive and negative allosteric sites on CB1 and CB2 receptors could lead to the treatment of diseases such as chronic pain, cancer, anxiety, depression, schizophrenia and metabolic, neuroinflammatory disorders (67).

2.1 ENDOCANNABINOID SYSTEM AND CNS

Cannabinoids engage with various neurotransmitters including acetylcholine, dopamine, serotonin, and more. These interactions help to explain some of the effects that THC has on our body. Tachycardia and malnutrition alongside dry mouth are caused by THC's impact on acetylcholine release and conversion (68). The reason for the antiemetic properties of the cannabinoid system is due to its interactions with serotonin (69). The effects of neuroprotective cannabinoids on animals come from their ability to reduce the overproduction of glutamine, block the entry of calcium into cells, and provide antioxidant benefits to the vascular system (70,71).

2.2 ENDOCANNABINOID SYSTEM AND IMMUNE SYSTEM

Immune cells possess CB1 and CB2 receptors and have the capability to generate, secrete, mobilize, and metabolize cannabinoids. The expression of cannabinoid receptors in immune cells varies. Cell's level of expression is impacted by its immunostimulation and activation state (72). In mice lacking fatty acid amide hydrolase (FAAH), an enzyme that breaks down anandamide, levels of anandamide in the CNS and PNS rise, reducing inflammation and indicating a role for endocannabinoids in the immune system (72,73).

Hemp smoke has been found to cause cancer and mutation in rodents through in vivo and in vitro studies. This is because cannabinoids affect the cell cycle at the mesophase stage, the humoral immunity of rodents, and reduce the body's resistance to infection (74). However, not all studies conducted lead to deregulation of the immune system (75).

2.3 ENDOCANNABINOIDS AND THE REPRODUCTIVE SYSTEM

Chronic administration of THC in large quantities in animals has been shown to lead to reduced testosterone production, reduced sperm production and motility and disruption of the ovulatory cycle (76).In humans, however, this cannot be accurately supported due to a lack of studies. In animals, relevant studies have found that neonatal weight is reduced when the mother uses cannabis during pregnancy (77). The results from epidemiological studies in humans are equivocal (78).

2.4 THE ENDOCANNABINOID SYSTEM AS A THERAPEUTIC TARGET

The medicinal application of cannabinoids can be traced back to the 1800s, with the cannabis plant being utilized since ancient eras for alleviating rheumatism, stomach issues, and mental illnesses. Given that the endogenous cannabinoid system is crucial in regulating various physiological processes in both the brain and peripheral system, it is also involved in controlling the immune, cardiovascular, reproductive endocrine systems, and energy metabolism (79).

Cannabinoids may serve as alternative treatments for mood disorders and anxiety, replacing traditional antidepressants. Activation of cannabinoid receptor 1 (CB1R) plays a role in the reward system process (80), suggesting CB1R could be a promising new drug target for treating mood and anxiety disorders. The endocannabinoid system controls the communication of neurotransmitters like dopamine and glutamate, influencing emotions and behavior (81).

CB1R has a high density in the brain, in areas responsible for mediating reward such as the Hippodamus. CBD was found to exhibit anxiolytic activity when it was observed to reverse the psychotic and anxiogenic effects of Δ9-THC. Preclinical studies have demonstrated that CBD may act as an anxiolytic and that it reduces fear and limits the negative effects of chronic stress (82).

CBD is thought to regulate fear and anxiety through interaction with serotonin 5-HT1A, the TRPV-1 receptor and, to a lesser extent, CB1R (82). Activation of the serotonin 5-HT1A

receptor (5-HT1AR) by CBD has been implicated in the regulation of fear and prevention of chronic stress (82). Another proposed mechanism of action by which CBD may produce anxiolytic effects is the upregulation of hippocampal AEA, an endogenous cannabinoid with anxiolytic properties (83).

2.4.1 CANNABINOIDS AND ANXIETY

The connection between cannabis and anxiety has not been widely talked about in society. One potential explanation could be that this medication is prohibited in a majority of countries, leading patients with anxiety to be hesitant about revealing their cannabis consumption (84). Moreover, anxiety can stem from cannabis consumption or be a withdrawal symptom in cases of cannabis dependency, raising questions about the existence of cannabis addiction, a topic that has sparked controversy (85). Individuals who consume cannabis are at a higher risk of experiencing elevated levels of anxiety compared to those who do not use the substance. Several researches have confirmed the connection between marijuana and anxiety. A new study found that adolescents with cannabis dependence have higher levels of psychological distress and anxiety (86). Cannabis may induce a brief and intense anxiety episode resembling a panic attack in individuals who do not use the drug. In individuals with a normal weight, consuming a high dose (>5 mg D9-THC) of cannabis results in experiencing fear and anxiety. At higher levels of dosage, panic attacks and feelings of fear might arise (87,88). These signs frequently appear when cannabis is consumed in large amounts and when individuals are in unfamiliar or tense surroundings (89).

The comorbidity of cannabis use and anxiety has been little studied. Relevant studies have shown that chronic cannabis use is associated with a higher prevalence of comorbidity with anxiety disorders (90). It has also been observed that several individuals experienced anxiety disorders before the first symptoms of cannabis addiction, which indicates that some of these individuals were using cannabis as a self-prescribed anti-anxiety medication (91).

2.4.2 ASSOCIATION BETWEEN ANXIETY AND CANNABIS USE

There are many theories that try to explain this relationship. Acute anxiety can be enhanced by cannabis use in the following ways:

a. The main psychoactive component of cannabis D9-THC, due to its effect on serotonin, noradrenalin (92,93) GABA and glutamate (94) causes symptoms of anxiety. Although there is relatively little information on the interactions between the serotonergic and endocannabinoid systems, there is some emerging evidence for a complex interaction between the two systems, the full extent of which is not yet clear (92,95). Animal studies (92,96,97 have shown that cannabidiol (CBD), which induces anxiolytic effects, does so by acting on the 5HT1A receptor.

b. Acute intoxication can lead to anxiety secondary to decreased cognitive function and blurring of consciousness. Cannabis can lead to persistent anxiety disorders through sustained dysregulation of endocannabinoid systems (anandamide) (98). New human and

animal studies suggest neurotransmitter or hormonal effects of cannabis on subsequent states of anxiety and mood, particularly in adolescents.

Anxiety leads to cannabis consumption. Anxiety disorders in adolescents lead to subsequent cannabis use (99). This may be explained by the fact that people with high levels of anxiety and those with anxiety disorders use cannabis as a means of self-medication. In other words, many people claim to use cannabis to relax and cope with anxiety.

2.5 SUSPENSION OF NAUSEA AND VOMITING:

Cannabinoids display anti-nausea effects in animal experiments. The CB1 receptor can be found in the cholinergic nerve endings of the myenteric and submucosal plexus in the stomach, duodenum, and colon; the reduction of gut movement by cannabinoids occurs by preventing the release of acetylcholine in these regions. (100)

Evidence also suggests that cannabinoids impact CB1 receptors in the brainstem's dorsal-pulmonary-gastric complex, which regulates the vomiting reflex. Endocannabinoids and their inactivating enzymes are found in the gastrointestinal tract and may play a role in controlling vomiting (101).

THC works by impacting the pathways responsible for vomiting through receptors in the nucleus of the solitary tract in the area of the terminal wing, thereby alleviating chemotherapy-induced nausea. It could also counteract the impacts of drugs that stimulate 5-HT3 receptors and cause vomiting (102).

2.6 EUPHORIA, COGNITION AND BEHAVIOUR:

Cannabinoids have been demonstrated to have a distinct impact on individuals' behavior, with the primary characteristics being a feeling of euphoria and calmness. Excessive use of cannabinoids is linked to decreased performance in a range of cognitive tasks, including memory impairment and distorted time perception. At low levels, D9-THC acts as a stimulant before causing sedation, while at high levels it only induces sedation. This indicates that there is a distinct mechanism of activity at varying levels of agonist concentrations.

2.7 APPETITE STIMULATION:

The endocannabinoid system could act as a biological controller of hunger. For instance, endocannabinoids and CB1 receptors can be found in the hypothalamus, the area of the brain that regulates food consumption, with leptin decreasing levels of hypothalamic endocannabinoids, a key appetite-suppressing hormone. However, CB1 receptors are also present in nerve terminal cells and adipocytes, so they may also be involved in appetite regulation.

2.8 CHRONIC PAIN DUE TO CANCER

Most studies have shown that most patients seeking cannabinoid treatments cite pain relief as their main motivation. Cannabis is now replacing conventional pain medications in people suffering from arthritis and side effects from chemotherapy (103). THC and CBD have anti-inflammatory and analgesic properties, suggesting that cannabinoids are effective for treating inflammatory pain (104).

CB1 receptors, which are based in the central nervous system, are found in high concentrations in brain regions that regulate algesic processing, in a similar distribution to opioid receptors. Cannabinoids also interact with mast cell receptors, thus inhibiting the release of substances produced by inflammation and enhancing its treatment and control (105).

However, although medical cannabis or cannabinoids are increasingly being prescribed for the treatment of chronic pain, the latter remain controversial due to the suspected or known risks associated with cannabis use.

2.9 CANNABINOIDS AND CANCER

The exact role of cannabinoids in the development of cancer is still not fully known. Numerous cancer cells, such as those in skin cancer, show controlled levels of CB receptors and abundant endocannabinoids, but there is no connection with the expression levels in the original tissue. The concentration of endocannabinoids and the expression of receptors also impact the level of aggression in tissues. Research in mice has shown that the lack of CB receptors results in reduced skin cancer caused by UV light exposure (106). According to this information, CB2 plays a role in promoting tumor growth signals from the human epidermal growth factor (HER) receptor, and an increased expression of CB2 makes individuals more susceptible to developing leukemia after being infected with viral leukemia (107,108).

Activation of cannabinoid receptors may hinder cancer progression, so the transmission of endocannabinoid signals could potentially have anti-cancer effects. Sources suggest that cancer cell lines and human tumors show an increase in enzymes that break down endocannabinoids (109). In mice, research has demonstrated that inhibiting CB1 expression speeds up the growth of intestinal adenoma, while activating it decreases its development (110). Yet, the higher amount of endocannabinoids still decreases the formation of pre-cancerous growths in the colons of the mice under investigation (111).

Many cannabinoids ranging from phytocannabinoids such as THC to synthetic cannabinoids such as JWH-133, inhibit proliferation, metastasis and angiogenesis in various cancer models (112). However, there are anecdotal reports stating that under certain conditions t cannabinoids may be protumorigenic (113). The main action of cannabinoids is that they inhibit the proliferation of cancer cells and induce apoptosis. CB1 and CB2 receptor agonists stimulate apoptosis in glioma cells through induction of de novo ceramide synthesis (114).

Ceramide activates the pathway related to stress signaling. This results in a rise.

a. The p8 protein (Nupr1) controls stress levels.

b. a transcriptional regulator implicated in tumor complexities,

c. ATF4, the transcriptional activating factor, C/EBP homologous protein CHOP and

d. homologue 3 of tribbles, also known as TRIB3, is shown in Figure 8 (115).

Certain stimuli can trigger ER stress as a cell's reaction (116). Ca2+ deficiency, viral infection, or specific anticancer medications are some of the possible causes. Translation is halted, misfolded proteins are degraded, and the ER's protein disposal function is restored as a result. Failure of this response can result in activation of the intrinsic apoptosis pathway due to ER stress (116).

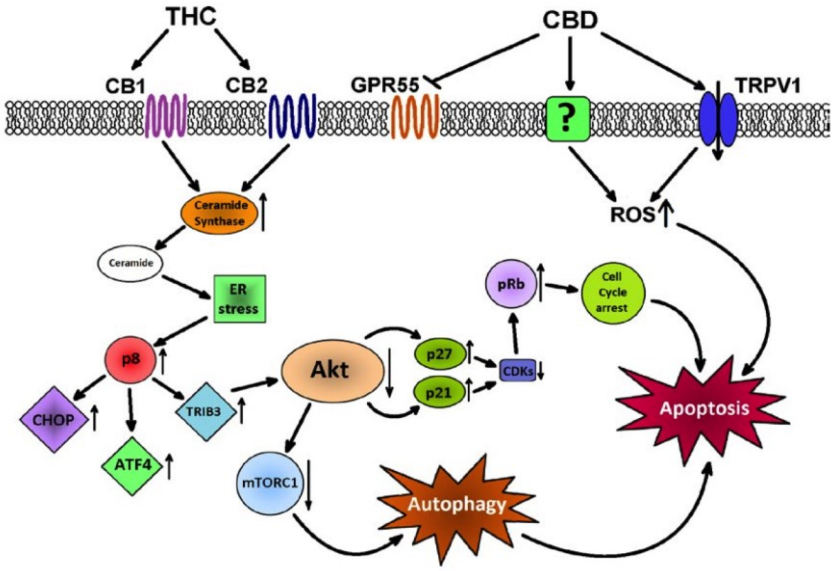

Figure 9: The known mechanisms responsible for the induction of apoptosis by cannabinoids

Triggering the pathway governed by p8 causes the blocking of protein kinase B, which then inhibits the mammalian target of rapamycin 1 (mTORC1) complex, ultimately causing cell death induced by autophagy (117).

Autophagy occurs after apoptosis during the process of cell death induced by cannabinoids. Blocking autophagy with cannabinoids also blocks apoptosis, and blocking apoptosis prevents cell death through autophagy (189). Cannabinoids have been shown to induce autophagy in cancer cells such as glioma and melanoma (117).

ER stress caused by cannabinoids in liver cancer triggers activation of AMP-activated protein kinase and calcium/calmodulin-dependent protein kinase kinase 2, serving as an additional factor in autophagy-induced cell death (118).

Nevertheless, non-psychoactive cannabinoids also exhibit anti-cancer properties. CB1 receptors are not directly impacted by these cannabinoids and the functioning of this system is not completely understood. Currently, the main suggested way they work is by promoting the production of reactive oxygen species (ROS), which then causes apoptotic cell death through autophagy (119,120).

Another significant variation proposes that CBD stops the degradation of anandamide (AEA) and results in higher concentrations of endocannabinoids by acting as a FAAH inhibitor (121). The majority of studies indicate that CBD and other non-psychoactive cannabinoids function without directly activating CB receptors. CBD's interaction with various receptors, such as GPR55, is crucial for its effectiveness. For example, both cannabidiol and cannabigerol exhibit anti-cancer properties by serving as strong blockers of TRPM8 receptors (122). Nevertheless, there are claims suggesting that CBD induces cancer cell apoptosis by directly or indirectly activating the CB2 receptor (123).

2.9.1 CANNABINOIDS: ANGIOGENESIS AND METASTASIS OF CANCER

CBD has been shown by studies to act against cancer cells and exhibits anti-proliferative and other properties. It inhibits cell growth and migration of cancer cells, induces apoptosis of cancer cells and affects angiogenesis (124).

Cannabinoids demonstrate other significant properties. They hinder the formation of new blood vessels by obstructing the VEGF pathway. In glioma, skin, and thyroid carcinoma, they decrease the levels of vascular endothelial growth factor and its receptors (VEGFR1 and VEGFR2) (125,126). Furthermore, cannabinoids can inhibit tumor-driven growth of endothelial cells and, as previously stated, trigger their apoptosis, consequently impacting the development of the tumor vasculature (127).

The ability of cannabinoids to reduce spinal tumors, prevent metastases, and inhibit the spread of cancer cells in vitro, particularly in breast cancer, is attributed to their modulation of extracellular proteases and inhibitors (128). Preventing the reported effects can be achieved by pharmaceutical blocking of ceramide biosynthesis and p-protein expression (128). Studies have shown that CB receptor agonists have antiangiogenic and antimetastatic properties, in addition to their antiproliferative effects, by promoting ceramide biosynthesis and regulating pathways related to p8-protein. These inductive traits are common to cannabinoids, which do not trigger CB receptors.

2.10 CANNABINOIDS AND EPILEPSY

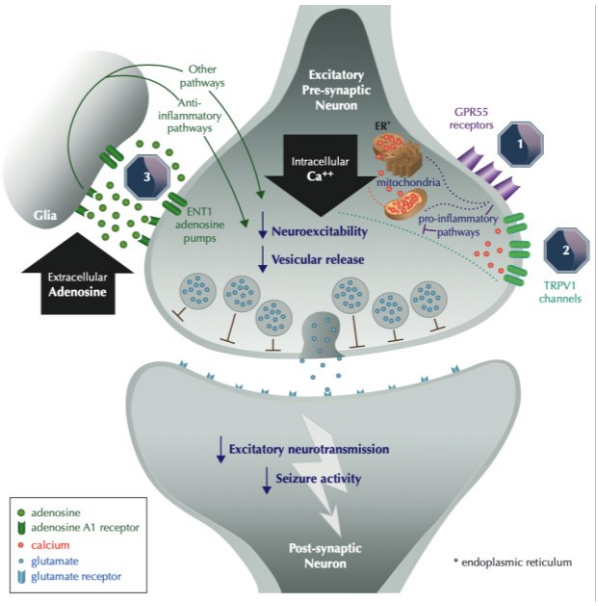

Figure 10: Mechanism of CBD action in epilepsy.

Cannabis has been utilized for many years to address different medical conditions like epilepsy. Preclinical research currently backs up the use of cannabinoids in treating epilepsy. Limited clinical studies support the use of cannabinoids in treating epilepsy in humans.

Research has been done on how cannabinoids and the endocannabinoid system can help with seizures. Specifically, cannabinoids have been proven to have a neuroprotective function in situations of sudden seizure onset

Both CBD and THC have anticonvulsant properties. The former operates via a CB1-related mechanism while CBD operates via a more intricate mechanism (129). THC's function involves activating the CB1 receptor and inhibiting DSI/DSE and MSI/MSE signals, thus regulating the endocannabinoid system (130). It has been demonstrated to impact the regulation of the threshold of temporal lobe epilepsy seizures (131). CBD demonstrates a greater range of versatility than THC in relation to how it produces anticonvulsant effects (132). CBD acts as an inhibitor of CB1 receptors and a negative allosteric modulator. CBD's ongoing impact on epilepsy suggests that these effects may be influenced by different pathways that do not rely on the CB1 receptor. CBD acts as a booster for the 5HT1A receptor, in addition to the transient receptor potential of ankyrin type 1 channel, and the transient receptor potential of vanilloid type 1 and 2 channels. CBD acts as a blocker at the GPR55 receptor and TRPM8 channel, and also inhibits fatty acid amide hydrolase which degrades anandamide (133). Following the administration of Kainic acid to induce seizures, there was a 20-minute increase in anandamide levels, whereas seizures induced by pilocarpine resulted in a 2-AG rise just 15 minutes later (134).

In response to seizure activity, endocannabinoids are produced and released as needed (134). Seizures that are a result of kainic acid in animal models were found to be reduced in severity following activation of CB1 receptors. However, removal of these receptors leads to increased apoptosis of prostictal cells. Conversely, overexpression of these receptors leads to reduced cell death and reduced severity of seizures (135). In the hippocampus, epileptiform activity is cepically regulated by this receptor (CB1), as it regulates anticonvulsant and neuroprotective effects in acute seizures. Specific activity in the hippocampus causes an increase in CB1 receptor expression in the dentate gyrus and CA1-3 (136). This compensatory response is distinguished between excitatory and inhibitory interneurons. The former, i.e., excitatory glutaminergic interneurons cause an increase in CB1 expression whereas inhibitory GABAergic interneurons cause a decrease in CB1 expression. Thus, these changes act by reducing the excitability of the hippocampus (136). The CB1 receptor homeostasis that is subject to disruption probably plays a role in the development of choroidal seizure disorders (137). This has been suggested by additional tissue samples from individuals with epilepsy that showed decreased CB1 expression in glutaminergic neurons and increased expression in GABAergic neurons.

Epilepsy sufferers have reported minimal side effects from CBD, as the latter has been reported to have antipsychotic and anxiolytic effects with no side effects. Drug metabolism has to do with CBD but detailed study of patients regarding possible interactions between drugs is necessary and important to prevent adverse allergies. Understanding the importance of studies and research on CBD for epilepsy treatment and the overall role of cannabinoids in treating the disease is essential for the future. Further research is required to explore the potential benefits of other cannabinoid derivatives (aside from CBD and THC) in managing epilepsy.

2.11 MULTIPLE SCLEROSIS

This disease is neurological in nature and is considered to be autoimmune. It has a negative impact on the central nervous system and affects a large part of the population. This demyelinating disease leads to a severe impairment of nerve signal transmission between the brain and the spinal cord causing loss of myelin (138). The symptoms observed are convulsions , bladder dysfunction and some mental problems.

It has been noted by MS patients that cannabinoids can treat many of the symptoms of the disease.

2.12. CANNABINOIDS HUMAN TOXICITY

Cannabinoid poisoning most often occurs through inhalation. Cannabinoid toxicity is usually due to overuse and abuse or unintentional ingestion of cannabis. Accidental overuse can occur with edible marijuana products due to over ingestion during the prolonged, unpredictable time it may take for maximum effect (139). Greater availability due to legalisation and commercialisation has led to new cannabis preparations such as baked goods, various candies, hashish and oils. Despite the wider availability of cannabis, the most

significant toxicity of cannabinoids is probably due to the abuse of synthetic cannabinoids, which are known to have more adverse effects.

Human toxicity includes, among others, cerebral infarction, infections or psychomotor changes and even testicular cancer. Perhaps many of these may be due to pollutants. However, there are difficulties in establishing a direct causal relationship between cannabis and outcomes, as users often smoke and use other drugs at the same time.

2.12.1 PATHOPHYSIOLOGY

Cannabinoids, either endogenous or exogenous, act on specific cannabinoid binding receptors (CBs), cannabinoid binding receptor 1 (CB1) and cannabinoid binding receptor 2 (CB2). CB1 is mainly located centrally, but is also present in the periphery, while the opposite is true for CB2, which is mainly located peripherally, but is also found centrally. CB1 receptors are primarily involved in the central effects of cannabinoids, which include effects on learning, cognitive memory, emotion, movement, sensory perception, and nausea, as well as the psychoactive properties associated with cannabinoids (140).

CB2 receptors are located peripherally and are thought to influence inflammation and immune regulation. Cannabinoid receptors are G-protein-coupled receptors that inhibit adenylyl cyclase and thus cyclic AMP, which affects calcium and potassium channels, leading to an overall decrease in intracellular calcium and extracellular potassium concentrations. This then leads to reduced neurotransmission. However, depending on the specific CB site and the specific G-protein involved, CB1 stimulation can lead to inhibition or stimulation of several neurotransmitters, including acetylcholine, L-glutamate, gamma-aminobutyric acid, dopamine, norepinephrine and 5-hydroxytryptamine. This neurotransmitter modulation may contribute to the central and peripheral effects seen in cannabinoid toxicity. The physiological effects of synthetic cannabinoids may vary depending on the specific molecule in play, as many synthetic cannabinoids are continuously produced and are often contaminated with other products. Chemically speaking, synthetic cannabinoids and traditional cannabinoids such as THC are very different. Unlike THC, which is a partial agonist at the CB1 receptor, synthetic cannabinoids are full agonists. They clearly show greater affinity for CB receptors. Therefore, the effects of synthetic cannabinoids may be much more potent than THC, thus increasing the effects observed physiologically and toxicologically.

2.12.2 TOXICOKINETICS

The toxic effects of cannabinoids are secondary to overstimulation of the endocannabinoid system by exogenous cannabinoids. This overstimulation of the endocannabinoid system leads to the aforementioned irregular neurotransmitter modulation that can lead to toxicity. The absorption kinetics of cannabinoids and THC depend on the route of exposure, with inhalation reaching peak serum concentrations in less than thirty minutes, while ingestion peaks concentrations approximately 2 to 4 hours (or more) after consumption. The duration of toxicity secondary to inhalation and ingestion is approximately 2 to 6 hours and 8 to 12 hours, respectively. The volume of distribution of THC is about 3 litres per kilogram and

after exposure it is eventually concentrated in fat due to its high fat solubility. Chronic exposures lead to increased accumulation in fat.

THC crosses the placenta and can accumulate in significantly increased concentrations in breast milk. The hepatic cytochrome p450 system mainly metabolizes THC into many metabolites, mostly inactive. The major active metabolite of THC is 11-hydroxy-delta-9-tetrahydrocannabinol, which is further degraded to numerous inactive metabolites, including 11-nor-delta-9-tetrahydrocannabinol-carboxylic acid (THC-COOH), which is detectable in urine, as excretion occurs both via faeces and urine over a period of hours to days, with more prolonged elimination depending on the timing of use. The toxicokinetics of synthetic cannabinoids is less predictable, as the specifically abused compound may vary and adulteration is not uncommon.

2.12 LEGISLATIVE FRAMEWORK

Cannabinoids are the most commonly used illicit substances worldwide. It is estimated that 1 in 8 young people aged 15-34 years used cannabis recreationally in Europe in 2017, while in 2018 an estimated 17.5 million people of the same age in Europe used cannabis. In fact, it is estimated that around 1 % of adults aged 15-64 years use cannabis daily or almost daily. However, at the national level in different European countries, they range from about 1 % to about 20 % of young people.

There is strong concern in Europe about the impact of cannabis consumption, as legalisation of its use as a medicine may lead to increased use.

2.12.1 EUROPEAN LEGISLATION ON CANNABIS

The permissibility of marijuana for medical or recreational purposes differs from country to country. Nevertheless, the legality of drugs in most countries is determined by international conventions such as the United Nations Single Convention on Narcotic Drugs (1961), the 1971 Convention on Psychotropic Substances, and the 1988 Convention against Illicit Traffic in Narcotic Drugs and Psychotropic Substances.

In November 2020, the European Court of Justice published a judgment stating that cannabidiol (CBD) extracted from the cannabis plant should not be considered a narcotic substance under the 1961 United Nations Single Convention on Narcotic Drugs. The 1961 United Nations Single Convention on Narcotic Drugs is the basis for all national drug control legislation. Following this decision, the European Commission noted in a recent press briefing that cannabidiol should not be considered a narcotic substance within the meaning of the 1961 United Nations Single Convention on Narcotic Drugs and that cannabidiol can be classified as a food, provided that the other conditions under the European Union Food Safety Regulation are met. Also, a recent review by the World Health Organization's Expert Committee on Drug Dependence, 2018-2019, states that cannabidiol (CBD) "has no potential for abuse and cannot cause dependence" (141).

Penalties for non-serious cannabis-related offences have been reduced and criminal penalties for possession of cannabis have been abolished. However, to date, these policy

changes have taken place in the general context of maintaining the prohibition on the supply of cannabis and not creating a regulated market for cannabis for recreational use.

Countries such as Chile, Canada, Cyprus, Cyprus, Greece, Norway, have legalised the use of cannabis for medical purposes. Others have more restrictive laws and allow specific cannabis herbal preparations.

2.12.2 LEGISLATIVE FRAMEWORK IN GREECE

The legal framework regarding the production, exploitation and circulation of medicinal cannabis preparations is now defined in Greece. The movement of cannabis (and its resin) from Schedule A' of Law 3459/2006 on "Narcotics", which concerns prohibited drugs, to Schedule B', which concerns pharmaceutical substances, was legislated by the Joint Ministerial Decision (CMD) No. G5c οικ. 49690/2017 (Government Gazette 2238/B/29-6-2017). In this way, cannabis is now legally recognised in Greece as a medicinal substance with therapeutic benefits and not as a psychotropic substance with dangerous health consequences. Medicinal cannabis is the drug whose only active ingredient (i.e. active substance) is that of varieties of the species Cannabis Sativa L, with a tetrahydrocannabinol THC content of more than 2% (THC>0.2%).

Since then, the circulation of preparations or proprietary preparations, in any pharmaceutical form, containing more than 0.2% THC is allowed, with a marketing authorisation and an accompanying special (two-line red) prescription. The medicinal properties of tetrahydrocannabinol (THC) include its anti-inflammatory, analgesic, antimicrobial and neuroprotective properties. Also,

it is an appetite stimulant, has anti-nausea and anti-emetic effects, and reduces the intensity of muscle spasms. At the same time, it seems to contribute to the apoptosis, especially of cancer cells. The administration of cannabis to a patient is justified only in the following cases:

1) severe and objectively excessive pain

2) Oncological diseases - cases of acute and severe acute and severe pain

3) Rheumatoid arthritis

In addition, with Law 4523 /2018, the production, possession, transport, storage and supply of raw materials and substances of cannabis varieties of Cannabis Sativa L, with a tetrahydrocannabinol (TCH) content of more than 0.2% (THC > 0.2%), as well as the establishment and operation of a processing plant for the processing and production of finished medicinal cannabis products for the sole purpose of either supplying the state monopoly and making them available for medical purposes or exporting them, were approved on a uniform basis.

The first legislative initiative on the therapeutic use of cannabis was taken in 2013 and in 2018 the law was extended. More specifically, the law passed in 2018 enables the following:

A. Easy access for our country's patients to finished medicinal cannabis products that are produced domestically.

B. To natural and legal persons to cultivate hemp varieties for the processing of raw materials and, in general, of these substances for the exclusive purpose of producing finished medicinal hemp products in Greece, ensuring the appropriate space for cultivation and the establishment of a processing plant.

C. Promotion of investment initiatives and creation of new jobs in the sectors of cultivation, processing, quality control and export of medicinal hemp products, taking advantage of the favourable climatic conditions and comparative advantages of the country.

D. There should be economic benefits for the state from the export of finished medicinal cannabis products and the taxation of economic activities in the sector, given that the global market for the cultivation and processing of medical cannabis is in the early stages of development and therefore supply is significantly below demand.

E. Broadening the production base for Greek farmers, especially through cooperative schemes, creating opportunities for other crops to produce innovative products, while introducing the philosophy of green entrepreneurship in Greek agriculture.

CHAPTER 3: HOW DO THE CANNABINOIDS ACT IN THE HUMAN BODY

3.1 RECEPTORS

Endocannabinoids work primarily through receptors CB1 and CB2, along with others such as TRP. CB1 and CB2 are G protein-coupled receptors (GPCR) that are associated with G proteins from the Gi and Go categories (142). Their activation inhibits adenylate cyclases and particular voltage-dependent calcium channels, while also activating various MAP kinases and inward rectifier potassium channels, with minor differences depending on the cell's properties. Therefore, the activation of CB1 or CB2 receptors results in influencing different cellular functions like synaptic function, gene transcription, and cell mobility.

CB1 receptors are plentiful in the CNS, especially in the cortex, basal ganglia, hippocampus, and cerebellum (143). The majority of CB1 receptors are found at axon terminals and on the proximal parts of axons, while the active zone is not densely populated according to a study (144). The cortical and hippocampal CB1 receptors are highly concentrated in cholecystokinin (CCK)-positive endoneurons (low threshold spike interneurons) (145,146), and are also present in glutamatergic neurons at lower but still functionally important levels (147).

CB1 receptors can be found abundantly in both the dorsal and ventral striatum in a variety of medium spiny neurons, with a high expression on the direct pathway axons as they travel towards the substantia nigra through the globus pallidus (148). CB1 receptors in the cerebellum can be located in parallel and climbing fibers, as well as in basket cells (149). Although CB1 has been found on numerous neurons, several independent groups have also reported the functionally significant presence of CB1 in glial components (150).

Unlike CB1 receptors, CB2 receptors are not as commonly found in the central nervous system but are primarily located in microglia and vascular components (151). The participation of cannabinoid CB2 receptors is observed in numerous physiological processes, impacting immune reactions. CB2 is predominantly expressed by certain neurons during pathological conditions, such as in cases of nerve injury (152). It is interesting that CB2 receptors can be induced and their expression increases by 100 times when tissue is injured or during inflammation (153).

CB2 receptors have been discovered to have a significant role in CB1-mediated signaling by forming functional heteromers. Studies have shown that even with low levels of expression, CB2 receptors play a key role in signaling through CB1 receptors by attenuating the cellular response. Moreover, the expression of CB2 receptors is elevated in response to specific pathological conditions (154).

The psychoactive actions of cannabis appear to be coordinated mainly through CB1 receptors. An example is that the CB1 receptor is densely expressed throughout the cortex and hippocampus and this explains why cannabis smoke causes impairments in memory and learning. Also, this receptor is dense in the basal ganglia and cerebellum and this explains the motor disorders that marijuana causes. Finally, its expression in the amygdala explains the emotional effects and malaise, while its lack from areas of the central brain that control respiratory functions explains why cannabinoids do not induce respiratory depression (155).

The receptors act after G proteins have been activated, and depending on the system of intracellular mediators that has been followed, cause inhibition of adenylate cyclase, increase in potassium channel conductance or decrease in calcium channel conductance (156). Metabolic receptor potential channels, i.e., TRP channels, are ion channels in the plasma membrane in many animal cells. Cannabinoid lipids in the skin are presented either as endogenous or exogenous and are capable of regulating numerous sensation-related phenomena such as homeostasis etc. It has been accepted that they are mediated by metavoloreceptor cannabinoid receptors. However, in a developing organism it has been shown that TRP channels function as ionotropic cannabinoid receptors.

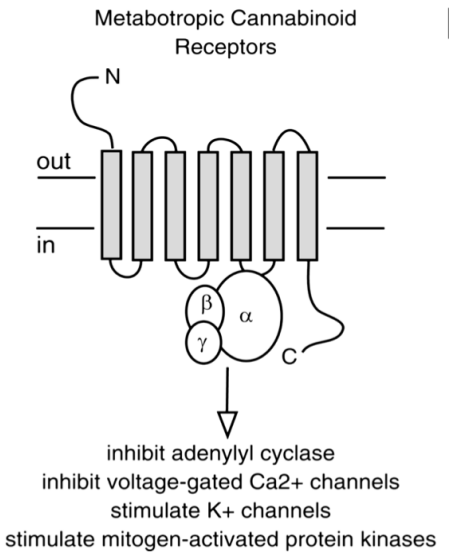

Figure 11: Metabolotropic cannabinoid receptors

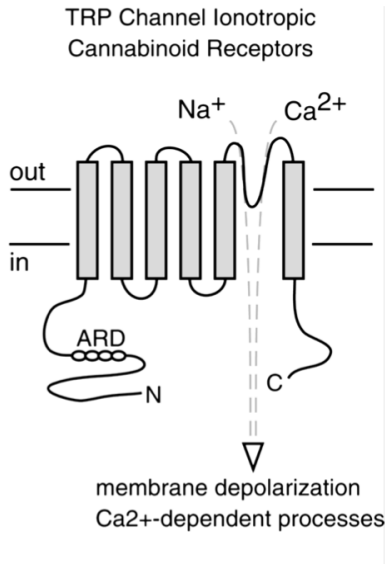

Figure 12: Iontotropic cannabinoid receptors

In addition to this many TRP channels are involved in cutaneous processes involving the initiation of pain perception, itching, regulation of hair follicles and regulation of dermatitis (157).

Metavolotropic receptors are composed of 7 transmembrane proteins, from which the signal is transported via heterotrimeric G proteins. TRP channels have 6 different domains. The 4 subunits form a functional channel, which is a channel for influx of sodium and/or calcium ions.

3.1.1 CB1 RECEPTORS AND DOPAMINE

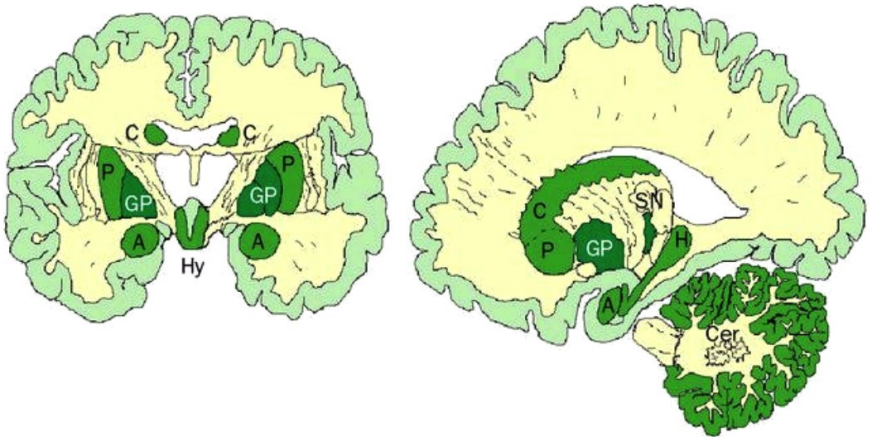

Figure 13: CB1R expression in the adult brain varies by region. Different intensities of green colour represent the concentrations of CB1R per region. The concentration of CB1 is very high in the macula (GP) and the sulcus (SN), moderate in the cerebellum (Cer), hippocampus (H), caudate nucleus (C), cortex (P), hypothalamus (Hy) and amygdala (A), and low in the cortex and very low in the white matter.

Studies on animals have demonstrated how amphetamine interacts, leading to an increase in the release of dopamine and THC (158). These research findings determined that the behavioral impact of amphetamine was either heightened or diminished based on the amount of THC administered. This caused the scientists to determine that dopamine is 'a top choice for the way Δ9-tetrahydrocannabinol works'. This is confirmed by the fact that THC has a significant impact on the dopamine system, leading to both positive and negative effects.

The hormone that regulates the endocannabinoid system is dopamine (159). CB1Rs and the endocannabinoid ligands anandamide and 2-arachidonoylglycerol (2-AG) abound in dopaminergic pathways and enter the striatum (160). Dopamine transport is regulated by the former, acting as a feedback system t o presynaptic gamma-aminobutyric acid (GABA) nerve terminals. CB1Rs and D2Rs are expressed in the neural elements of the nervous system. AC is inhibited as either receptor is activated, since both receptors cooperate with Gi protein.

Dopamine is released after stimulation of Anandamide and 2-AG, which occurs in the nucleus accumbens (NAc) shell. However, this state of stimulation is blocked by the CB1 antagonist rimonabant. This leads to the conclusion that CB1receptorsare affected by the otopaminergic effects of endocannabinoids. THC has responsive properties, which are potentiated by biased signal transduction mechanisms fr om the CB1R. These properties are induced by increasing dopamine release and rapidly activating dopaminergic neurons. There has been evidence that acute and frequent exposure to THC affect the dopaminergic system differently each time.

Cannabinoids interact with specific cannabinoid receptors that are located in the brain region and have several physiological effects (161). CB1 receptors are highly localized in regions that are related to cognition, memory, anxiety, pain perception etc (162,163), while

CB2 receptors are found in the spleen and other surrounding tissues (164). These binders are normally associated with receptors that appear to be family of anandamides (165) which are produced by the arachidonic acid, related to prostaglandins.

Due to its low water solubility CBD is poorly absorbed by the gastrointestinal system and therefore its phamacokinetics vary. CBD is very rapidly distributed in tissues and has a large volume of distribution. Due to its high lipophilicity it is distributed in adipose tissue (166). It is biotransformed in the liver via a hydroxylation reaction to form the metabolite 7-OH-CBD and other secondary hydroxy metabolites (167). The biotransformation of CBN involves hydroxylation at carbon 11 leading to the major metabolite. The major metabolites are 11-hydroxy-CBN and 8-hydroxy-CBN. Because CBN has an additional aromatic ring in its molecule, it is biotransformed much less and much more slowly than D9- THC (168). Its excretion is relatively slow, with 35% being eliminated through the faeces and only 8% being excreted in the urine (169).

3.2 CANNABINOIDS MECHANISM OF ACTION

The actions of endocannabinoids can vary. They are under strict control which depends on biosynthetic and more on hydrolytic enzymes. This control is perfected by distinct transporters which enable the movement of endocannabinoids across the plasma membrane and intracellularly. The receptors, enzymes and transporters form the endocannabinoid system, leading to the timely delivery of the correct endocannabinoid to the target. This system allows endocannabinoids to act as highly sophisticated signals, capable of mutual interactions and cross-checks (170,171), and collectively act as a summary switch that determines the threshold of neuronal excitability, affecting various physiological states and neurological disorders such as epilepsy.

The endocannabinoid system is highly complex so any disruption in signalling from compounds such as phytocannabinoids can lead to unpredictable and potentially harmful side effects.

Membrane phosphatidocholines give rise to endogenous cannabinoids through cleavage by phosphodiesterases, forming the fatty acid ethanolamines and fatty acid esters that act as endogenous cannabinoids (172,173). The production of natural cannabinoids relies on calcium and can be boosted by activating cyclic adenosine monophosphate (cAMP) simultaneously (174). It is understood that cannabinoids are produced and released when needed. They are extracted from the fissure through facilitated transport (175) and degraded inside the cell by the FAAH enzyme into fatty acids and ethanolamine or ester (176,177). They can be added to phospholipids afterward. In addition to natural substances that activate receptors, certain fatty acid esters do not directly activate cannabinoid receptors but they do increase the impact of naturally occurring cannabinoids (178). Cannabinoids in the synaptic cleft interact specifically with cannabinoid receptors, causing numerous effects. CB1 is predominantly found in the central nervous system, whereas CB2 is located in the periphery (179,180). CB1A, a cloned version of the CB1 receptor, differs from CB1 at the amino-terminal end (181). Protein (179,182) inhibits adenylyl cyclase activity when receptors are activated. When the CB1 receptor, but not CB2, is activated, potassium A currents are inactivated, the potassium uptake channels that are associated with G-protein are activated,

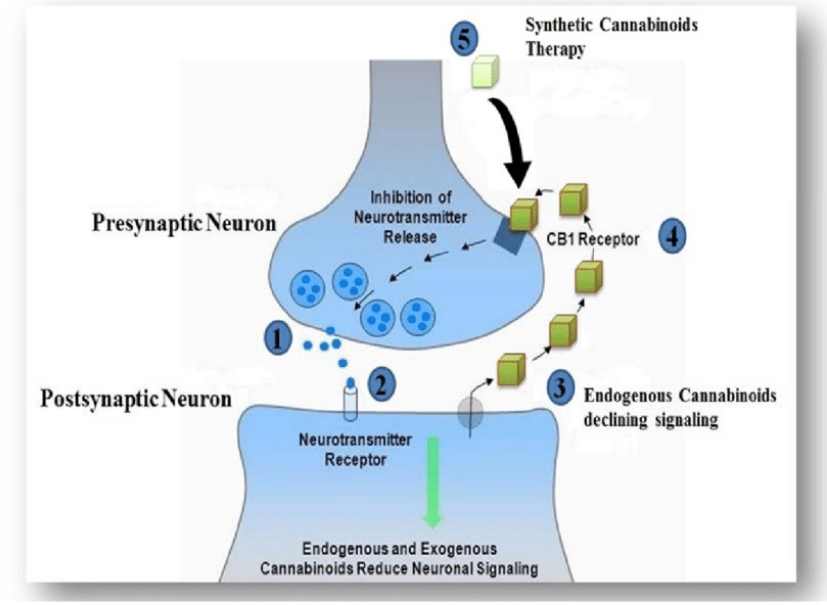

Figure 14: Mechanism of action of the cannabinoids at pre and postsynaptic terminal

the calcium channels are inhibited and finally the protein kinase is activated (183,184). Most cannabinoid agonists are not selective for CB1 and CB2 receptors (183). One example is the endogenous cannabinoid palmitoylethanolamide (PEA), which specifically targets the CB2 receptor (185). SR14176A is the most well-known selective CB1 antagonist (186). When administered alone, it can produce effects opposite to cannabinoid agonists (187). This indicates that naturally occurring cannabinoids are functioning in these systems or the CB1 receptor is consistently active and SR 141716A works as a reverse antagonist to decrease the receptor activity. Studies indicate that the CB1 receptor promotes constant activity in both transfected cells and cells that naturally express the CB1 receptor (187,188). Research has also demonstrated that the CB2 antagonist (SR 144528) acts as an inverse agonist (189).

More precisely, CBD does not strongly bind to CB1 and CB2 receptors and acts as a non-agonistic regulator of CB1. When THC is in the system, CBD works against it, leading to a decrease in the negative effects of THC. Additionally, CBD acts as a stimulator of the serotonin 5-HT1A receptor and another receptor called vanilloid type 1, possibly clarifying the anti-inflammatory and neuroprotective properties of CBD. It blocks the reuptake and enzymatic breakdown of the body's own cannabinoid anandamide, leading to higher levels. CBD could improve adenosine receptor signaling, preventing adenosine deactivation, leading to the potential outcome of CBD's pain-relieving and anti-inflammatory characteristics (190,191,192).

THC acts as a partial activator of CB1 and CB2 receptors, resulting in psychoactive and muscle-relaxing effects (193). It presents significantly higher anti-inflammatory properties compared to hydrocortisone (194) and interacts with multiple receptors, such as GPR18 (195), while also acting as an antagonist on the 5-HT3A receptor (196).

3.3. ENDOCANNABINOID SYNTHESIS

In general, endocannabinoids have characteristics that differ from classical neurotransmitters. Neurotransmitters are known to be synthesised in presynaptic neurons and stored in vesicles to be released after neural activation and subsequent calcium influx. However, endocannabinoids are synthesised from membrane lipids in postsynaptic neuronsafter the calcium influx that follows neuronal activation. The synthesis of anandamide depends mainly on the activity of phospholipase D, whereas the synthesis of 2-AG involves phospholipase C. Also, endocannabinoids diffuse immediately into the synaptic cleft and are not stored in vesicles (197).

Arachidonic acid is contained in Anandamide and 2-AG, but what differs is the way it is synthesised in each case but the way it is degraded. Also, the enzymes involved are different (198). More specifically, the former is produced mainly by N-arachidonoylphosphatidyl ethanol (NAPE), whereas the opposite is true for 2-AG from 2-arachidonoyl-contaminating phospholipids, and basically from arachidonoyl-contaminating phosphotidyl inositol bis-phosphate (PIP2). It is also reported that one function of 2-AG is to serve as an endogenous ligand for cannabinoid receptors. However, it is one of the important metabolic intermediates in lipid synthesis and is also the major arachidonic acid substance in the synthesis of prostaglandins (199). It follows that the production and cleavage of 2-AG have to do with functions that are not related to the endocannabinoid system.

Anandamide is produced in various ways based on the specific region of the brain in which it is found. Research has shown that activating D2-like dopamine receptors leads to higher levels of anandamide in the striatum, and blocking CB1 receptors can reduce the excess movement induced by a D2 receptor agonist (200). Many studies have indicated that changes in anandamide levels are linked to schizophrenia, which may aid in understanding the causes of the illness. Four routes have been proposed for the production of anandamide: NAPE-PLD, NAPE-phospholipase C (PLC) with phosphatase (201), simultaneous hydrolysis of the acyl groups by phospholipase B, ABHD4, followed by hydrolysis by GDE1 (202), and hydrolysis of one acyl group, leading to the release of anandamide by the activity of a lysophospholipase NAPE-PLD (203).

The initial route pinpointed for the production of anandamide within cells is the breakdown of NAPE by NAPE phospholipase D (204). The deletion of NAPE-PLD gene causes various changes in anandamide levels as reported in references 205 and 206. The distribution of NAPE-PLD only partially coincides with the distribution of CB1 receptors. Another way anandamide is produced is by breaking down the NAPE phosphodiester bond with a NAPE-selective phospholipase C (PLC), followed by dephosphorylation of the resulting phosphoanandamide (201), leading to the release of anandamide. Extensive research has been conducted on this pathway in immune cells. Nevertheless, the other two have been mentioned in expressing systems, so it is important to explain their function in anandamide production (203).

The routes for producing anandamide are simple compared to those for producing 2-AG. The majority of 2-AG is generated through step-by-step breakdown of an arachidonoyl-containing precursor PIP2 (usually 1-stearoyl-2-arachidonoyl-sn-glycerol) (207) by PLCβ and then by DAGL (208). Following activation of receptors that induce PLC (e.g., receptors like I metabotropic glutamate, M1 or M3 muscarinic, orexin A, etc.), the initial

pathway is triggered. Two isoforms of DAGL, known as DAGLα and DAGLβ, have been identified (209). DAGLα was identified as the primary producer of 2-AG crucial for synaptic plasticity in the adult mouse CNS according to research (210). Anatomical research shows that mGluR5 and DAGLα receptors are closely located near each other in dendritic spines, where they interact with presynaptic CB1 receptors (211,212). DAGLα seems to be the primary dominant enzyme responsible for producing 2-AG in the adult central nervous system. On the other hand, DAGLβ can help produce synaptic 2-AG in specific circumstances (213). It also has a significant role in producing 2-AG during an immune response (214). The breakdown of the inositol phosphatidyl precursor by a phospholipase A, along with hydrolysis of the phosphate ester and lysophospholipase C, serves as an alternative route for 2-AG synthesis. Nonetheless, the significance of this pathway in the brain remains uncertain.

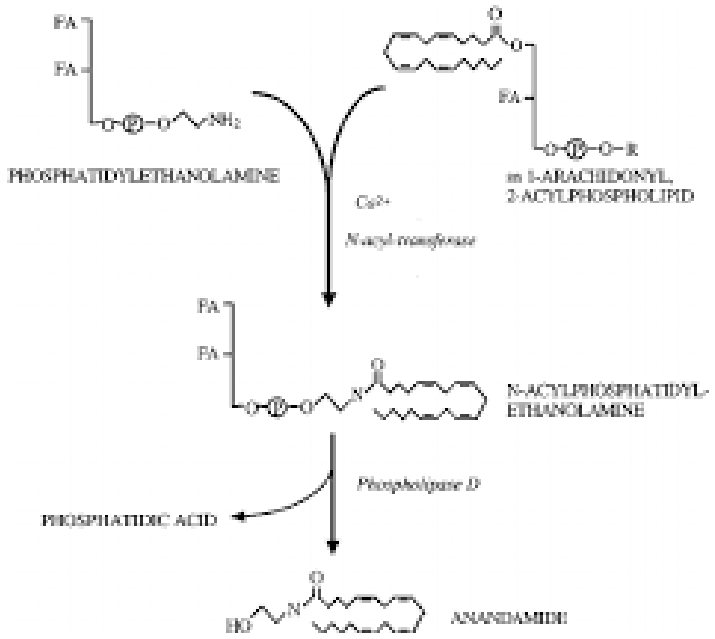

Figure 15: Anandamide biosynthesis.

3.4 ENDOCANNABINOID DEGRADATION

The degradation of anandamide in the CNS is done first by the enzyme fatty acid amino hydrolase (FAAH) (Fig. 2B) (215). This one, degrades multiple fatty acid amides, like palmitoyl, oleoyl ethanol amide etc. This means that there are many therapeutic aspects because FAAH when it is been inhibited the levels of these ethanol amides are been increased (216,217).

The other pathway of anandamide degradation involves oxidation by cyclooxygenase-2 (COX-2) to produce prostamides (218). These components have separate actions and are independent of cannabinoid receptors. They are also important components that help in the treatment of intraocular hypertension but also have their own pharmacology which is unique (218). Due to the different structure of arachidonic acid and anandamide, the presence of COX-2 inhibitors causes inhibition of anandamide oxidation , without however affecting

the production of prostaglandin (219). Besides, COX-2 is selective compared to acyl ethanolamides and binds to anandamide, and when inhibited causes a more selective mode of enhancing anandamide compared to FAAH inhibition (220).

Another method by which anandamide can degrade is via N-acylethanolamine-hydrolyzing acid amidase (NAAA) (221). Inhibiting FAAH may influence how anandamide is metabolized, leading to alterations in cellular functions aside from cannabinoid receptor involvement. Three enzymes that break down 2-AG are monoacylglycerol lipase (MGL) and the hydrolases ABHD6 and 12 (222). COX-2 oxidizes 2-AG while FAAH hydrolyzes it. These enzymes are found in various areas within the cell, regulating the breakdown of 2-AG in different cellular regions. MGL is a widely recognized enzyme found in the synaptic terminals of the mature central nervous system (223). Inhibiting MGL results in increased levels of 2-AG stimulating cb1 receptors in the human CNS. Moreover, there is a reduction in the amount of arachidonic acid used in the production of prostaglandins. Inhibition of MGL reduces inflammation caused by prostaglandins, whereas ABHD6 can be found in the dendrites and dendritic spines of excitatory neurons in the cortex (224). Blocking ABHD6 results in enhanced 2-AG signaling via CB1 receptors in the CNS. Moreover, ABHD12 enzyme also has hydrolytic functions and is involved in the degradation of 2-AG in the brain. Although its role in 2-AG metabolism in living organisms is not well understood, it plays a significant part in the degradation of long-chain lysophosphatidylserines (225).

The importance lies in the central nervous system's metabolism of COX-2 for 2-AG. 2-AG signaling through CB1 receptors leads to the inhibition of COX-2, as indicated by research studies (226,227). Certainly, a significant oxidative metabolite of 2-AG, PGE2-glycerol ester (PGE2-GE), boosts synaptic plasticity and triggers hyperalgesia (228,229). Alterations in COX-2 levels could have a notable impact on the functioning of the central nervous system. The reason for this is that 2-AG works via CB1 receptors to inhibit synaptic transmission and neuronal excitability, while PGE2-GE is stimulating.

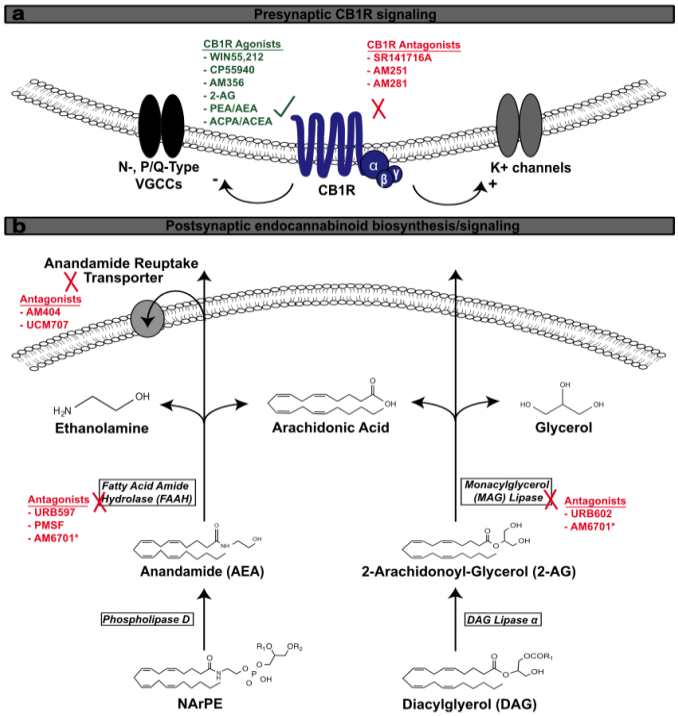

Figure 16: Biosynthesis, degradation, and signaling of endocannabinoids. (A) Presynaptic cannabinoid type 1 receptor (CB_1R) signaling. (B) Postsynaptic endocannabinoid biosynthesis/signaling. NArPE = N-arachidonoyl phosphatidylethanolamine; DAG = 1-acyl, 2-arachidonoyl diacylglycerol; VGCC = voltage-gated calcium channels; PEA = palmitoylethanolamide; ACPA = arachidonylcyclopropylamide; ACEA = arachidonyl-2'- chloroethylamide; PMSF = phenylmethylsulfonyl fluoride

3.5 SIGNAL TRANSDUCTION

The CB1 and CB2 receptors belong to the large family of Gi/o-type guanine regulatory protein (G protein) receptors. Both CB1 and CB2 exhibit a 48% similarity (230) in genetic sequence and both have the ability to block adenylyl cyclase and trigger mitogen-activated protein kinase (MARK) pathways (230,231); activation of CB1 receptors also leads to the release of G-proteins that interact with various ion channels. The receptors increase A-type and inwardly rectifying K+ currents and inhibit N- and P/Q-type Ca2+ currents (232). Connector CB1 coordinates the channels and this mechanism is one of the most important as it mediates cannabinoid-induced synaptic plasticity. When Gi/o Gβγ mediated inhibition of Ca2+ entry is activated in combination with inhibition of cAMP/PKA phosphorylation of ion channels (233). This receptor stimulates the Gs-associated priotins and is stimulated by accessory priotins (234). It is commonly believed that multiple pieces of evidence indicate that the combined impact of cannabinoids cannot be completely attributed to their interaction with CB1 and CB2 receptors alone, but that other G-coupled receptors also play a role and need to be controlled (235). A possible candidate is the orphan G-protein-coupled receptor 55 (GPR55) (236), also known as "CB3". Although some groups have presented

contradictory findings about its classification as a genuine cannabinoid receptor, Δ9-THC and CBD are the ones that attach to this receptor. Other non-G-protein-coupled receptors that may directly influence cannabinoid signaling include the transient receptor potential vanilloid type 1 calcium channels (TRPV1). Endocannabinoids also engage with nuclear peroxisome proliferator-activated receptors α and γ that play a role in regulating lipid and glucose metabolism, along with inflammatory reactions. There is mounting evidence suggesting that cannabinoids reduce immune responses.

Within the brain, the polymeric functional molecules of CB1 receptors are situated. CB1 is found in various parts of the brain in the form of multimeric homodimers. The former are deemed as the originating state of CB1 receptors. CB1 and CB2 create heteromers together. Furthermore, there is evidence indicating that heteromers can also develop between CB1 and other G-coupled receptors, such as D1 and D2, orexin, μ-opioid, and adenosine. The physiological function of these heteromeric G-protein receptors remains unclear, however, indications point towards their significance in conditions like Parkinson's disease and addiction. Interactions and transduction occur through the receptors and non-receptor tyrosine kinases when CB1 is activated. Further analysis is required to understand the functioning and interactions of receptors, yet the current data offers insight into the complexity of cannabinoid signaling and hints at potential targets for cannabinoid pharmacotherapy research.

3.5.1 INHIBITION OF ADENYLIC CYCLASE

Cannabinoids like Δ9- THC have the ability to inhibit the action of adenylate cyclase in a stereoselective, dose-dependent, reversible manner (237). Because of this we have been led to believe that the actions of cannabinoids are coordinated by membrane receptors that are bound to G proteins. The receptor involved in the attenuation of cAMP production induced by cannabinoids is CB1Cannabinoids are found in specific regions of the brain where they act as strong inhibitors of adenylate cyclase, with a higher concentration of CB1 receptors. In nerve cells grown in a lab setting and in the brain of mammals, the inhibition observed in adenylate cyclase can be blocked by pertussis toxin (237). The participation of a inhibitory G-protein has been effectively shown. Therefore, there is a reverse relationship between Gi/o proteins and cannabinoid receptors, as pertussis toxin hinders the separation of the a and b/g subunits of these G proteins through covalent ADP-ribosylation, thereby blocking the inhibition of adenylate cyclase by G proteins. Overall, adenylate cyclase is controlled similarly to other Gi protein-associated receptors and is responsive to both divalent cations and guanine nucleotides.

Cannabinoids do not inhibit adenylate cyclase and this is not mediated by nocannabinoid receptors. It has been shown that no interaction has taken place between cannabinoid compounds with a2-adrenergic, M4-muscarinic, or d-opioid receptors. This is because the effect on neuroblast cells induced by h9-THC is not inhibited by antagonists of these receptors (237).

3.5.2 CALCIUM CHANNELS

Anandamide and other cannabinoid agonists inhibit the L, N, Q, P-type taseo-controlled calcium channels. This suggests that a G0 protein is the direct mediator of this response. (238,239). Inhibition of calcium channels through stimulation of presynaptic CB1Rs leads to a reduced likelihood of neurotransmitters being released from presynaptic nerve terminals because membrane unfolding and exocytosis are blunted. (240,241) The physiological effect of this inhibition is dependent on the cell populations that are activated. In summary, inhibition of calcium channels by cannabinoids can lead to various biological responses. However, activation via CB2Rs receptors, does not affect calcium channels. From the studies it was found that adding the upper effective concentrations of muscarinic receptor agonists, a2-adrenergic receptor, dopamine D2 receptor, GABAB and opioid receptors did not lead to an increase in the maximal inhibition of cAMP accumulation, which is achieved with cannabinoids. This implies that CB1 and other G protein-coupled receptors in the same neuron are cooperating. It also follows that they share a short mechanism with each other. Nevertheless, in spite of their absence of additivity to adenylate cyclase activity inhibition, GABAB agonists and cannabinoid agonists evoke a fully afferent response to stimulation of low-Km GTPase activity in cerebellar granule cells (242). Not only does this finding indicate that diverse pools of G proreins are stimulated by different receptors but also that they tend to share a common effector system, that is, the adenylate cyclase.

3.5.3 POTASSIUM HYDROXIDE CHANNELS

When CB1Rs receptors are activated, the activity of G-protein coupled inwardly rectifying potassium channels (GIRK/Kir), the A-type potassium channels, is enhanced and the opening of D-type potassium channels is inhibited. These are due to decreased phosphorylation of potassium channels which is caused by inhibition of the action of A-type protein movement (243).

3.6 ENDOCANNABINOIDS AS RETROGRADE SYNAPTIC MESSENGERS

Endocannabinoids, specifically 2-AG, have been suggested in experiments to act as a retrograde messenger (244). The suggestion stems from the fact that CB1 receptors are located presynaptically and can hinder synaptic transmission, while enzymes linked to endocannabinoid synthesis are located postsynaptically. Additionally, endocannabinoid production is enhanced by postsynaptic activity, especially through an increase in intracellular calcium and the activation of Gq/11-linked G protein-coupled receptors.

In terms of synaptic plasticity involving endocannabinoids, three forms of synaptic plasticity have been investigated. This refers to endocannabinoids that act as retrograde messengers. These refer to depolarization-induced suppression of inhibition (DSI)/depolarization-induced suppression of excitation (DSE), metabotropic-induced suppression of inhibition (MSI)/metabotropic-induced suppression of excitation (MSE) (also known as synaptically-evoked suppression of inhibition/excitation (SSE/SSI) (245) or

endocannabinoid-mediated short term depression (eCB-STD) (244)), and endocannabinoid-mediated long term depression (eCB-LTD).

3.6.1 DSI/ DSE

DSI transiently suppresses inhibitory input to a neuron after intense activation (repetitive action potential or step depolarization) (246). Similarly, DSE is exactly the same but affects excitatory inputs. It is a general finding that inhibitory synapses are more sensitive to depolarization-induced suppression of synaptic transmission than excitatory synapses (247).

What happens at many inhibitory synapses is that the receptors are activated by endocannabinoids (248,249), which affects how they function, especially when exposed to THC. GABA or glutamate are inhibited in their release from DSEs/DSIs.. In addition to this, when CB1 receptors are activated, peptide release is inhibited, for example CCK found on positive terminals of CB1 receptors (250).

In DSI, strong depolarization of a neuron that is postsynaptic causes temporary suppression of inhibitory transmission in that neuron. A similar phenomenon involving excitatory transmission is DSE, i.e., depolarization-dependent suppression of excitatory transmission, which have been extensively studied in the hippocampus and cerebellum. The mechanism that many investigators argue is that depolarization of the post-synaptic cell causes an increase in intracellular calcium that activates alpha diacylglycerolipase (DAGL), which immediately cuts the acyl chain at one position of diacylglycerol (DAG), producing 2-AG. The inhibition of calcium channels and possibly the inhibition of the vesicular release mechanism leading to suppressed synaptic transmission is the result of 2-AG, which travels continuously with (possibly by diffusion or via an undefined vector) towards the presynaptic terminal. This is also where the presynaptic CB1 receptors bind. 2- AG is then degraded either by MGL and/or cyclooxygenase-2 and DSI (and DSE) are driven to termination. The implications of the involvement of COX-2 in DSE termination may be therapeutic (251,252,253).

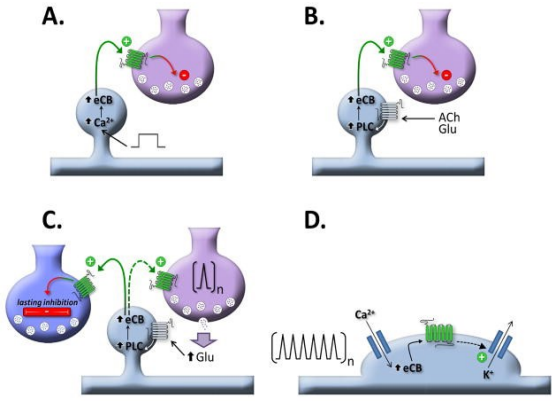

Figure 17: Forms of endocannabinoid-mediated synaptic plasticity and endocannabinoid mediated cell-autonomous regulation of excitability)

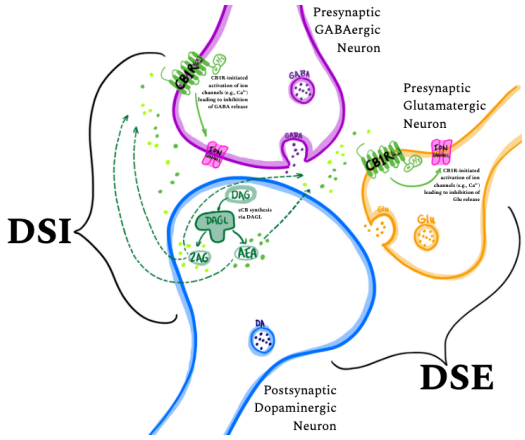

Figure 18: DSI/ DSE

3.6.2 MSI/MSE

Metabolic suppression of inhibition (MSI) or stimulation (MSE) is a further version of the transient modification of synaptic transmission by endocannabinoids. In this context, m obilizati o n o f a p osynaptic Gq/11-bound GPCR stimulates PLCbeta, leading t o DAG pr oducti o n. DAG is cut by DAGL immediately to 2-AG, and this in turn cleaves the synapse to activate presynaptic CB1 receptors that block synaptic transmission. Any appropriately positioned postsynaptic Gq/11-linked GPCR can express MSI/MSE. The metabotropic group I glutamate receptors (i.e. mGluR1 and mGluR5) and the muscarinic receptors M1 and M3 are the most common receptors involved in MSI/MSE (254,255).

Endocannabinoids mediate and create a form of short-term plasticity which is Metatropic-induced suppression of arousal. This is caused by engagement of a postsynaptic Gq/11-coupled GPCR and activation of a phospholipase Cβ. Phospholipase C then produces diacylglycerol. Diacylglycerol in turn is deacylated by diacyl glycerol lipase to yield 2-AG. Finally, it is diffused presynaptically. Its purpose is to activate CB1 receptors and thus suppress synaptic transmission.

MSI/MSEs are triggered by a variety of Gq/11-linked GPCRs and include mGluR1, mGluR5, M1, M3, orexinA, CCKA, and α1 adrenergic receptors (244). The calcium sensitivity of PLCβ1 (256) results in a cooperative synergy between depolarization-induced and metabotropic-induced suppression of inhibition/stimulation. As a result, both of these displays of reverse synaptic plasticity can function as a requirement for the stability of Gq/11-induced signaling and postsynaptic depolarization or calcium influx (257,258).

While DSI/DSE and MSI/MSE may seem unrelated, they have the ability to collaborate. In other words, a brief depolarization that slightly activates the Gq/11-coupled receptor leads to an increase in intracellular calcium levels, which then promotes PLCbeta activity. This results in elevated levels of DAG production, possibly higher DAGL activity, leading to increased 2-AG production. Therefore, endocannabinoids act as contributing links between depolarization and activation of metabolic receptors.

3.7 INTERACTIONS BETWEEN THC AND ENDOCANNABINOIDS

THC and endocannabinoids interact with CB1 receptors. THC and anandamide under certain conditions such as at low receptor density or in restriction of post-receptor effectors (259), antagonize CB1 receptor signaling induced by 2-AG. In some systems, however, THC and andamide act as effective CB1 antagonists.

When people consume cannabis, what happens to their brain?

Immediate responses to cannabis have to do with both agonism and antagonism of CB1 receptor signalling. Even repeated, very high doses of the CB1 receptor antagonist, rimonabant, reduce subjective measures of feeling "high" and suppress the tachycardia that cannabis induces (260).

Taking rimonabant orally does not cause deprivation syndrome in people who have consumed moderate doses of THC (261). However, after chronic high-dose THC intake in

rodents, rimonabant causes deprivation syndromes (262). The low efficacy of THC and the sparse receptor occupancy induced by occasional human cannabis use compared to what can be achieved experimentally (263) or with high THC cannabis strains (264) may result in milder and more direct effects in the population. The use of strong antagonists of cannabinoid receptors leads to the occurrence of undesirable psychiatric effects (265). In general, the interactions of THC, CB1 and endocannabinoids are very complex and even more so than simply THC occupying CB1 receptors as an agonist.

3.8 THE EFFECT OF CANNABIS ON NEUROTRASMITTER SYSTEMS

Tetrahydrocannabin affects the endogenous cannabinoid and dopamine systems through receptors. Cannabinoid receptors occur in high concentration in the cerebral cortex. These brain regions in which they are localized include those areas associated with schizophrenia that affect dopamine synthesis and uptake (266). Studies on the neurotoxicity of THC have been conducted in animal models and suggest that THC increases dopamine levels in a variety of brain regions, including striatal and prefrontal regions (267). Animal studies have demonstrated more irreversible residual effects in pre-adolescent rats after years of exposure to THC compared to more mature rats. THC is an agonist of the CB1 cannabinoid receptors. Cannabis acts through its action on these very GABA receptors and glutamic acid, which regulate the excitability of midbrain dopamine neurons and pyramidal cells in the prefrontal cortex. THC exacerbates dopaminergic imbalances and increases dopaminergic tone in the striatal areas of the brain, which when administered repeatedly, reduces dopamine levels in the prefrontal areas of the brain through sensitisation processes resulting in the expression of psychotic disorder (267,268).

Repeated THC use alters the function of the prefrontal cortex and affects dopamine signalling through activation of CB1 receptors (267). However, because most evidence is based on animal studies, the effects of endocannabinoids are not yet fully understood in humans. No relationship between postsynaptic striatal dopamine receptors and cannabis use has yet been reported (269), and pretreatment with the dopamine receptor antagonist haloperidol did not alter the behavioural effects of delta-9-THC. On the other hand, study (270) has found that chronic cannabis use is associated with a reduced ability to synthesize presynaptic dopamine in the striatum, which suggests a complex relationship between cannabis and changes in dopamine availability.

3.9 CANNABINOIDS AND SEROTONIN

The serotonin receptor is found throughout the central nervous system, is bound to Gq/11 and is expressed in pyramidal neurons of the prefrontal cortex. Under normal conditions 5-HT2ARs are involved in the regulation of many functions mainly mood and impulse control. Therefore, alterations in the expression of these receptors have been implicated in drug addiction (271).

Studies have shown that modification of serotonergic signalling by cannabinoids underlies their effects on behaviour such as stress. Both systems, endocannabinoid and serotonergic, regulate processes and behaviors such as sleep, body temperature and emotional processes.

Endocannabinoids and phytocannabinoids, control the release of serotonin (5-HT) in terminal areas as well as the activity of serotonergic neurons. Cannabinoids are both inhibitory and excitatory and may have an indirect or direct effect on 5-HT activity.

In vivo studies have demonstrated that agonism of CB1 receptors diminishes 5-HT production in the hippocampus. Serotonin release, on the other hand, is enhanced in the NAc by the systemic administration of a CB1 agonist, whereas immediate application to the terminals in the NAc decreases release. Enhanced 5-HT release in the NAc contributes to rewarding influences of Δ9-THC (272).

Research has demonstrated that blocking CB1 receptors boosts the release of 5-HT. A study on animals discovered that D9-THC has a positive impact on depression. However, this only occurred when administered for 5 days, like with traditional serotonin reuptake inhibitors like citalopram. Additionally, repeated doses of Δ9-THC increased the spinal activity of the DR 5-HT neurons and the postsynaptic activity of the 5-HT1a receptor in the dorsal hippocampus, similar to what happens with typical antidepressants. Cannabis has antidepressant properties when used for an extended period. An examination of the existing research suggests that using cannabis, especially in large amounts, could lead to a higher likelihood of developing depressive disorders (271,273,274,275).

Indirect modulation of 5-HT2AR expression by synthetic cannabinoids has been proposed as a mechanism that causes psychosis and the regulation of 5-HT2AR by synthetic cannabinoids is mediated by the CB1R receptor. A typical example is the fact that synthetic cannabinoids increase the interaction of 5-HT2AR and dopamine receptors, which is associated with interactions such as in schizophrenia. If some antipsychotics (e.g. clozapine) act as 5-HT2AR antagonists and others e.g. fluoxetine, are useful in psychosis by upregulating 5-HT2AR, then it is true that synthetic cannabinoids create propsychotic effects by upregulating 5-HT2AR in a specific way in a specific region of the brain (276,277,278).

3.10 CANNABINOIDS AND NOREPINEPHRINE

Another common pharmacotherapeutic target of psychiatric disorders affected by cannabinoid signalling is the noradrenergic system. When noradrenergic signaling is disrupted it has an effect on mood disorder such as depression. As in the case of serotonin, when noradrenergic and cannabinoid activity is increased, mood improves. However, when the two systems are activated together, mania can be induced. This leads to the conclusion that these two systems interact or share common pathways (279).

Most of the CB1 receptors can be found in the postsynaptic norepinephrine terminals and presynaptic glutamate terminals. CB1 receptors found in GABA terminals that are presynaptic (280) are key to how the body responds to external cannabinoids acting as agonists. Stimulation of CB1 leads to a rise in noradrenergic neuron activity likely by

releasing inhibitory tone. In general, the interaction of cannabinoids with the noradrenergic system has important functions in both physiological and behavioral responses (279).

3.11 VIABILITY AND SELECTIVITY

The viability of non-cancerous cells remains unchanged. However, in some cases it is increased by cannabinoids. The latter cause apoptosis in untransformed cells, especially those that proliferate rapidly. The proapoptotic properties of cannabinoids are associated with activation of either CB1 and/or CB2 receptors as in glioma, whereas in other types of cancer cells e.g. pancreatic cancer, they rely only on activation of CB2 receptors.

What we need to investigate is how the activity of cannabinoids is influenced based on their concentration. Cannabinoids exhibit stimulating effects at nanomolar concentration in vitro, but display inhibitory effects at micromolar concentration. Experiments conducted in vitro demonstrated that low levels of CB receptor agonists caused a rise in the growth of specific cancer cells, with the involvement of ADAM17 metalloprotease activity, EGFR activation, and subsequent triggering of ERK and Akt pathways. The amount of THC used in the experiments matched the level of THC found in the blood after smoking or taking THC orally (281,282,283).

CHAPTER 4: WHERE THEY ARE LOCATED

Cannabinoids are found in biological materials, i.e. blood, urine, aqueous humour, saliva and hair. The **table 2** below summarises the results of a study of 5 case studies where drug users were found dead.

The samples were blood from the femoral vein and heart, urine from the gall bladder, cerebrospinal fluid, liver, lungs, etc. From this it was found that the gall bladder fluid showed the highest concentrations of all analytes except THC, which was found in maximum concentration in muscle tissue. However, its metabolites could hardly be detected in the ethical tissue, but were abundant in the bile.

Table 2
Cannabinoid concentrations in body fluids (ng/mL) and tissues (ng/g); –: not detectable, *: referring to the total gastric contents (ng), positive: positive finding ≤ the lower limit of quantification.

Specimen/Analyte	THC	CBD	CBN	11-OH-THC	THC-COOH	THC-COOglu
Case 1						
Femoral blood	Positive	Positive	Positive	Positive	221	–
Heart blood	1.4	1.5	–	1.1	18	193
Bile	14	58	221	92	990	11,160
Liquor	–	–	–	–	Positive	–
Gastric contents*	Positive	Positive	–	0.7	–	–
Urine	–	0.5	–	–	6.3	66
Brain	1.1	3.7	–	–	5.5	–
Liver	–	13	5.1	4.1	–	79
Lungs	5.6	5.1	–	1.1	6.9	46
Muscle	40	32	3.6	–	Positive	–
Kidneys	2.4	4.1	7.9	–	9.0	393
Case 2						
Femoral blood	0.7	Positive	–	–	–	–
Heart blood	1.2	–	–	0.5	Positive	296
Bile	4.9	63	11	105	285	635
Gastric contents*	5.7	–	–	46	–	–
Urine	–	Positive	–	2.2	Positive	63
Brain	5.8	6.7	3.1	–	–	–
Liver	1.1	1.1	4.4	–	Positive	71
Lungs	4.8	3.6	–	–	Positive	Positive
Muscle	6.2	3.7	2.1	–	Positive	–
Kidneys	2.6	5.7	1.9	–	Positive	Positive
Case 3						
Femoral blood	–	–	–	–	–	–
Heart blood	0.6	Positive	–	–	9.2	100
Gastric contents*	1.6	1.7	–	268	178	–
Brain	–	–	–	–	–	–
Liver	0.8	1.4	–	1.6	Positive	68
Lungs	0.8	1.3	–	–	–	–
Muscle	3.2	3.1	–	–	–	–
Kidneys	–	2.3	–	–	Positive	124
Case 4						
Femoral blood	5.8	Positive	–	1.7	18	36
Heart blood	1.9	Positive	–	Positive	14	25
Bile	6.3	–	–	20.3	420	9,332
Liquor	–	–	–	–	–	20
Gastric contents*	130	–	210	760	Positive	1220
Brain	–	1.0	–	–	–	–
Liver	–	2.1	3.0	Positive	Positive	87
Lungs	1.4	1.3	–	–	–	–
Muscle	8.9	0.8	–	–	Positive	–
Kidneys	–	1.6	4.3	–	–	83
Case 5						
Femoral blood	2.5	–	–	1.7	13.7	–
Bile	4.8	Positive	–	54	234	8,284
Brain	–	1.8	–	–	4.4	–
Liver	–	–	–	Positive	8.0	518
Lungs	1.2	2.4	–	–	98	165
Muscle	7.3	6.3	–	–	Positive	–
Kidneys	–	1.3	2.2	–	37	212

Table 2: A preliminary investigation on the distribution of cannabinoids in man.

4.1 CANNABINOIDS IN THE BRAIN

Cannabinoids are present in the brain. When smoking, THC is absorbed rapidly with a bioavailability of 18-50% and reaches the peak level. After inhaling smoke containing cannabinoids, THC can be found in the bloodstream within a few seconds, reaching its highest level in the blood 3-10 minutes later (284). Nevertheless, the levels of THC drop from 84.3-162.2 μg/L to 1-4 μg/L within 3-4 hours (285).

The difference in the impact of THC and its blood levels is elucidated by the compound's pharmacokinetics. The quick absorption linked to inefficient elimination and retention of THC in neutral fat tissues, along with the blood-brain barrier restricting plasma levels, is an evolutionary safeguard mechanism to shield the brain from exposure to fat-soluble toxins (286).

THC is converted to 11-OH-THC, which is later metabolised to THCCOOH. In plasma the level is easily reduced and brain concentration increases. When administered intravenously, at the time of peak psychoactivity, in the brain THC is at about 1%. Brain tissue is perfused very rapidly and THC is immediately absorbed into neutral fat. THC is then, slowly and restrictedly transported to the brain. The THC metabolite (11-OH-THC) enters the brain more rapidly and at an increased concentration compared to THC (287). This easily leads to the conclusion that this metabolite contributes significantly to the psychoactive effects of THC.

The transport of THC and CBD to the brain has not yet been clarified and described with certainty. This is explained because of the many routes of administration, such as the respiratory route through smoking or orally through food that may contain cannabinoids. The highest systemic bioavailability of THC occurs when cannabis is smoked (288). Regarding the nose-to-brain route for cannabinoid delivery, CBD is the substance that has been investigated. Its absorption from the nose is very rapid. Cyclodestrins are used to increase the permeability of cbd to the brain, since they interact with epithelial membranes in the nose and can open tight junctions (289).

4.2 CANNABINOIDS IN BLOOD

With regard to analytical approaches for the determination of cannabinoids in human matrices, the most commonly emerging human samples have been found to be blood (whole blood, plate and serum), urine for hair. Each time the time elapsed since the last cannabinoid ingestion should be taken into account, because it is unlikely from a certain point onwards that all cannabinoids can be detected. For example, after consumption of cannabis and after 10-12 hours THC cannot be detected. The compounds that cannot be detected have a short half-life in this matrix and this makes this sample suitable for analysis only in cases of acute intoxication.

The compounds analysed in blood are 11-hydroxy-D9-THC (THC-OH) and 11-nor-9-carboxy-D9-THC (THC-COOH), i.e. the major metabolites of THC.

4.3 CANNABINOIDS IN ORAL FLUID

Because of the ease and speed of collection of oral fluid and the difficulty of adulteration, it is a sample for the determination of synthetic cannabinoids (290). In order to detect cannabinoids in oral fluid, recent consumption must have taken place. The quantification of 28 synthetic cannabinoids in mouthwash has been performed by LC-MS/MS (291).

4.4 CANNABINOIDS IN SERUM

This sample provides the possibility to detect directly the JWH family, instead of their metabolites. However, serum is not the most ideal sample to be used for such analyses due to the larger sample amount required. This is not a problem for other biological samples such as urine.

4.5 CANNABINOIDS IN HAIR

The foreign substances are absorbed by the hair cells that grow, through passive diffusion from the blood, and thus become trapped in the keratin cavity without being further metabolized. This enables the hair to be analysed after some time, maybe even some months. In addition to this, the drugs are shoveled into the hair and in turn the hair is a stable tissue, compared to other samples such as urine. Finally, hair is not so affected by adulterants and short term abstinence, it is stored for a long time and does not require refrigeration.

However, there are factors such as hair colour and length, individual characteristics and ethnicity or even cosmetic treatments can alter the concentrations of cannabinoids and thus complicate the analysis.

4.6 CANNABINOIDS IN URINE

Urine is one of the most widely used biological fluids in crime toxicology. The compounds generally analysed in urine are those analysed in blood, i.e. the main metabolites of THC. THC-COOH undergoes glucuronidation and can be found in urine as the most abundant metabolite (both in conjugated, THC-COOH-gluc, and unconjugated form). However, many monohydroxylated and carboxylated compounds have been identified in user urine, which are presumed to be metabolites of synthetic cannabinoids (292). Their detection is not always possible as synthetic cannabinoids are biotransformed into many metabolites, which are not yet known. In addition, there is a lack of metabolic reference standards (293).

4.7 DISPOSAL

Once THC is introduced into the human body, it attaches to plasma proteins, predominantly low-density lipoproteins, with a minor amount found in red blood cells (294). On the other hand, the metabolite 11-hydroxy-THC binds more firmly. Research has discovered that only a tiny fraction, less than 1% of a given dose, actually makes its way to the brain. 65% of inhaled THC and its metabolites are eliminated through feces, while 20% are excreted through urine. Within five days, around 80-90% of the entire dose is eliminated (296). The

metabolites detected in urine are acidic, such as 11-nor-9-carboxy- THC. Similarly, those found in faeces are acidic and neutral; the most abundant are 9-carboxy-THC (29%) and 11-hydroxy-THC (21%).

The highest levels are located in the heart and adipose tissue. These levels exceed those in plasma by about ten to one hundred times higher (297). THC easily penetrates the blood-brain barrier. After 72 hours, the body fat and spleen become the primary distribution sites for THC in animals, where it is retained for an extended period, as opposed to the immediate distribution found in the liver (298). This information has naturally raised significant worry about the potential effects of THC staying in fatty tissues for a long time. Nevertheless, THC does not stay in the brain and is released slowly from the brain's fatty tissue. Due to the minimal levels reached, psychological effects cannot be generated. With consistent usage, THC builds up over time (299).

4.8 METABOLISM

Cannabinoid metabolism occurs in the liver and generates different metabolites based on the methods of administration. The metabolism of THC is compartmentalized and involves allylic oxidation, epoxidation, decarboxylation, and coupling (300). Cannabinoids are important targets of the enzyme P450 mixed oxidase (CYP) enzyme, and in humans, the primary site of hydroxylation is carbon 11, which is facilitated by CYP 2CP (299). When THC is broken down by hepatic microoxidases, the resulting metabolite 11-hydroxy-THC is quickly produced, with its levels in the plasma corresponding to how long the drug remains effective.

The main acid metabolite of THC is psychoactive 11-nor-9-carboxy-THC, excreted in urine and monitored in urinalysis cases in forensics (301). Peak levels in the blood are detected 1.5 to 2.5 hours post smoking. Numerous oxidizing side-chain portions are identified, and additional oxidation of alcohols results in carboxylic acid portions (300). Following oxidation, the metabolites appear as conjugated glucuronides in urine, but their impact is not significant (300).

Limited information is available on CBD metabolism in humans. Hydroxylation has been found to be localized at all side chain positions, and many secondary dihydroxylated metabolites have been identified (302). The polar metabolites formed for CBD are more numerous than those of THC (300).

CHAPTER 5: METHODS FOR DETERMINATION OF CANNABINOIDS IN HUMAN URINE

5.1 ANALYTICAL METHODS FOR THE DETERMINATION OF CANNABINOIDS IN HUMAN URINE

As cannabis is the most widely consumed drug worldwide, the use of marijuana has increased significantly in recent years. The increasing use of marijuana both medically and recreationally leads to health risks for users from both cannabinoids and tobacco containing toxic substances. Biological recording of cannabinoid exposure from individuals who are active and passive users is a sensitive detection and a broad linear range. In recent years various methods for the analysis of THC, OH-THC, COOH-THC, CBD and CBN in urine samples have been applied for this purpose, including methods using liquid or gas chromatography (LC or GC) in combination with either single quadrupole mass spectrometry (MS) or tandem mass spectrometry (MS/MS). The reported limits of detection (LODs) for these analysers range from 0.2 to 5.0 nanograms per millilitre (ng/mL). Given the relatively high exposure levels resulting from active marijuana smoking, these LODs meet the needs respectively for the desired detection rates.

In addition to the above, synthetic cannabinoids, which pose a challenge in forensic and clinical laboratories, are identified or quantified by minimal methods in urine. Metabolites of synthetic cannabinoids detected in urine are relatively difficult to identify considering that there are few studies identifying which metabolites are formed in humans and that the reference standards of metabolites fall short of the availability of parent compounds. (303).

Several methods are commonly used for the determination of cannabinoids in human urine, each with its own advantages and limitations. Here are some of the most common methods:

- **Immunoassays:** Immunoassays, such as enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (LFIA), are rapid and relatively inexpensive methods for screening cannabinoids in urine. These tests rely on the specific binding of antibodies to cannabinoids or their metabolites. While immunoassays are useful for initial screening due to their speed and simplicity, they may lack specificity and can produce false positives or false negatives.

- **Gas Chromatography (GC):** Gas chromatography coupled with flame ionization detection (GC-FID) or mass spectrometry (GC-MS) has been traditionally used for cannabinoid analysis in urine. GC separates cannabinoids based on their volatility and polarity. While GC-MS offers high specificity and sensitivity, it requires derivatization of cannabinoids prior to analysis and may not be suitable for thermally labile compounds.

- **High-Performance Liquid Chromatography (HPLC):** HPLC coupled with ultraviolet (UV) or diode array detection (DAD) is another method for cannabinoid analysis in urine. HPLC separates cannabinoids based on their chemical properties and is suitable for non-volatile and thermally labile compounds. However, HPLC may lack sensitivity compared to LC-MS/MS and may require longer analysis times.

- **Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS):** LC-MS/MS is considered the gold standard for cannabinoid analysis in urine due to its high sensitivity, specificity, and multiplexing capability. LC separates cannabinoids chromatographically,

and MS/MS provides accurate identification and quantification based on the mass-to-charge ratio of ions. LC-MS/MS offers advantages in terms of sensitivity, specificity, and throughput compared to other methods but may require more extensive sample preparation and higher initial investment.

The choice of method for cannabinoid determination in human urine depends on factors such as sensitivity, specificity, throughput, cost, and the required level of accuracy and precision for the intended application. LC-MS/MS is commonly preferred for its superior performance in terms of sensitivity and specificity, especially in forensic and clinical settings where accurate quantification is essential.

5.2 USE OF ILLEGAL CANNABINOIDS

The use of cannabinoids illegally is a situation that society cannot ignore. In recent years, there has been a dramatic increase in the consumption of cannabinoids, which has innumerable consequences for society. It is therefore important to know all aspects of the phenomenon, such as the levels of use, where there is more systematic use or trafficking and new trends. In this way, the necessary measures against the use of cannabinoids will be taken. Data from surveys of different population groups, combined with statistics from reports of use of illicit substances such as cannabinoids, give the authorities an overview of the situation in each country. However, because these surveys take place in urban centres, since this problem is considered to be predominantly urban, combined with other factors such as the limited time frame in which they are conducted, the need has arisen to find new ways of approaching the issue that will provide more objective and unbiased data on the use and types of drugs in circulation.

5.3 EPIDEMIOLOGY OF WASTE WATER

As technology and science develops and evolves, through analytical chemistry, the above problem finds its solution. It is now possible to detect even compounds present in small quantities in a sample. A new idea proposed by Daughton for estimating the total consumption of illicit substances is based on measuring the concentration of these substances and their metabolites in urban wastewater collection centres (304). Many analytical techniques and methods have been used to detect and quantify a variety of illicit compounds, such as cannabinoids.

The reverse calculation method is based on the fact that the metabolic excretions of a human user of such substances contain a quantity of the substance or some of its metabolites. Therefore, the collection of excreta can give a very good picture of the proportion of the population using drugs and the quantities and types of substances consumed (304).

5.4 DETECTION OF DRUGS AND THEIR METABOLITES IN WET SEWAGE

By means of the inverse calculation method, the concentration levels of illicit substances such as cannabinoids (parent compound and metabolites) in liquid effluent can be calculated and thus the initial quantities of substances consumed (expressed in g/day or doses/day) can be calculated. Then, based on data on compound metabolism and excretion by the human body, it is possible to estimate the percentage of the population using drugs, depending on the population served by the wastewater treatment plant, with results expressed finally in g/day per 1000 inhabitants or doses/day per 1000 inhabitants. It is important and necessary to carry out stability studies of the substances under wastewater conditions. So far from the studies that have been carried out it is assumed that drugs and their metabolites are chemically stable from the point of elimination until they reach the wastewater treatment plants, during sampling and storage. However, stability experiments that have been performed show that not all compounds are stable (305,306). Therefore, extensive stability experiments should be performed in the future that take into account various factors that may affect the decomposition of compounds in aqueous effluents, sampling and storage. If the decomposition cannot be considered negligible, then the measured concentrations of the drug substances and their metabolites should be corrected before they can be used to estimate drug use. An important factor in converting drug concentrations into drug-using population percentages is to have a good understanding of the metabolic pathway of each substance in the human body.

5.4.1 INVERSE ANALYTICAL EQUATION FOR TETRAHYDROCANNABINOL (THC)

Estimates of THC consumption are based primarily on concentrations of the metabolite THC-COOH . Studies have found that for every mg of THC inhaled, 6 μg (0.6%) of THC-COOH is excreted through the urine. Knowing also that the THC/THC-COOH molar ratio is 0.91, it is possible to estimate the percentage of the population using a cannabis product (305).

5.5 METHODS OF SAMPLE PREPARATION PRIOR TO THE LC-MS/MS METHOD

Most drugs, such as cannabinoids, found in wastewater are in concentrations of the order of ng/L. This means that a preconcentration is required prior to analysis by LC-MS/MS. The method most commonly used is solid phase extraction (SPE). This method requires a high sample volume, i.e. 50-1000mL.

To obtain good recovery and low limits of detection and quantification with this method, it is important to remove material that may interfere with the detection of the target substances. The most common pretreatment method used is quite simple and results in a sample with a high percentage of organic matter that can interfere. However, a more complex pre-treatment which will result in purer samples takes a lot of time and there is likely to be a loss of target substances. It is necessary prior to sample treatment to filter the solution to

remove solid particles and then adjust the pH according to the requirements of each SPE method.

5.6 METHODS OF ANALYSIS OF SAMPLES

Liquid chromatography (LC) coupled with mass spectrometry (MS) is used to determine cannabinoids and their metabolites in liquid effluents.

The separation of analytes is usually performed either by high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UPLC). Based on the literature, separation by these methods is performed in reverse-phase columns using a moderately polar mobile phase, usually a mixture of water and organic solvent. The organic solvents commonly used for eluting analytes from chromatographic columns are methanol and acetonitrile. The aqueous mobile phase usually consists of water or ammonium formate or acetate (1-50 mM) and is acidified with formic or acetic acid (0,05-0,1 %) to improve the ionisation of the target substances in the positive potential ionisation method.

The ionisation of psychotropic substances and their metabolites is performed by electrospray ionisation (ESI). The majority of compounds ionized best at positive potentials, with the exception of cannabinoids which showed satisfactory ionization at both positive and negative potentials. The main disadvantage of using ESI is the sensitivity which it exhibits to the influence of the organic matter substrate present in the samples, resulting in the inhibition or enhancement of the ionization of some compounds. It has been observed that the ionisation rate of drugs and their metabolites in environmental aqueous solutions decreases significantly as the complexity of the organic matter substrate increases, such as in samples from raw sewage effluents. The substrate matter present in such samples has been estimated to be able to inhibit ionization at rates of 30-94%. To counter such phenomena, in addition to the application of selective extraction to obtain purer samples, internal standards, such as deuterated analogues of the compounds to be analysed, are also used.

Mass spectrometers that have been used in the determination and quantification of drugs in wastewater are of the tripole quadrupole (QqQ), ion trap (IT) and hybrid quadrupole and linear ion trap (QLIT) type.

5.7 CHROMATOGRAPHIC METHODS

Chromatographic methods are used for the analysis of complex samples. By means of this analysis, inorganic or organic substances in mixtures can be determined, since it is based on differences in the boiling point, complexity or electrical charge of the components. In each chromatographic separation, the sample is dissolved in a mobile phase (liquid or gas) and then passes into the static phase on the chromatographic column. The components that are more strongly retained in the static phase move slowly during the flow of the phase movement, while the components that are more weakly retained by the static phase move faster and are eluted first. Taking into account this difference in the mobility of the sample

components, it is expected that separation into separate zones will take place, on the basis of which qualitative or quantitative determination will follow.

5.7.1 ANALYTICAL TECHNIQUES

The extraction of the sample is followed by the determination of the substances (cannabinoids) using an analytical method. Liquid (LC) and gas chromatography (GC) are commonly used for cannabinoids identified in human urine.

In gas chromatography, a very high temperature is used on the sample when injecting it into the chromatograph to pass it into its gaseous form. However, heating also causes decarboxylation of acids which leads to incorrect quantification of the substances. However, to avoid this and to substitute the functional groups of substances, i.e. carboxyl groups and hydroxyl groups, it is necessary to produce the sample. The generation results in the formation of more stable and volatile compounds and increases the sensitivity of the method. Mass spectrometry (MS) or coupled mass spectrometry (MS/MS) is used for the detection of substances.

High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique used to separate, identify, and quantify components in a mixture. It is widely used in various fields such as pharmaceuticals, environmental monitoring, food safety, and clinical research. In HPLC there is a basic principle, where a mobile and a stationary phase must be available. The first one is A liquid solvent that carries the sample through the column while the second one is A solid or liquid phase that is fixed in place inside the column. The sample components interact differently with the stationary phase, leading to their separation. Liquid chromatography is very often used because it shows high sensitivity and reduced sample preparation time. In contrast to gas chromatography, in liquid chromatography there is no decomposition of the emerging substances and thus the sample can be analysed immediately after extraction, thus eliminating the production step. Compounds requiring production have high polarity, are non-volatile, thermally unstable and with production the detector signal is enhanced. At the end of the production process a product is obtained which has a better chromatographic behaviour than the two initial products (i.e. the analyte and the production reagent). In compounds with functional groups such as -COOH, -OH, -NH, -SH, the generation is crucial as they can easily form hydrogen bonds with each other and with the column components. Through alkylation, acylation or silanation, derivatives of these compounds are formed, resulting in a modification of the molecular structure and therefore easier analysis using GC. The modification of the structure causes changes in the molecular weight of the compounds and therefore the differences in their fragmentation in GC/MS.

The temperature of the injection point, the flow of the carrier gas and the column temperature program are the parameters that affect the analysis of substances in gas chromatography.

Liquid-liquid extraction is used to isolate substances from the biological substrate with an appropriate solvent system and pH adjustment. The substance undergoes partitioning between the organic and aqueous phases to achieve an accelerated equilibrium partitioning between the two phases. This is achieved by keeping the substances in their neutral form and preventing their ionisation by proper pH adjustment.

Solid phase extraction is also an important method of extracting analytes prior to chromatography. It exhibits high reproducibility, easy automation and reduced solvent content. The following are basic principles of solid phase extraction.

α. Activation of extraction columns.

β. Sample loading and holding the analyte in the adsorbent material.

c. Rinsing the columns and removing unwanted contaminants.

δ. Washing the analyte with the appropriate elution solvent.

5.8 METHODS OF EXTRACTIOB AND SAMPLE PREPARATION

The extraction method should offer high recovery, appropriate selectivity, be simple and reproducible. In order to carry out the method correctly every time, i.e. to increase sensitivity and reduce potential interferences from the sample matrix, sample preparation is very important. Samples derived from human urea almost always require a hydrolysis step and this is because most cannabinoid metabolites are bound in the glucuronide form.

5.8.1 EXTRACTION METHOD

The extraction method chosen each time plays an important role when analysing biological fluids. The extraction should be simple and reproducible, selective and with high recovery. Also, the parent material should not be reduced in its effect. For this purpose, i.e. to avoid erroneous quantification, the internal standard is used. When cannabinoids are analysed, secondary analogues of the substances analysed shall be used as internal standards.

The extraction methods used in the analysis of cannabinoids are mainly liquid-liquid extraction (LLE) and solin phase extraction (SPE). In both methods, the sample is eluted with an appropriate solvent. This solvent is then either diluted with another immiscible solvent (LLE) or the target compound is adsorbed onto solid phase solids (SPE).

The first method requires large amounts of extraction solvents and achieves less purification of biological material, which makes it difficult to quantify substances at lower levels. In contrast, the second extraction method shows high reproducibility and requires a smaller amount of solvents than LLE. In this method, the target compound is highly concentrated and therefore this method is ideal for liquid samples with very low concentration of the target compound, as is the case in body fluids. In urine samples, SPE can be applied directly to the sample without a prior extraction step.

Various solvents can be used to extract the components. These solvents can be polar, such as methanol and ethanol, or less polar, such as benzene and petroleum ether. Tethyst is widely used in cannabis analysis, since it dehydrates both neutral cannabinoids and their acidic forms (307,308). N-hexane and diethyl ether have been used to capture cannabinoids of cannabis using pyrolysis-GC (309), while methanol:chloroform (9:1) has been reported

for metabolic profiling (310), as this solvent system recovers almost all cannabinoids. When acid forms of cannabinoids are analysed, special care is needed with sample handling, because if elevated temperature is used it may lead to decarboxylation of the acidic compound and result in a neutral form.

Urine appears to be promising as a sample material, since not only is collection non-invasive, but metabolites can be detected up to 12 days or more after drug use. In both blood and urine, the target of analysis is usually 11-nor-9-carboxy-Δ9-THC, which, in urine, is the major compound present in both conjugated and unconjugated forms. Alkaline solutions such as 0,1 m sodium hydroxide and 0,1 m potassium hydroxide are usually used for hydrolysis of samples. Enzymatic cleavage can be used with urine samples to hydrolyse ether-bound glucuronides that cannot be cleaved by alkalis (311).

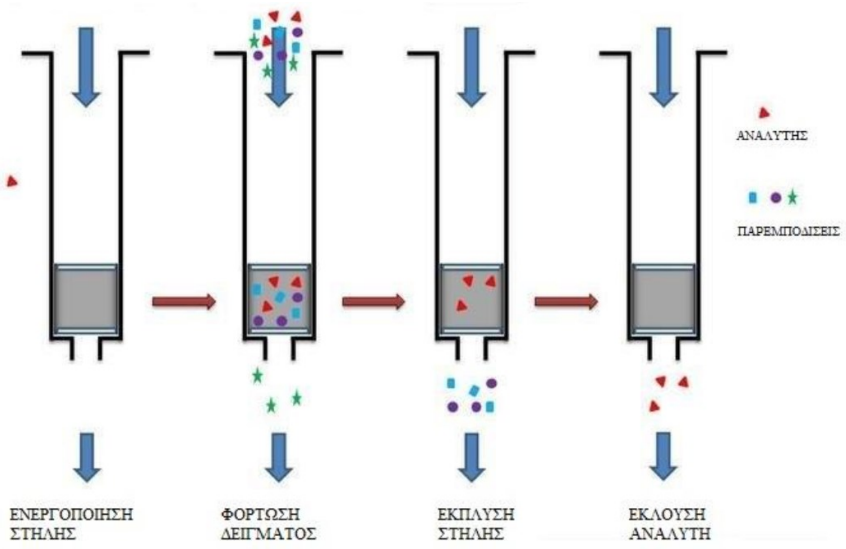

Figure 19: Schematic illustration of the solid phase extraction process.

5.9 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

In gas chromatography the components of the sample are separated according to their distribution between the mobile gas phase and the liquid or solid static phase. After evaporation, the sample is passed to the head of the chromatographic column and the elution is carried out by means of the carrier gas, which is inert and constitutes the mobile phase. By this method, the volatile components can be detected. The time taken for the component to elute in the chromatographic column is called the retention time and is measured at the peak maximum of the peak. Gas chromatography can be dynamically coupled with a mass spectrometer (GC-MS), which can be used to obtain a qualitative determination of the total composition of the sample, as well as a quantitative determination, i.e. the proportion of its components. It is characterised by very high sensitivity, selectivity and reliability of its results. The flow rate from the capillary columns is low enough for the ionisation chamber of the mass spectrometer to receive the effluent from the column outlet directly. The mass spectrophotometer detects a predetermined m/z value so the chromatogram shows the peaks

of only the substances consisting of the specific ions. At each new m/z value a new signal is recorded by the detector corresponding to a retention time. Finally, after the elution of all components has been completed, the chromatogram is extracted. However, the problem encountered when analysing with this method is that the sample components in the chamber or columns can undergo thermal degradation. One solution to this issue is to reduce the temperature used (312).

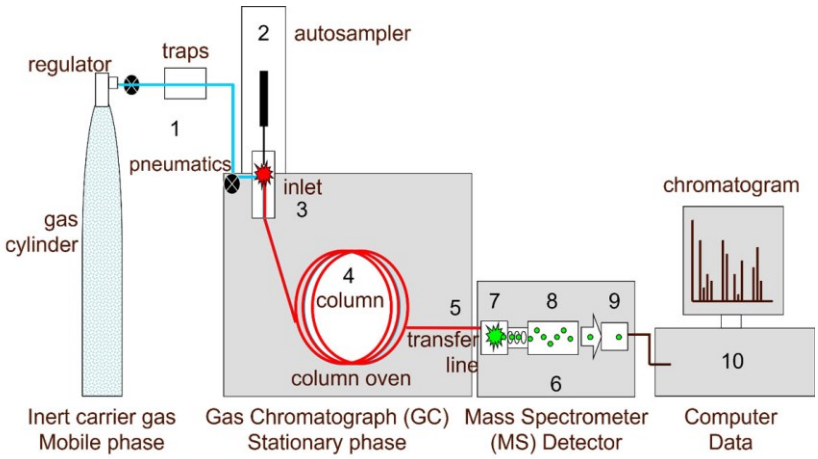

Figure 20: Standard GC-MS layout diagram.

5.10 FLAME IONISATION DETECTORS (FID)

The flame ionisation detector is the most common detector associated with GC. It has high sensitivity, large linear range, low noise and therefore the operator can easily perform the analyses. However, it is a destructive method because the sample is burned and constantly needs consumable gases and buffers. In non-combustible gases such as H2O, CO2, SO2 and noble gases, this detector does not respond (312).

5.11 DEVELOPMENT OF GC METHOD

To determine cannabinoids, the gas chromatograph is combined with various types of detectors, most notably a mass spectrometer (MS) (313,314), which operates mainly by electron impact (EI). In addition to this, other detectors such as a flame ionisation detector (FID) are also used (315).

Proper sample preparation is necessary for gas chromatography–mass spectrometry (GC–MS) analysis, as it has a significant impact on analyte isolation from complex matrices, including urine. Urine has similar distribution and higher detection windows in comparison with blood and oral fluid but requires a simpler pre-treatment. Micro extraction by packed sorbent (MEPS) is a miniaturization of solid-phase extraction (SPE) that is mostly applied for drug measurements. The main distinction between the two techniques is that in MEPS, the sorbent material is inserted directly into a syringe rather than being placed in a separate column as occurs in SPE. Additionally, the quantities of solvents and samples are significantly reduced in MEPS. This approach, in combination with techniques like GC–MS, is a powerful tool for screening and for the determination of several compounds in biological samples (316).

For the determination of cannabinoids by gas chromatography, a step of derivatisation is required to achieve substitution of the side functional groups of the analytes, in particular the hydroxyl group and the carboxyl group. This is required in cannabinoids due to the fact that the chromatographic behaviour of the analytes having functional groups is optimised and the volatility of these substances is increased and thus the method acquires increased sensitivity. In addition, the production stage is important because at the point of injection of the sample into the gas chromatograph, high temperatures are developed to convert the sample into a gas phase and thus decarboxylation of the acids present such as THCA, CBGA to the corresponding cannabinoids i.e. D9-THC, CBD etc. takes place. This has resulted in several studies in which an error in the quantification of analytes is made since the total percentage of substances consisting of the decarboxylated substance and the corresponding acid is measured (313). So in order to increase the stability of analytes and more so of acids during determination by gas chromatography, derivatization is essential. To carry out this step, various generation reagents such as N,O-bis (trimethylsilyl)-trifluoroacetamide (N,O-bis (trimethylsilyl)-trifluoroacetamide, BSTFA) (317), N-methyl-N-(trimethylsilyl)-trifluoroacetamide, MSTFA (318) and others have been used. This technique leads to a high sensitivity and selectivity, as well as detection of more compounds than liquid chromatography. In contrast to gas chromatography, in liquid chromatography this step is omitted since no high temperatures are developed which would destroy the acids, so they are identified immediately.

GC analysis is suitable for plant cannabinoids. However, it is limited to the determination of the quality of cannabis for smoking, if used directly, since it can only provide information on decarboxylated cannabinoids, such as Δ9-THC. GC uses high temperatures to decarboxylate compounds, and in this way the true metabolic record of the plant is not reflected in the chromatogram. In order to determine the acids that may be present in the extract, derivatisation should be performed, in which cannabinoid acids and their neutral forms are separated. In fact, the levels of the neutral forms determined in this way can be used to assess the quality of the sample, since in fresh material the neutral form is barely present, whereas poor storage at high temperatures causes decomposition of the acids. For the determination of cannabinoids in human urine samples, this method offers high resolution and is faster than HPLC. The concentration of Δ9-tetrahydro- cannabivarin in the cannabis plant is low, but the corresponding acid metabolite can be easily detected in the human urine of cannabis users by GC-MS analysis (319). An alternative method is GC with a temperature-programmed vaporiser (PTV) to improve the sensitivity of the analysis of cannabinoid metabolites when there is a sample size limitation. Through the PTV the analytes are separated from the sample as the sample is introduced and the solvent is eliminated through the separation outlet. The analytes with a higher boiling point are retained and then transferred to column years by heating.

5.12 DEVELOPMENT OF HPLC METHOD

Unlike GC, in HPLC no decomposition of cannabinoids occurs during analysis. This implies that the acidic forms of cannabinoids are analysed directly for phenotype determination (320,321). The high sensitivity of HPLC can be achieved more efficiently by using thermal spray-MS, but results may show high variability due to spray instability (322). The liquid

chromatograph is coupled to various detectors such as mass spectrometer (MS) (323), coupled mass spectrometry (MS/MS) (324), ultraviolet UV detector and others.

Key parameters for effective separation are the choice of column, mobile phase and flow rate. It is a method that offers easy quantification, good sensitivity and the ability to separate non-volatile compounds and compounds belonging to the same homologous series.

Quantification, the process of determining an unknown concentration in a known solution, involves a series of steps starting with the injection of known concentrations of the fixed solution. The analysis gives a series of peaks associated with the concentration of the compound injected, and the resulting diagram is called a chromatogram. The time it takes for a compound to elute is characteristic and unique to the compound, the height of the peak formed can be correlated and can determine the concentration of each compound in solution (69).

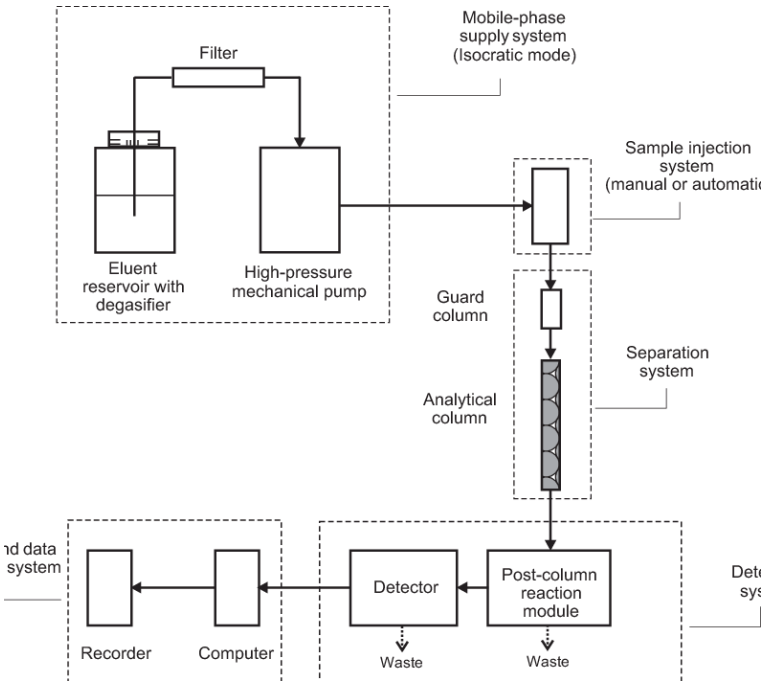

Figure 21: Standard HPLC instrument diagram.

5.13 DETECTORS

In order to make a correct measurement and to obtain a correct qualitative and quantitative characterisation, a correct choice of detector to be connected to the HPLC and operating under its optimum conditions must be made. This way the chromatogram obtained will be correct, with clean peaks.LC detectors are usually simple analytical instruments adapted to flow cells to measure low concentrations. There are two types of detectors appropriate for an LC system, those that respond to a basic property: such as refractive index, dielectric constant, the values of which are affected by the presence of the eluted components, and detectors that respond to the properties of the eluted components: such as UV absorption or fluorescence, a property that has no mobile phase.

5.14 LIQUID CHROMATOGRAPHY - MASS SPECTROMETER DETECTOR (LC/MS)

To achieve satisfactory separation and good detection sensitivity, LC is effectively coupled to mass spectrometers. The LV/MS system is able to distinguish substances whose chromatographic peaks coincide, i.e. it provides high selectivity. Also, it can give a footprint of a certain eluent instead of relying on elution time as with conventional HPLC, and finally the combination can even provide information on molecular weight, structure and accurate quantitative analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful analytical technique commonly used for the determination of cannabinoids in urine samples. LC-MS/MS offers high sensitivity, specificity, and selectivity, making it an ideal technique for the analysis of cannabinoids in urine samples, even at trace levels. It's widely used in forensic toxicology, clinical research, and drug testing applications. This method is used for the qualitative determination of cannabinoids and some of their metabolites and the most important advantage of the method is the fact that the sample preparation is not complex, but is a time saving. Non-volatile substances and thermosoluble compounds do not require various derivatisation techniques as in other methods. This method offers high sensitivity and detection of cannabinoids and their metabolites in human urine. An overview of the process is summarizes in the **Table 3**, below:

STEP 1	STEP 2	STEP 3	STEP 4	STEP 5
SAMPLE PREPARATION	**LIQUID CHROMATOGRAPHY (LC)**	**MASS SPECTROMETRY (MS)**	**DETECTION AND QUALIFICATION**	**DATA ANALYSIS**
Urine samples are typically pretreated to remove any interfering substances and to concentrate the cannabinoids present. This might involve processes such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE).	The prepared sample is injected into a liquid chromatography system. In LC, the sample is passed through a chromatographic column where the cannabinoids and other compounds are separated based on their chemical properties. The mobile phase (solvent) carries the sample through the column.	After separation by the LC system, the individual cannabinoids are introduced into the mass spectrometer. Here, they are ionized, fragmented, and sorted based on their mass-to-charge ratio (m/z). Tandem mass spectrometry (MS/MS) involves using two mass analyzers in series, where the first analyzer selects a specific ion (parent ion), fragments it, and then the second analyzer detects the resulting fragments (daughter ions). This process provides higher selectivity and sensitivity.	The ions generated in the mass spectrometer are detected and analyzed. By measuring the abundance of specific ions corresponding to the target cannabinoids and their fragments, the concentration of each cannabinoid in the urine sample can be determined. Calibration curves generated from standards of known concentrations are typically used to quantify the cannabinoids present.	The data collected from the LC-MS/MS analysis is processed using specialized software. This involves identifying peaks corresponding to the target cannabinoids and integrating the peak areas to determine their concentrations in the urine sample.

Table 3: Steps for LC-MS/MS.

Comparing LC-MS/MS with other methods for cannabis determination in human urine, several factors come into play:

- **Sensitivity and Specificity:** LC-MS/MS typically offers higher sensitivity and specificity compared to other techniques such as immunoassays. This means LC-MS/MS can detect cannabinoids at lower concentrations and provide more accurate identification and quantification of specific compounds, reducing the likelihood of false positives or false negatives.

- **Multiplexing Capability:** LC-MS/MS allows for the simultaneous analysis of multiple cannabinoids and their metabolites in a single run, offering higher throughput compared

to techniques like gas chromatography (GC). GC typically requires separate runs for each compound, increasing analysis time and resource requirements.

- **Quantitative Accuracy:** LC-MS/MS provides accurate quantitative results through the use of calibration curves and standards of known concentrations. Other methods, such as immunoassays, may offer rapid screening capabilities but lack the precision and accuracy required for quantitative analysis.

- **Sample Matrix Effects:** LC-MS/MS is less prone to interference from complex sample matrices, such as urine, compared to techniques like immunoassays. Immunoassays can be influenced by cross-reactivity with other compounds present in urine, potentially leading to false results.

- **Sample Preparation Requirements:** LC-MS/MS methods for cannabis determination in urine may require more extensive sample preparation compared to immunoassays. However, this sample preparation step helps to reduce matrix effects and improve the reliability of results.

In summary, while LC-MS/MS may require more extensive sample preparation and higher initial investment compared to some other methods, its superior sensitivity, specificity, multiplexing capability, and quantitative accuracy make it the preferred choice for many applications requiring cannabis determination in human urine. Other methods may offer advantages in terms of speed or simplicity but may sacrifice accuracy and reliability. The choice of method depends on the specific requirements of the analysis and the resources available.

5.15 DERIVATIZATION

Derivatization helps to improve analysis, reduces the tail of polar compounds, helps to improve analytical performance, and increases the detectability and stability of compounds (325). When cannabis extracts are introduced into G, the extraction port is decarboxylated and thus only decarboxylated cannabinoids can be directly measured by GC. Besides, in the human body cannabinoids are metabolized to polar compounds with a high boiling point or high breakdown point. This makes GC unsuitable for direct analysis. The introduced functional groups can be derivatised to convert cannabinoids or their metabolites into more stable and volatile compounds. Derivatization can be used to enhance the detectability of a compound by introducing a group with high electron affinity causing an increase in ionization yield and enabling a sensitive analysis using an electron capture detector.

Generativity can also be used to improve the detection of compounds when other analytical techniques are used. Cannabinoid derivatisation to produce fluorescent derivatives can be advantageously used prior to analysis by HPLC or TLC (Thin layer chromatographic analysis). Bypassing the derivatisation step means a shorter pre-treatment process.

5.16 THIN LAYER CHROMATOGRAPHIC ANALYSIS (TLC)

It is a liquid chromatography technique for the detection of complex mixtures. The procedure followed is similar to that of column chromatography. It has a high resolution and is flexible, since several static phases can be used, it is very fast and quite sensitive, as it requires small amounts of sample to perform the analysis.Corneal ammonium thiocyanate has been suggested for detection of cannabinoids. This technique has high sensitivity and selectivity and is used for the detection of metabolites of cannabinoids. However, it does not compete with other methods for quantitative analysis, since it requires an additional step for quantification.

5.17 DEVELOPMENT OF THE ULTRA-HIGH PRESSURE LC (UHPLC) - ELECTROSPRAY IONISATION (ESI) METHOD IN COMBINATION WITH MS/MS.

One of the most important methods for the analysis of human urine for the detection of cannabinoids is the ultra-high pressure electrospray ionisation method in combination with MS/MS. This method is widely used in all scientific fields as well as in forensic science. Methods such as this, fast, rapid and ideal for the analysis of complex samples, with high sensitivity and selectivity, are necessary because the concentrations of various compounds in biological samples are much lower than in the past. However, the preparation of samples is an obstacle, especially when this requires manual extraction techniques.

Cannabis is one of the most widespread illicit drugs worldwide and its use is detected by determining the presence of the main psychoactive component of marijuana, THC, or its metabolites in biological fluids. The most important metabolite is THC-COOH either in free or in the guanidine form (326,327)

One of the challenges in measuring cannabinoids in urine and their metabolites is that the high lipophilicity of these compounds can lead to significant adsorption on the materials used for sample preparation, i.e. the tips and well plates.

The thermal instability of these analytes in urine is another issue that requires special attention in all sample collection, transport, storage and analysis procedures. Stability tests conducted in a study with enriched urine at room temperature (~25 °C) showed that >80% of total CBD, CBN and THC was lost after 24 hours.

At 4 °C, approximately 10-30% of total CBD, THC and CBN decomposed after 3 days, while no obvious losses of total OHTHC and COOH-THC were observed.

Available data indicate that urine samples should always be kept frozen during storage and shipment. For analysis, samples should be prepared well in advance and analysed within 1 day. The high-throughput automation system for liquid processing and rapid UHPLC-MS/MS analysis presented in a study would better meet these needs to maintain the quality of analytical results.

One of the main advantages of this method, in addition to the wide applicable quantitative ranges, high throughput preparation and rapid analysis, is the considerable sensitivity

necessary for effective monitoring and assessment of SHMS exposure. In a study conducted, LODs and limits of quantification (LOQs) were determined by preparing and analysing four low-concentration urine pools (0.005, 0.010, 0.025 and 0.050 ng/mL) over a 3-month period. This was most likely the result of the tandem hydrolysis (enzyme-alkaline hydrolysis) process. When incubating the hydrolysis at higher temperature, the analytes can be partially degraded due to their thermal instability; meanwhile, they can also be partially bound to the added enzyme (E. coli.) due to high lipophilicity and then precipitate by centrifugation. Despite the loss of mass during sample preparation, the detection sensitivity achieved by this method for urine samples was 10-100 times higher than the values (0.2-5.0 ng/ml) reported in the literature. (328)

CHAPTER 6: CONCLUSION

In this master thesis, we have thoroughly examined the mechanisms of action of cannabinoids within the human body and explored various methods for their determination in human urine. Cannabinoids, particularly tetrahydrocannabinol (THC) and cannabidiol (CBD), exert their effects primarily through interaction with the endocannabinoid system (ECS). This complex system, consisting of endogenous cannabinoids, receptors (CB1 and CB2), and metabolic enzymes, plays a critical role in maintaining homeostasis. The primary psychoactive component of cannabis, delta-9-tetrahydrocannabinol (THC), primarily binds to CB1 receptors, which are abundant in the brain and central nervous system. This interaction leads to the modulation of neurotransmitter release, affecting various physiological processes, including pain perception, mood regulation, appetite, and immune response. The therapeutic potential of cannabinoids is substantial, yet their psychoactive properties, particularly those of THC, pose challenges for medical use and necessitate robust detection methods for regulatory and clinical purposes.

Cannabinoids are a class of substances that are numerous and complex. The properties of cannabinoids (analytical, anti-inflammatory, curative) have proven to be excellent and promising for the treatment of serious diseases. However, over time cannabinoids have been used for purposes beyond the spectrum of medicine. They are quick, cheap, attractive and extremely easy to get hold of, namely as a means of recreational use, leading individuals to become addicted.

The pharmacokinetics of cannabinoids involves their absorption, distribution, metabolism, and excretion. Upon consumption, cannabinoids are rapidly absorbed into the bloodstream, with THC typically peaking within minutes if inhaled, and within hours if ingested orally. Once in the bloodstream, cannabinoids are distributed to various tissues, including the brain, where they exert their effects. Metabolism occurs primarily in the liver through the cytochrome P450 enzyme system, producing a variety of metabolites, some of which remain biologically active. The excretion of these compounds occurs mainly through feces and urine. Understanding the pharmacokinetics and pharmacodynamics of cannabinoids is essential for optimizing their therapeutic use and managing potential side effects, particularly in medical contexts where precise dosing is critical.

Cannabinoids regulate the CNS through their receptors, namely CB1 and CB2, while the best known endocannabinoids are Anandamide and 2-AG. Cannabinoids such as CBD were extracted from cannabis. This one has no psychoactive effects. The molecule associated with psychotropic effects in humans is THC, whose effects are blocked by CBD. Certain activities cause anandamide production and this then activates cannabinoid receptors. Anandamide is broken down by various enzymes such as FAAH.

When used for medical purposes, cannabinoids through specific pathways help reduce pain from cancer, treat nausea and vomiting and also help fight cancer. The action of cannabinoids is coordinated by enzymes and receptors that play an important role in each pathway of synthesis, absorption and elimination by the human body. It is important to note that as they enter the body they can be found in many biological fluids such as saliva, blood and tail, and even hair.

Regarding the legislative framework in Greece for medicinal cannabis, it has gone from a prohibited substance to a medicinal substance. In fact, in 2013 Greece began allowing the

therapeutic use of cannabis, with the law being extended until 2018. The changes in legislation combined with the trend towards alternative treatment of diseases through the use of cannabis is slowly leading to the rapid growth of the medicinal cannabis sector. However, the use of cannabis does not offer the desired results.

In cancer the role of cannabinoids is not fully clear and this is because upregulated expression of CB receptors and high levels of endocannabinoids are observed in many cancer cells. Cannabis has been used to treat epilepsy for years. Preclinical studies support the role of cannabinoids in epilepsy, but clinical studies are limited. Both CBD and THC have anticonvulsant properties, working through different mechanisms. The endocannabinoid system plays a role in controlling seizures, with CB1 receptors regulating hippocampal activity. Epilepsy patients have found CBD to have minimal side effects, with reported antipsychotic and anxiolytic effects. Detailed drug metabolism studies are essential to prevent adverse reactions and allergies. Research on CBD and other cannabinoids for epilepsy treatment is crucial, as well as exploring additional derivatives' potential roles.

The analysis of cannabinoids in human urine is essential for various applications, including clinical diagnostics, forensic investigations, and compliance with drug policies. Urine testing is advantageous due to its non-invasive nature and the relatively long detection window for cannabinoids. This thesis has evaluated several analytical techniques, focusing on immunoassays, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Immunoassays offer rapid and cost-effective screening but lack specificity and may result in false positives. GC-MS and LC-MS/MS, on the other hand, provide high sensitivity, specificity, and the ability to quantify multiple cannabinoids and their metabolites simultaneously. Among these, LC-MS/MS is emerging as the preferred method due to its superior performance in complex biological matrices and lower limits of detection.

A very common sample for analysis to determine whether cannabinoids have been used is human urine. In order to make a qualitative determination of cannabinoids in a urine sample many analytical methods are used, such as HPLC, LC-MS/MS and GC. In many cases these samples in order to be analysed have to undergo an additional treatment, such as extraction of the sample or even derivatisation.

As the therapeutic use of cannabinoids continues to grow, so does the need for advanced analytical techniques and a deeper understanding of their mechanisms of action. In conclusion, understanding the intricate mechanisms of cannabinoid action and developing reliable methods for their detection in human urine are crucial for advancing both therapeutic applications and regulatory frameworks. The continued evolution of analytical techniques, particularly the refinement of mass spectrometric methods, promises to enhance our ability to monitor cannabinoid exposure accurately and to elucidate their pharmacokinetics and pharmacodynamics in diverse populations. Future research should focus on standardizing detection protocols, expanding the scope of cannabinoid metabolites studied, and exploring the clinical implications of long-term cannabinoid use. By integrating insights from pharmacology and analytical chemistry, we can better harness the benefits of cannabinoids while mitigating potential risks.

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