Development and application of a LC-MS/MS method for the analysis of plasma bioavailabilities of different cannabinoids after the administration of *Cannabis sativa L.* extracts and Marinol™

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Basel, Schweiz

Basel, 2008

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Prof. Dr. Hans-Peter Hauri

Dekan

To my parents

Evi and Werner Grauwiler

“Curiosity always comes first when a problem needs to be solved.”

Galileo Galilei

Acknowledgements

I would like to thank André Scholer, PhD, for giving me the opportunity to prepare my thesis in the clinical chemical laboratory at the University Hospital Basel. His expert knowledge has been a great support for my work. I was always encouraged to invest maximum effort due to his excellent ability to motivate resulting in important outcomes for further investigations. Furthermore, I would like to thank him for the many opportunities to participate in international congresses.

A special thank goes to Professor Jürgen Drewe for supervising this work and his support during the whole thesis. I am grateful for his great ideas, his constructive and exciting discussions and for the chance to realise them into practice.

I would like to thank my colleagues for creating a helpful and friendly atmosphere. I am thankful to Urs Gyr, Yvonne Bottazzini, Madeleine Lützelschwab, Axel Regeniter, MD, Stefan Sturm, PhD and the whole "Tox" team for their help during my whole thesis.

I am also beholden to the laboratory 411 of the University Hospital Basel, Switzerland. The working atmosphere in the laboratory meetings was always very convenient and constructive.

My gratitude goes to Luisa Baselgia, Michael Bodmer, MD, Martin Eichler, PhD and Luca Spinedi, PhD, for their support and the good time we spent together during the work with the test persons.

A great thank goes to all the volunteers who participated in the clinical study.

Furthermore, I would like to thank Markus Lüdi, PhD and the company Cannapharm AG, Burgdorf, Switzerland for the financial support of the clinical study.

I would like to thank Professor J. Huwyler for overtaking the co-Referee and Professor P. Hauser for being a further expert of the thesis committee.

My deepest thanks go to my family and my closest friends for their love, encouragement and support during my PhD.

List of Abbreviations

APCI	Atmospheric Pressure Chemical Ionisation
CBD	Cannabidiol
CID	Collision Induced Dissociation
CYP	Cytochrome P450 System
DAD	Diode Array Detection
FDA	Food and Drug Administration
IS	Internal Standard
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LLOQ/LLOD	Lower limit of Quantification/Detection
11-OH-THC	11-hydroxy-Δ9-tetrahydrocannabinol
P-gp	P-glycoprotein
MS-MS	Tandem Mass Spectrometry
R.S.D.	Relative Standard Deviation
RT	Retention Time
SPE	Solid-Phase Extraction
THC	Δ9-tetrahydrocannabinol
THCA-A	Δ9-tetrahydrocannabinolic acid A
THC-COOH	11-nor-9-carboxy-Δ9-tetrahydrocannabinol
TPGS	D-α-tocopheryl polyethylene glycol 1000 succinate

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1. Summary and Aims of the thesis

A lively interest in the cannabis plant can be verified for a long time. As a drug in the traditional medicine, different pieces of the cannabis plant were used against a palette of diseases such as pain (head- and stomach-ache), menstrual problems and diarrhoea. Further, it was used as a sedative and to induce sleep [1].

At the beginning of the 20th century, a scientific interest for cannabis has emerged. Research was done to detect the pharmacokinetic and pharmacodynamic effects of cannabis. The discovery of the endogenous cannabinoid system opened a broad field for research. This system gradually helped to better understand the molecular mechanisms of the cannabis effects. Links to other modulating or regulatory systems in our body are now possible [2].

The special applications of cannabis in traditional medicine, have to be clinically investigated with the scientific knowledge of today. Some applications are already established. The United States Food and Drug Administration (FDA) approved Marinol^{TM} (a soft gelatine capsule containing THC dissolved in sesame oil) to treat nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional therapies. The antiemetic effects are comparable to conventional therapy such as domperidone [3]. Furthermore, the FDA approved Marinol^{TM} to treat appetite loss associated with weight reduction in people with acquired immunodeficiency syndrome (AIDS). It was shown in recent studies that THC or cannabis preparations have a promising potential as a releasing factor, in moving disorders and as a pain reducer in patients suffering from multiple sclerosis. Therefore further research is required.

Worldwide, the use of natural cannabis products for medical purposes is practically not allowed. In contrast, drugs containing synthetic cannabinoids like dronabinol, a synthetic THC are often exempt from these restrictions. Synthetic products, however, have a disadvantage: they do not contain a well-balanced combination of active substances which can be found in the natural cannabis plant. Patients having

consumed natural cannabinoids for their medical therapy report more adverse effects after the administration of synthetic cannabis preparations [4, 5]. The principle of phytotherapy is the treatment with a mixture of bioactive compounds. The idea is that a complex pathophysiological process can be influenced more effectively and with fewer adverse effects by a combination of several low-dosage extract compounds than by a single isolated compound [6]. Therefore, it is important to develop and clinically investigate oral cannabis extract formulations to prove pharmacodynamic and pharmacokinetic properties and to compare them with the existing synthetic THC products and against placebo is important.

The aim in project one of the present work (publication 3, chapter 3.3) was to approve the performance of an open, randomised, single-center, three-periods cross-over study with different, standardised *Cannabis sativa L*. extract capsule formulations and Marinol^{TM}, to analyse the pharmacokinetics and pharmacodynamics of the cannabinoids and to evaluate the best *Cannabis sativa L*. extract capsule formulation in this clinical phase I study for a possible future implementation as a new, concomitant medication in cancer, HIV and AIDS therapies.

In the first study part, the heating-effect on the relative content of cannabinoids in the *Cannabis sativa L*. extract capsule formulation was assessed. Data were compared to the commercial formulation Marinol^{TM}. The reason for this is that in naturally grown *Cannabis sativa L*., up to 95% of the occurring total cannabinoids (THC_{tot}) are in the form of ∆9-tetrahydrocannabinolic acid A (THCA-A). By heating, THCA-A is quantitatively decarboxylised to phenolic THC [7]. Although THCA-A is described as pharmacologically inactive and devoid of psychotropic effects [7], reports of popular medicinal use of unheated cannabis or cannabis preparations show pharmacological effects often accompanied with a lower rate of adverse effects (anecdotal reports). It also possesses some anti-inflammatory and analgesic effects [8]. Recently, it was shown that unheated cannabis extracts were able to inhibit tumor necrosis factor alpha in macrophage culture and peripheral marcrophages after LPS stimulation [9]. In the second study part, the effect of different *Cannabis sativa L*. extract capsule formulations, containing different concentrations of TPGS, on the bioavailabilities of different active cannabinoids was assessed. The reason for this is that the enteral

absorption of cannabinoids under optimal conditions would be up to 95%, but due to the extensive liver first-pass metabolism and the poor solubility the effective, measured bioavailability is very low (10-20%) [10]. Further the activity of P-glycoprotein (P-gp), a membrane efflux transporter also expressed in the intestine, may reduce the oral bioavailability of cannabinoids. Therefore, it is important to increase the bioavailabilities of oral drugs with substances possessing absorption enhancement, drug solubilising and inhibiting effects on P-gp. Previous work has shown that D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) has an accelerating effect on gastrointestinal transit and a modulating influence on drug absorption in humans [11]. A P-gp inhibition could be demonstrated [12-14].

Further study endpoints were a) to assess the relative bioavailabilities of THC and its metabolites assessed as area under the plasma concentration/time curve from time T = 0 h extrapolated to infinity (AUC(0-∞)), b) to assess the relative tolerability and safety of six different oral formulations of 20 mg THC_{tot} (THC and THCA-A), c) to assess the effect of six different oral formulations of 20 mg THC_{tot} on psychomotor function assessed as simulator assisted evaluation of driving ability, d) to assess repetitive heart rate, blood pressure and a visual analogue scale (VAS) for psychotropic effects.

The pharmacokinetics of the cannabinoids was highly variable between the subjects. Due to this variability, no statistically significant differences between the AUC of the different forms could be detected, neither in part I nor in part II of the study. Addition of different amounts of TPGS resulted in an increase in relative bioavailability of the sum of cannabinoid metabolites (THC + 11-OH-THC + THC-COOH + CBN) to 122.5% (7.5% TPGS), 134.9% (0.5% TPGS) and 135.9% (5% TPGS) compared with the AUC of the unheated extract (=100%) in study part I. The administration of cannabis extracts as well as the addition of TPGS leads to a qualitatively different pattern of cannabinoid metabolites. After administration of the unheated extract, a significantly higher proportion of THC AUC and a significantly lower THC-COOH AUC of all cannabinoids were observed compared to the heated extract or Marinol™. After administration of the synthetic Marinol™, no plasma concentrations of CBD could be detected. This was expected, since THC is not converted to CBD *in vivo* and is found

only in cannabis plants. Heating of extracts decreased the proportion of CBD significantly. The future approach will address further research. Further, clinical studies with the 0.5% or 5% TPGS *Cannabis sativa L*. extract capsule formulations may be helpful. The study should be placebo controlled and later tested in the future patient group.

In the present work, only the pharmacokinetics of the study are described, evaluated and discussed. The pharmacodynamic results are reported in two separate publications.

The aim in project two (publication 1, chapter 3.1) was the development of a sensitive high-performance liquid chromatographic separation method with tandem-mass spectrometry detection for the simultaneous detection of THC and its major metabolites 11-OH-THC and THC-COOH as well as the components CBD and CBN in human EDTA-plasma and urine. Optimal conditions for the analysis method, such as extraction procedure, matrices, column, quality controls, wavelength, mobile phases, run time, optimal separation (gradient, retention times), temperature, voltages, vacuum and internal standards, resulting in the best sensitivity and selectivity, were developed in preliminary experiments. The validation of the method was performed according to the FDA Good Laboratory Practice guidelines, containing linear measuring range, quantification, lower limit of quantification (LLOQ), lower limit of detection (LLOD), quality controls, precision, accuracy, recovery, stability and matrix effects. In conclusion, the described high-performance liquid chromatographic separation method with tandem-mass spectrometry detection showed a satisfactory overall analytical performance well suited for applications in medical science. The combination of SPE/LLE, LC and APCI-MS/MS represents an attractive alternative to the well-established technique of GC-MS.

In project three (publication 2, chapter 3.2), the sensitivity and specificity of two immunoassays (CEDIA, FPIA) were established in urinary samples from volunteers receiving oral synthetic THC or *Cannabis sativa L*. extracts. Urinary THC-COOH excretion was evaluated by the immunoassays with a cut-off value of 50 ng/ml as

well as the described LC-MS/MS method (gold standard) with a cut-off value of 15 ng/ml. It was demonstrated that LC-MS/MS is an excellent confirmation method for immunoassays allowing the qualitative and quantitative detection of many cannabinoids. The ROC analysis indicated that the FPIA test discriminates better between users and non-users than the CEDIA test. The results of both immunoassays show that the National Institute on Drug Abuse (NIDA) set general immunoassay cut-off of 50 ng/ml is possibly not applicable for analysis of samples from persons consuming the *Cannabis sativa L*. extracts orally instead of smoking. It has to be discussed, whether a lower cut-off value would be advantageous. It is supposed that metabolite concentrations differ strongly depending on the route of application. The amount and appearance of different metabolites may disturb the immunoassay methods. The hydrolysation procedure showed a total transformation of the THC-COOH-glucuronides to THC-COOH confirmed by the nearly 100% agreement of the concentrations in the different samples analysed with the two immunoassays and the LC-MS/MS comparisons. The glucuronide is automatically detected together with THC-COOH and it is direct de-glucuronated in the APCI unit of the detector.

The present work is structured into a theoretical and a publication section. The theoretical section gives an overview about cannabis, mass spectrometry, assay validation and GLP-guidelines related to the aspects used in the work of this thesis. The publication section describes the results of the investigations, submitted for publication to different scientific journals.

2. Introduction

2.1. Biological Part

2.1.1. Cannabis sativa L.

2.1.1.1. Botanical characteristics [15-17]

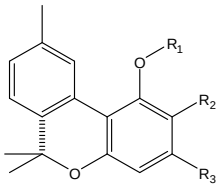
Cannabis sativa L. belongs to the family of cannabaceae and grows in all warm and temperate zones, except in the tropical rain forest. It is an annual plant, which can grow up to 5m. When planted closely together, the plants are variously branched or even unbranched. The cannabis plant is covered with tiny hairs. The leaves and branches are paired at the bottom and arranged alternating higher on the stem. The leaves have a delicate stem which is about 6 cm long. They are pinnate in a dactyloid form. The 5 to 9 leaflets are mostly narrowly lanceolate. They are coarsely sawed and toothed and have a long protracted pointed tip. Cannabis is a dioecious plant which means that the flowers are either female or male. Female plants are very leafy up to the top while male plants have fewer leaves on the inflorescence which are much further apart. The glands of the female flowers, secret drops of resin, which are produced under hot conditions. Male plants die after the pollination. In the absence of male plants, female ones produce more flowers covered with THC producing glands. A function of the resin glands is the protection of the plant from animals. The greyish or brownish fruit is physically an achene. It is ellipsoid, smooth, slightly compressed, about 2.5 - 5 cm long and the diameter is 2 - 3.5 cm.

2.1.1.2. Chemical constituents of Cannabis [15, 18]

More than 480 compounds were detected in *Cannabis sativa L*. of which more than 65 are cannabinoids (“phytocannabinoids”). Cannabinoids are specific and characteristic components of the Cannabis plant. They are nitrogen free, quite lipophilic, phenolic and have a C_{21}-skeleton. Cannabinoids are derived from a monoterpene and a phenol or a phenolic acid. According to the difference in the

terpene part of the molecule, cannabinoids can be classified in five types as well as some minor cannabinoid classes: the cannabigerol, the cannabichromene, the cannabidiol, the tetrahydrocannabinol and the cannabinol type. The cannabinol type is an artefact formed by oxidation of biogenic cannabinoids. This type is produced during storage or extraction. Structures of the cannabinoid types are presented in *Figure 1*.

Cannabigerol type

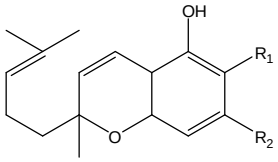


R_1 = H or COOH

R_2 = C_3- or C_5-sidechain

R_3 = H or CH_3

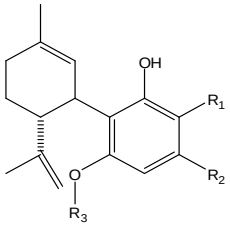
Cannabichromene type



R_1 = H or COOH

R_2 = C_3- or C_5-sidechain

Cannabidiol type

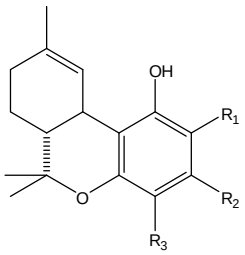


R_1 = H or COOH

R_2 = C_1-, C_3-, C_4- or C_5-sidechain

R_3 = H or CH_3

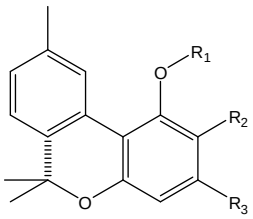
Tetrahydrocannabinol type



R_1, R_3= H or COOH

R_2 = C_1-, C_3-, C_4- or C_5-sidechain

Cannabinol type



R_1 = H or CH_3

R_2 = H or COOH

R_3 = C_1-, C_3-, C_4- or C_5-sidechain

Figure 1: Structures of the different cannabinoid types

The characteristic smell of the hemp plant is caused by an essential oil produced by the circular glands. This oil contains phenylpropane derivatives (e.g. eugenol, cis- and trans-anethol) and mono- and sesquiterpenes (e.g. humulene, α- and β-pinene,

limonene, β-caryophyllene, caryophyllene oxide). Furthermore, it contains small amounts of other phenolic compounds (e.g. dihydrophenanthrene derivatives, spiroindane, dihydrostilbene), flavonoids, amino acids, sugars and nitrogen-containing compounds (amines, amides).

2.1.1.3. Cannabis products [15, 16, 19, 20]

a. Cannabis herb

The herb (“Marihuana”, “Marijuana”) consists of the dried leaves and blooms of the cannabis plant. The THC content is 1 - 25 %.

b. Cannabis resin

The brownish resin (“Hashish”) is produced by the circular glands of the female inflorescence. For recreational use, Hashish is often pressed into bars or cakes. The THC content of the resin is 2 - 30 %.

c. Cannabis oil of the resin

Cannabis oil is extracted from hashish with an organic solvent or distillation technique out of hashish. The THC content in the oil is up to 65 %.

d. Fatty oil of the seeds

The fatty oil, extracted from the seeds, is very expedient for food use, since it contains a lot of essential fatty acids. Usually it does not contain THC.

2.1.1.4. Legislation for Cannabis preparations [21, 22]

In almost all countries of the world, the production, possession, purchase, and import, as well as the trade and transfer of cannabis are legally forbidden. Excluded from these regulations and restrictions are the use of cannabis for research projects. Under certain conditions and upon request, pharmaceutical companies may be allowed to raise seeds of THC rich cannabis for research purposes. In most countries and with a few restrictions, the sale of seeds (also of THC rich sorts) is permitted. This applies also for scions, provided their THC content does not exceed the predetermined limits.

Worldwide, the use of natural cannabis products for medical purposes is practically not allowed. In contrast, drugs containing synthetic cannabinoides like Marinol™ are often exempt from these restrictions. Synthetic products, however, have a disadvantage: they do not contain a well-balanced combination of active substances which can be found in the natural hemp plant. Patients having consumed natural cannabinois for their medical therapy report more side-effects after the administration of synthetic cannabis preparations [4, 5]. It was speculated that this could particularly be the result of the absence of cannabidiol (CBD) in the synthetic product [4].

The United States Food and Drug Administration (FDA) approved Marinol™ to treat nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional therapies. The FDA also approved Marinol™ to treat appetite loss associated with weight reduction in people with acquired immunodeficiency syndrome (AIDS).

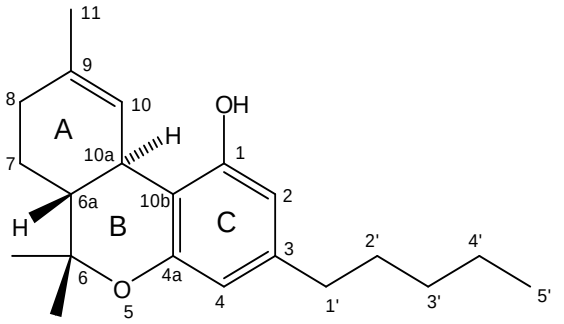
In Switzerland, all cannabis preparations underlay the legislation of narcotics and are listed in the category of the forbidden substances. Marinol™ is not registered. The Swiss Federal Office of Public Health (FOPH) can exempt cause if the cannabinoids/cannabis preparations are used for research projects or for a limited medical therapy (“compassionate use”). A special exemption for Marinol™ can be obtained if a person suffers from cancer, HIV, AIDS, multiple sclerosis or paraplegia. With this permission, the import of dronabinol capsules is allowed.

2.1.2. Cannabinoids

2.1.2.1. ∆9-tetrahydrocannabinol (THC)

2.1.2.1.1. Structure

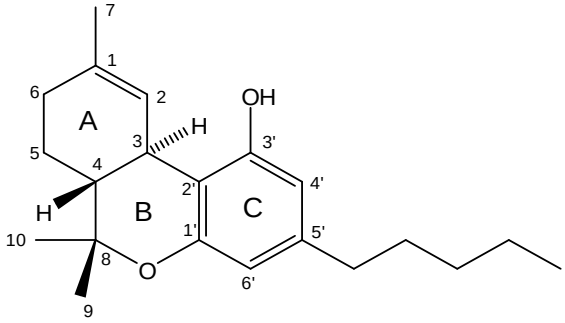
The structure of THC using two different numbering systems is presented in *Figure 2*. The common system resulting in ∆9-tetrahydrocannabinol is based on the dihydrobenzopyran-numbering according the IUPAC (International Union of Pure and Applied Chemistry) rules and the other, resulting in ∆1-tetrahydrocannabinol is based on the monoterpene-numbering. THC is the only biogenic, psychotropic substance without nitrogen in the structure. THC is psychotropic and pharmacologically active.



∆9-tetrahydrocannabinol

Dihydrobenzopyran-numbering

$C_{21}H_{30}O_2$



∆1-tetrahydrocannabinol

Monoterpene-numbering

$C_{21}H_{30}O_2$

Figure 2: Structure of THC

2.1.2.1.2. Physical and chemical data of THC

Chemical and physical data of THC are presented in *Table 1* [10, 23].

Table 1: Physical and chemical properties of THC, dronabinol respectively

Systematic name	(-)-*trans*-Isomer of the Δ^9-THC, (-)-Δ^9-*trans*-THC (dronabinol) Chemically: (6aR-*trans*)-6a, 7, 8, 10a-Tetrahydro-6,6,9-trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol
Synonym	Dronabinol
Molecular weight	314.5
Molecular formula	$C_{21}H_{30}O_2$
Boiling point	200 °C
Property to rotate plane polarised light	$[\alpha]_D^{20}$ -150.5°(c = 0.53 in $CHCl_3$)
UV max in ethanol	283 nm and 276 nm
Solubility	Highly insoluble in water (~2.8 mg/L, 23 °C) and 0.15 M NaCl (0.77 mg/L, 23 °C) High solubility in ethanol, methanol
Octanol:water partition coefficient	6,000:1
pK_a	10.6
Stability	Unstable in acidic solution ($t_{1/2}$ = 1h at pH 1.0, 55 °C) Sensitive to light and heat
Description	Yellow resinous oil, sticky at room temperature, hardens upon refrigeration, without smell, bitter taste, high lipophilic

2.1.2.2. Δ9-tetrahydrocannabinolic acid A (THCA-A) [24]

Δ9-tetrahydrocannabinolic acid A (THCA-A) (*Figure 3*) is the precursor of THC in hemp plants. During smoking, the non-psychoactive and pharmacologically inactive THCA-A is converted to THC, the main psychoactive component of marijuana and hashish. In *Cannabis sativa L*., 95% of THC consists of its precursor THCA-A [25]. The decarboxylation of THC acids to the corresponding phenols (*Figure 3*) occurs readily over time, upon heating, or under alkaline conditions.

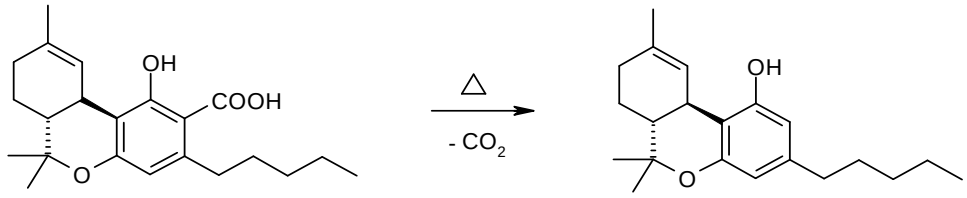


Figure 3: Mechanism of the decarboxylation of THCA-A to THC

2.1.2.3. Cannabidiol (CBD)

After THC, CBD (*Figure 4*) is the next most abundant cannabinoid substance that occurs in the blood and urine following ingestion of hashish, marijuana or cannabis. According to recent results [26, 27], the carboxylated precursors used in the biosynthesis of CBD and THC are both directly derived from cannabigerolic acid. The existence of the postulated enzyme CBD-cyclase catalysing the synthesis of THC via CBD has not been experimentally confirmed. The CBD/THC ratio is mainly dependent on the genetic background of the individual plant. There is some evidence that CBD is devoid of psychotropic actions and may even antagonise the psychotropic effects of THC [4]. Evidence emerged that CBD partially inhibits the CYP2C9-catalysed hydroxylation of THC to the psychotropically active 11-OH-THC. The probability for this inhibition is particularly high for oral intake because THC and CBD attain relatively high concentrations in the liver and in addition there is a high first-pass metabolism of THC [1, 28]. CBD does not bind to the known cannabinoid receptors and its mechanism of action is presently unknown [29].

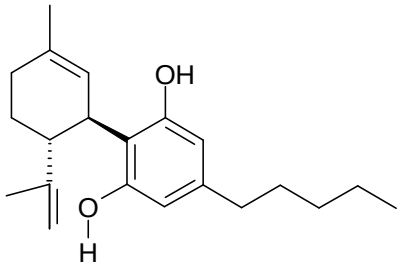


Figure 4: Cannabidiol

2.1.2.4. Cannabidiol acid (CBD-A)

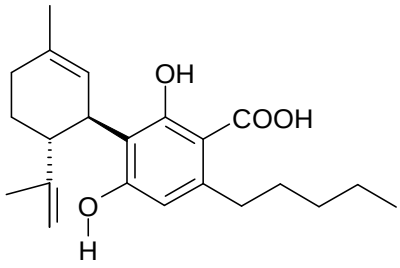


Figure 5: Cannabidiol acid

CBD-A (*Figure 5*) is a non-psychotropic and antimicrobial substance [30]. Decarboxylation of CBD acid to the corresponding phenol occurs readily over time, under heating or alkaline conditions [31]. CBD-A decarboxylates to CBD that undergoes a cyclisation to THC (*Figure 6*).

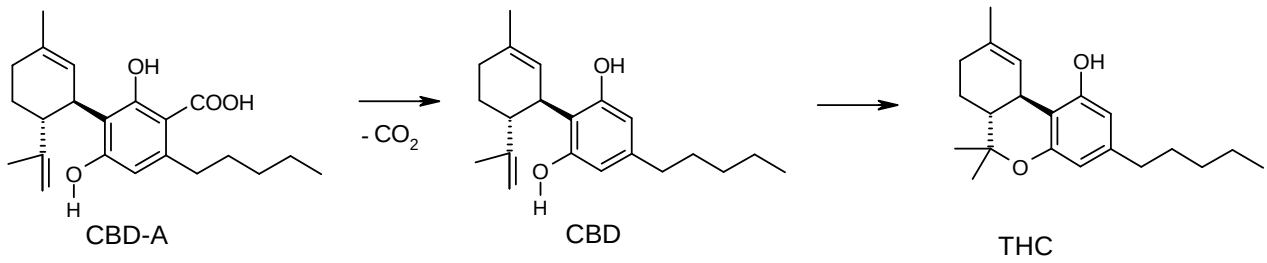


Figure 6: Decarboxylation of CBD-A to CBD and cyclisation to THC

2.1.2.5. The four cannabinoid types

Cannabinoids have four types: the drug type, the inter type, the fibre type and the propylisomer type (*Table 2*). The main cannabinoids of the fibre type are CBD and CBD-A and those of the drug type are THC and THCA-A [32].

Table 2: The four cannabinoid types, modified from [32]

TYPE	NAME	MAIN CANNABINOIDS	PSYCHOACTIVITY
I	drug type	THC/THCA-A	++
II	inter typ	THC/THCA-A, CBD/CBD-A	++
III	fibre-/seed type	CBD/CBD-A	(+)
IV	propylisomer type	Δ9-tetrahydrocannabi-varin/ -acid	++

2.1.2.6. **Dronabinol** [10]

Dronabinol is a synthetically produced THC. The structure of dronabinol is presented in *Figure 7*. dronabinol is a light yellow resinous oil that is sticky at room temperature and hardens upon refrigeration. dronabinol is insoluble in water and is formulated in sesame oil. It has a pKa of 10.6 and at pH 7.0 an octanol:water partition coefficient of 6,000:1. Marinol™ for oral administration are round and soft gelatine capsules containing 2.5 mg, 5 mg or 10 mg dronabinol. The United States Food and Drug Administration (FDA) approved Marinol™ to treat nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional therapiess. The FDA also approved Marinol™ to treat appetite loss associated with weight reduction in people with acquired immunodeficiency syndrome (AIDS).

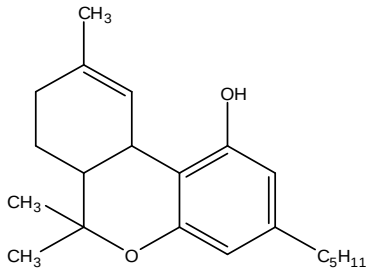


Figure 7: Dronabinol (Marinol™) $C_{21}H_{30}O_2$

2.1.3. Pharmacological and toxicological effects of THC

2.1.3.1. Pharmacokinetics

a. Absorption and distribution of THC [15, 33-35]

THC is rapidly absorbed after the inhalation of cannabis smoke. Rate of absorption depends on the inhalation technique and smoking frequency, resulting in very variable bioavailabilities (10-20%). The absorption after oral administration is more erratic because of a variable degradation of the drug by stomach acids and the extensive liver first-pass metabolism. Cannabinoids dissolved in a lipophilic vehicle like sesame oil can increase the absorption up to 95%. Due to the extensive liver first-pass metabolism, the measured bioavailability is very low (10-20%) [10]. THC administered as a prodrug (THC-hemisuccinate) in suppositories shows more constant- and about two-fold higher bioavailabilities compared to an oral application. Cannabinoids rapidly penetrate into the tissues (liver, heart, lung, gut, kidney, spleen, mammary gland, placenta, adrenal cortex, thyroid, pituitary gland, fat and lower concentrations in brain, testis and foetus) because of their high lipophilicity. Therefore, the distribution volume is about 10 L/kg. THC is strongly bound to plasma proteins (~97%). About 60% is bound to lipoproteins (the low-density fraction), about 9% to blood cells and the rest to albumin. The THC metabolite 11-hydroxy-Δ9-THC (11-OH-THC) is even more strongly bound (~99%).

b. Metabolism of THC [15, 28, 35, 36]

About 100 metabolites have been identified for THC so far. All cannabinoids are good substrates of cytochrome P450. The hydroxylation sides of THC are at C11 and C8 as well as at all positions of the alkyl side-chain. The preferred hydroxylation site in man is C11. It has been shown that cytochrome P450 isoenzyme 2C9 catalysis the formation of the psychoactive 11-OH-THC metabolite of THC and that cytochrome P450 3A is responsible for the hydroxylation at the 8β-position [37, 38]. Many of the hydroxyl groups undergo further oxidation after the hydroxylation. Carboxylic groups at C11 and C5' (alkyl side-chain) are formed. 11-OH-THC is further oxidised, probably by alcohol dehydrogenase and of microsomal alcohol oxygenase, to the intermediate aldehyde 11-oxo-THC followed by oxidation to 11-nor-9-carboxy-THC catalysed by a microsomal aldehyde oxygenase, a member of the CYP2C subfamily [37, 39, 40]. After glucuronidation of the carboxy group, the THC-COOH is the main metabolite excreted and found in urine [28]. Carbon atoms from the alkyl side-chain get lost after β-oxidation and related reactions of the C5'-acid. The predominant phase II metabolite of THC is the O-ester-glucuronide of THC-COOH. The formation of conjugates with long-chain fatty acids is another possible pathway. Although it is a phase II reaction, the lipophilicity is rather increased than decreased. It is possible that these conjugates with long-chain fatty acids may be retained within tissues for considerable periods of time because they are membrane constituents resembling compounds. *Figure 8* shows the main metabolic pathways for THC. The possible sites for the primary oxidation of THC are presented in *Figure 9*.

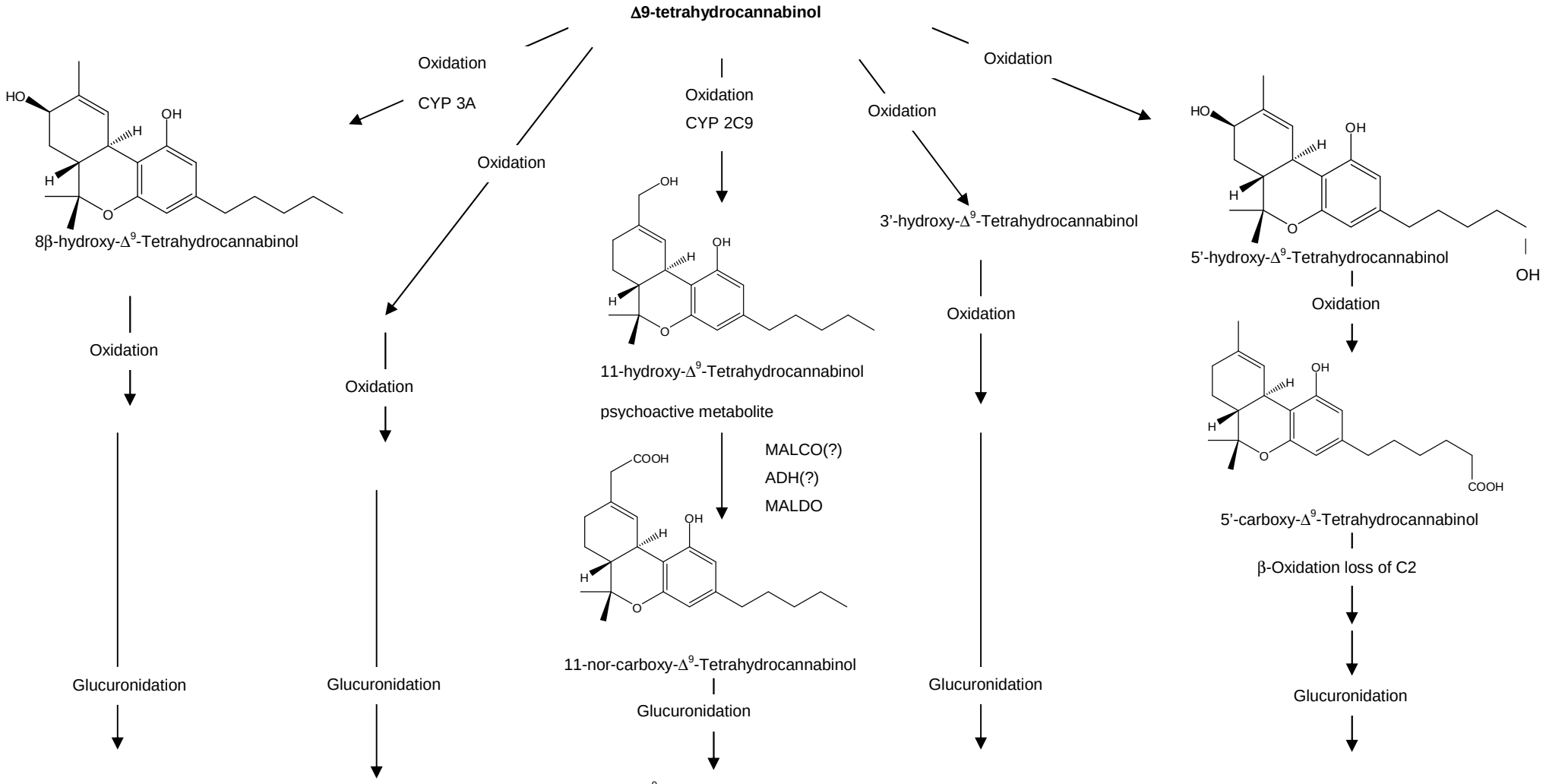


Figure 8: Main metabolic pathways for THC [24, 28, 41]

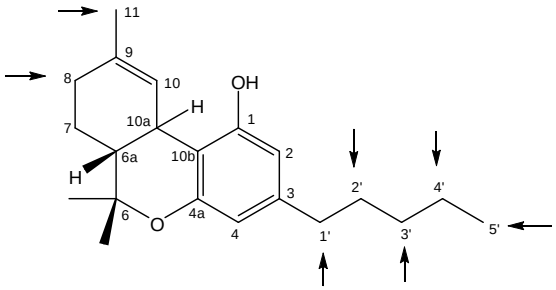


Figure 9: Sites for primary oxidation of THC

c. Excretion [15, 35, 36]

Elimination of THC from the plasma is rather slow because of the limited re-diffusion of THC from body fat and other tissues into the blood. The results of this phenomenon are low plasma levels. The literature describes very variable half-life periods from 1 to 4 days. The absolute elimination time could require up to five weeks. THC metabolites are excreted in urine (~20%) and faeces (~80%). The distinctive enterohepatic cycle is another reason for the slow elimination. Most urinary metabolites are acids. The major metabolites found in urine are THC-COOH and THC-COOH-glucuronide. Normalised to the drug creatinine ratio, THC-COOH and its glucuronide are used for the detection and monitoring of drug abuse.

2.1.3.2. Pharmacodynamics

a. The endogenous cannabinoid system, cannabinoid receptors [42, 43] and the structure-activity relationship (SAR) at the cannabinoid (CB) receptor [44, 45].

Two cannabinoid receptors are known in mammalian tissues. Both receptors are coupled to their effector systems through $G_{i/o}$ proteins. CB1 receptors are expressed in the spinal cord, in the brain and in certain peripheral tissues. It is supposed that some central and peripheral CB1 receptors, located at nerve terminals, modulate neurotransmitter release. CB2 receptors were found in immune tissues (e.g. leukocytes, spleen and tonsils). In the nineties, the discovery of CB1 and CB2

receptors was followed by the detection of endogenous cannabinoid-receptor ligands. Arachidonoylethanolamide (anandamide) and 2-Arachidonoylglycerol (2-AG) are the most important ones. The human body synthesises anandamide from N-arachidonoyl-phosphatidylethanolamine, which is itself made by transferring arachidonic acid from phosphatidylcholine (PC) to the free amine of phosphatidylethanolamine (PE). It is supposed that both agonists can act as neuromodulators or neurotransmitters. Anandamide and methanandamide activate vanilloid receptors on sensoric neurons [46]. Anandamide is an endogenous ligand for vanilloid receptors. Therefore, the vanilloid receptors represent possibly a cannabinoid CB3 receptor [46]. The cannabinoid receptors and their endogenous ligands are denoted as “the endogenous cannabinoid system” or “endocannabinoid system”. The endocannabinoid system is important for the regulation of processes in the central nervous system (neurotransmitter release). Furthermore, it regulates neurotransmitter release/action of autonomic and sensory fibres and it controls gastrointestinal, immunological and cardiovascular performance in the periphery. The links to neurotransmitter systems and all these functions are not yet fully understood. For both CB receptors, several selective synthetic agonists and antagonists have been synthesised. They are used for basic and clinical research. Possible biochemical pathways for THC and cannabinoids are presented in *Figure 10*.

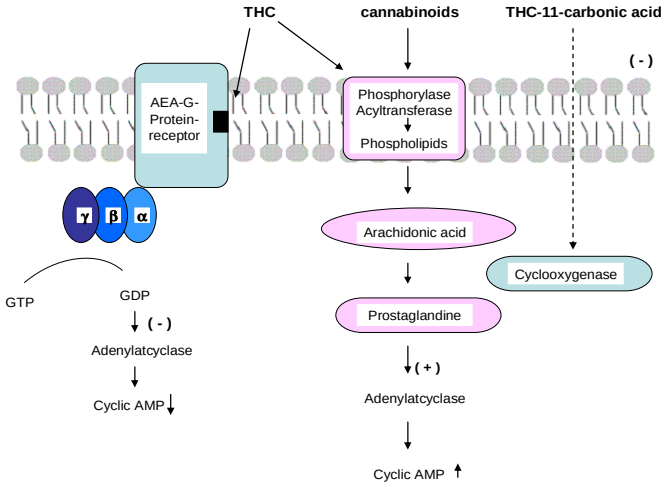


Figure 10: THC and cannabinoid effects on phospholipids- and arachidonic acid-dependent biochemical processes. THC binds to a G-protein receptor which affects adenylatcyclase-dependent processes (Reproduced after [47]).

The binding of THC to the CB1 receptor demands different essential structure-activity relationships. In most active cannabinoids, the dihydrobenzopyran-type structure with a hydroxylgroup at C1 and an alkyl group at C3 is present. The activity at the CB1 receptor gets lost as a result of a pyranring opening (forming cannabidiol-type compounds). The alkylic side chain with at least 5 carbon atoms at position C3 is of considerable importance: elongation and branching results in potentiation. It is essential that the phenolic group at the C1-position is free. The stereochemical trans-link of the two rings A and B at position C6a and C10a is important for the activity.

2.1.4. Therapeutic potential of THC [48]

As described in chapter 2.1.2.6. THC or cannabis preparations are well established in the therapy of nausea and vomiting associated with cancer and for appetite loss associated with weight reduction in people with AIDS as well as in Alzheimer's disease patients. Marinol^{TM} is registered and used in the USA for these indications. It was shown in recent studies that THC or cannabis preparations have a promising potential to combat spasticity, moving disorders and pain in patients suffering from multiple sclerosis. Some case reports show THC effects against pruritus, allergies,

inflammations, infections, depressions, epilepsy, anxiety, withdrawal syndromes and addiction. Furthermore, THC lowers intraocular pressure in glaucoma and is anti-asthmatic. Worthwhile research areas for a possible treatment with THC are high blood pressure, autoimmune diseases, neuroprotection and cancer fever [7, 49, 50].

2.1.5. Toxicology and adverse effects of THC

a. Acute toxicology of THC [51]

No deaths directly linked to toxic somatic effects of cannabis have been reported so far in human. With an oral LD_{50} dose in rats of 800 to 1900 mg/kg the acute toxicity of THC is very low. There were no cases of death due to toxicity following the maximum oral THC dose in dogs (up to 3000 mg/kg administered) and monkeys (up to 9000 mg/kg). Nevertheless, acute adverse effects after cannabis consumption are reported. Instead of a pleasant and relaxing feeling, dysphoric reactions sometimes accompanied with panic attacks and fear of death are reported. The ability to drive a car and cognitive functions like attention, reaction and memory are impaired. Further reported effects are sleepiness, dizziness, confusion and mental clouding.

b. Long-term toxicology of THC [51, 52]

The main described chronic effect after cannabis smoke inhalation (“joint”) is the destruction of the mucous membrane in consequence of pyrolysis products. It is evident that the cognitive functions and memory are decreased in heavy cannabis users. Cannabis can cause “temporal disintegration”. This term is described as a difficulty in regaining, coordinating and serially indexing memories. Perceptions and expectations that are relevant to the attainment of some goals are limited [53]. Vulnerable persons are at risk of a latent schizophrenia after cannabis consumption. The incidence of schizophrenia is not increased. Contradictory results are described about fertility and long-term toxic effects of the immune and endocrine system. No indication of an impairment of these functions is described in humans. As a result of

a cannabis consuming pregnant woman, the cerebral functions in the foetus can be impaired because of disturbances in the development of subtle cerebral functions [51, 52].

2.1.6. THC tolerance [54]

Frequent cannabis use quickly leads to a tolerance development. Tolerance is attributed to pharmacodynamic changes, probably based on receptor down regulation or receptor desensitisation. It can also be attributed to pharmacokinetic parameters like changes in absorption and metabolism, for example, modified biotransformation activities with regard to cytochrome P450. Mild withdrawal syndromes are reported by heavy chronic cannabis consumers after they cease taking the drug. The described symptoms are nervous agitation, hyperhidrosis and lack of appetite. The risk for cannabis abuse in the therapeutic context is low, the dependence is mainly a psychological and not a somatic effect.

2.1.7. Somatic adverse effects of THC [51]

Acute atropine-like adverse effects such as increased heart rate, hyposalivation reddening of the eyes and orthostatic hypotension, can occur after cannabis consumption. The risk of infections in the eyes and of the upper respiratory tract is increased by a decrease of lacrimation and hyposalivation. Nausea, headache and vomiting occur rarely.

2.2. Technical part

2.2.1. Urine drug testing and sample preparation

2.2.1.1. Specimen matrices and urine drug testing

Laboratory analyses of cannabinoids or other drugs have forensic, criminal and clinical utilisations. Urine, blood, saliva and hair are the common matrices for the detection of drugs. The collection of urine is easy. The drugs and their metabolites found in urine can be detected for a longer time frame after application compared to blood or saliva [55]. A hydrolysation procedure of the urine before the analysis to convert drug conjugates (glucuronides) to their free metabolites is advantageous for several analytical techniques. The rate of false positive or false negative results should be minimised in an analytical method. To achieve this objective, it is necessary to identify presumably positive specimens in a screening test. The principle of an immunoassay test is the use of antibodies binding to the drug. The selectivity is limited depending on the antibodies specificity in the immunoassay. False positive results can be caused if chemically similar structured substances of the assumed drug interfere with the test. A sample concentration below the accepted threshold or a dilution or adulteration of the sample to obscure the presence of a drug can lead to false negative results. Unfortunately, often in a screening test cross-reactive substances have to be considered [56]. Therefore, preliminary positive immunoassay results have to be confirmed by a second, more specific analysis method like mass spectrometry (MS) [55, 57].

Urine contains creatinine, a metabolic waste product removed from muscular tissue. It is filtered from the blood by the kidneys and excreted in the urine, nearly not secreted and not reabsorbed in the tubular system. This is the reason why the measurement of creatinine in blood and urine serve as parameters for the calculation of the glomerular filtration rate. Creatinine is excreted nearly constantly during the day, so it serves as a normalisation factor for urinary excretion of other substances. For measuring drugs excreted into the urine, the drug/creatinine ratio

is therefore often used as a normalisation factor if different measurements have to be compared [58].

2.2.1.2. Sample preparation

a. Liquid-liquid- and solid phase extraction

A pre-analytical isolation procedure to separate drugs from interfering substances in biological matrices is required in HPLC and LC-MS/MS. The main preparation techniques are liquid-liquid extraction (LLE) and solid phase extraction (SPE). These methods can be applied manually and automated. The manual (off-line) sample preparation can be adapted to every analysis but it is more time consuming than an automated (on-line) method. In an on-line method, the machine pipettes and washes the sample on its own. It is fast and easy. In LLE, the separation must be conducted at a pH at which the analyte is uncharged. Acidic drugs need an acidified aqueous phase, basic drugs a basified one. LLE is difficult to automate. It is time consuming and wastes large amounts of organic solvents compared to SPE. The organic solvents are often toxic and expensive. Solid-phase extraction (SPE) uses both a solid phase and a liquid phase to isolate an analyte from a solution. The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube. SPE is based on intermolecular forces (hydrogen bonding, dipole-dipole forces, ionic interactions, Van der Waal forces) of the analyte with the sorbent, the liquid phase and the sample matrix.

b. Reversed-phase SPE

Reversed-phase (RP) SPE chromatography separates organic solutes from a polar phase (generally aqueous) into a non-polar phase. The stationary phase of a RP SPE cartridge is derivatised with hydrocarbon chains. The affinity of the solute to the sorbent depends upon its hydrophobicity. The analyte is eluted with an organic

solvent (like acetonitrile, ethyl acetate or methanol) which disrupts the interaction of the analyte and the stationary phase [59]. The most common RP sorbents for SPE are chemically bonded silica phases [60-62]. Most widely used bonded phases are C_8 and C_{18} sorbents [63].

c. Ion exchange SPE

The separation in ion exchange sorbents is based on electrostatic interactions between the analyte of interest and the positively charged groups on the stationary phase. A pH at which the stationary phase and the sample are charged is needed for the ion exchange. Anion exchange sorbents are derivatised with positively or negatively charged functional groups. These sorbents retain negatively charged anions like acids. Strong anion exchange sorbents contain quaternary ammonium groups. They are permanently positive charged in aqueous solutions. Cation exchange sorbents are derivatised with negatively functional groups. They retain positively charged cations like bases. Strong cation exchange sorbents contain aliphatic sulfonic acid groups. They are always negatively charged in aqueous solutions [59].

d. Certify II cartridges for the extraction of THC-COOH

Certify II (Varian) are bonded silica mixed mode sorbent solid phase extraction cartridges used to extract acidic drugs like THC-COOH. Since this drug contains an acidic functional group, clean-up from urine samples can be optimised by using an anion exchange sorbent. Retention of acidic drugs on Certify II is initially achieved by non-polar interactions on the hydrophobic portion of the sorbent. Polar interferences can then be washed away with a basic buffer. This wash step also ensures that the COOH functional group is deprotonated, forming COO^-, which can then be retained on the anion exchange portion of the Certify II sorbent. After briefly drying the cartridge, non-polar and basic drugs can be removed with a non-polar solvent. Finally, the THC-COOH can be recovered by elution with a non-polar acidic solvent such as acetonitrile with 2% ammonia. [64].

2.2.2. LC-MS/MS conditions

2.2.2.1. Photo Diode Array (PDA)

The utilisation of a photo diode array detector allows the measurement of the absorbance of a component over the complete wavelength range at the same time. The photodiodes are arranged in parallel. Each array measures a small spectral area [65]. Photo diode array ultraviolet detectors record the absorbance of compounds at a wavelength range of 200-400 nm as they pass through the detector flow cell, allowing the on-line acquisition of UV spectra.

2.2.2.2. Overview of a LC-MS system

The coupling of LC and MS was a breakthrough in analytical technology. It is an alternative and complement to GC-MS [66]. The combination of selective and sensitive LC-systems with mass spectrometers and a simple sample preparation for LC may be the “gold standard” for bioanalytical application. The development of a chromatographic method is usually a compromise between desired resolution and analysis time. The reduction in the stationary phase particle size can result in the benefit to the chromatographic process [67]. The sample is injected by an injection port (e.g. autosampler) in the mobile phase stream delivered by the high-pressure pump and transported through the column where the separation takes place [68]. A gradient running solvent is normally used in a qualitative drug screening for the analysis of the large diversity of substances. For quantitative LC analyses of compounds identified by screening, sometimes it can be more efficient to use isocratic chromatography.

After the separation, the different, separated substances passes the MS detector system. It consists of an API source, an ion transfer capillary, tube lens and skimmer, ion optics, mass analyser and ion detection system. The sample ionisation takes place in the API source. The produced cations or anions are transmitted by the ion optics into the mass analyser. Selected ions are ejected from the mass analyser and

reach the ion detection system where they produce a signal. In *Figure 11* a schematic drawing of a liquid-chromatography mass spectrometer system is shown.

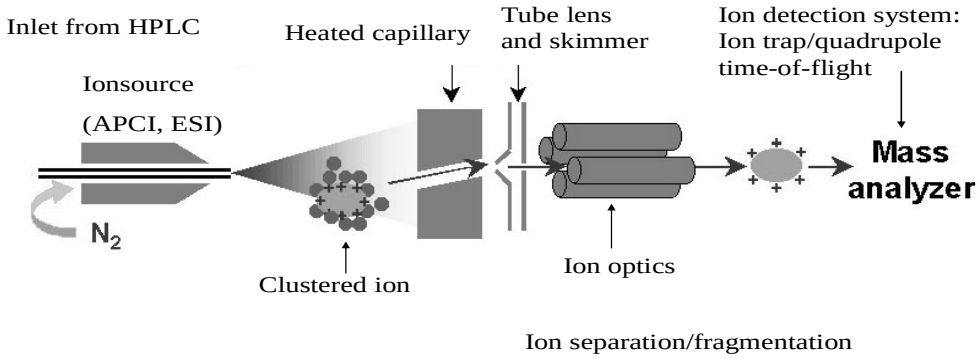


Figure 11: Schematic drawing of a liquid-chromatography mass spectrometer system (Reproduced after [69]).

2.2.2.3. API interfaces

The challenge of coupling two systems like LC and MS, one operating in liquid phase and the other in high vacuum, has been overcome by creating dedicated interfaces. Today the most widely used interfaces are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). APCI is generally regarded as the more robust ionisation method. It is less susceptible to signal suppression from co-eluting matrix components [70, 71] or to changes in variables such as changes in buffer and buffer strength. ESI is the softer and more versatile of the two methods enabling it to ionise very polar/non volatile molecules [70]. LC-MS interfaces remove the mobile phase and ionise the analyte. The collision-induced dissociation is followed by a thorough fragmentation (MSMS) of the compounds.

2.2.2.4. Atmospheric pressure chemical ionisation (APCI) and electro-spray ionisation (ESI)

APCI is suited for the analysis of molecules with a molecular weight up to 2'000 atomic mass units (amu) with a medium polarity and some volatility. The pH of the analyte in the gas phase and the solvent vapour play an important role in the APCI process because it works in the gas phase. The flow rate of solvent in APCI mode is typically high with 0.2 - 2 ml/min [72]. The APCI nozzle vaporises the sample solution in a high temperature (~500 °C). A needle is located near the end of the t ube. A high voltage is applied to the corona discharge needle. Electrons are produced (*Figure 12*). The electrons react with the liquid phase and the nitrogen gas. N_2 is delivered by the sheath and auxiliary gas. Reagent ions are formed through a series of chemical reactions. The reagent ions react with the sample molecules. The charge is transferred to the sample molecules which get ionised.

Figure 12: Atmospheric pressure chemical ionisation

Heat-labile compounds or high molecular weight compounds, which were previously not suitable for mass analysis, can be analysed by the soft ESI method. ESI can analyse any polar compounds which generate ions in solution. ESI allows the analysis of big molecules (>100'000 amu) like peptides and proteins due to the multiple charging. The flow rate of solvent in ESI mode is ranging from 1 to 1000 µl/min. The eluent from the LC system is sprayed through a capillary into the ESI source region. It is nebulised in a fine mist of droplets by a pneumatically assisted gas stream and a high temperature (*Figure 13*). The droplets are electrically charged at their surface when a capillary voltage is applied. Solvent evaporates from the droplets. The electrical charge density increases up to a critical point known as the

Rayleigh stability limit (the electrostatic repulsion is greater than the surface tension [73]). At this point, the droplet-clusters divide into smaller droplets [74, 75]. This mechanism is repeated several times. Sample ions are generated out of the cluster ions and move towards the entrance of the mass spectrometer by an electrical field. The ESI process is affected by droplet size, liquid surface tension, solvent volatility, surface charge and ion solvation strength. A good ESI process is prevented by large droplets with high surface tension, low volatility, strong ion solvation, low surface charge and high conductivity [75].

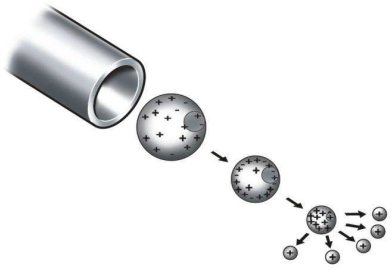


Figure 13: Electrospray ionisation

APCI and ESI work in the positive and negative ion polarity mode. A positive applied voltage generates cations, a negative one, anions. The ion-positive mode produces a stronger ion current for most molecules, especially for those with one or more basic (nitrogen) atoms. An exception to this rule are molecules with acidic sites (carboxylic acids or acidic alcohols). They produce strong negative ions. In general, more positive ions are produced but negative ion polarity can be more specific. The negative ion polarity mode often has less chemical noise compared to the positive one.

2.2.2.5. Ion transfer capillary and ion optics

The heatable (150-200° C) ion transfer capillary assists in desolvating the produced ions on their way to the mass analyser. A decreasing pressure gradient and electrostatic forces transport the ions to the skimmer region. First, they enter the tube lens. The ions are focused towards the skimmer through a dependent potential in the

tube lens. A collision with the residual solvent and with gas molecules can occur if the tube lens offset voltage is high. Fragments can be produced by this ion source collision induced dissociation (CID). The duty of the skimmer is to reduce the number of neutral molecules and large charged particles, because they would create detector noise. The produced cations or anions are transmitted from the API source by the ion optics (in our apparatus octapoles) into the mass analyser. The ion stream coming from the source is directed and focused by magnetic fields.

2.2.2.6. Mass analysis, detection modes and mass analysers

Mass analysis of ionised substances is performed by using one (LC-MS) or two mass analysers (LC-MS/MS). The most common instruments are ion traps and quadrupoles, less frequently sector field and time-of-flight instruments (TOF) are used.

Quadrupoles and ion trap mass-analysers can operate in the full scan mode or in the more sensitive selected-ion monitoring mode (SIM). They detect positive and negative ions. The modes can be combined in parent-ion scanning (scan mode in the first, SIM in the second analyser), daughter-ion scanning (DIS; SIM in the first, scan mode in the second analyser), constant neutral-loss scanning (CNLS; scan mode in both analysers) or selected-reaction monitoring (SRM; SIM in both analysers). The most common mode is the DIS, where a specific precursor is selected in the first quadrupole, fragmented in the collision cell and the products subsequently monitored in the second quadrupole. Another mode is multiple-reaction monitoring (MRM). It is the most powerful technique for the quantification of small amounts of analyte in complex matrices [76, 77]. The use of these scan modes is important in the analysis of drug metabolites which have a similar fragmentation behaviour and produce common fragment ions.

Mass analysing can be divided in four steps: Ion storage, ion isolation (SIM, SRM, and MS/MS full scan), collision induced dissociation (SRM and MS/MS full scan) and ion scan out.

The use of CID in a quadrupole, which is the most common ion activation method, was an important step in the development of LC-MS [78, 79]. The low-energy collisions are used in LC-MS/MS. The instrument consists of a first quadrupole, a collision cell and a second quadrupole for the production of fragment ions (*Figure 14*). In LC-MS/MS, the production of fragment ions is distinct from the ionisation step. The precursor and product ions are characterised independently by their m/z ratios [80]. Product ions are built by single or multiple collisions of the precursor ions with neutral gas molecules in the collision cell. Collisions between precursor ions and a neutral target gas like N_2 or Ar are followed by an increase in internal energy of the ion. A decomposition is induced which provides structurally informative fragments (product ions).

The linear quadrupole mass analyser is a mass filter. It consists of four hyperbolic or circular rods, which are placed parallel in a radial array [81]. A radiofrequency- and a direct current offset voltage are applied to the rods. An electrical field is produced that guides the desired ions along the axis of the quadrupole. The ions with an unwanted m/z ratio collide with the four square rods. The offset voltage is negative for positive ions and positive for negative ions during the ion transmission. The triple quadrupole is the most common mass spectrometer.

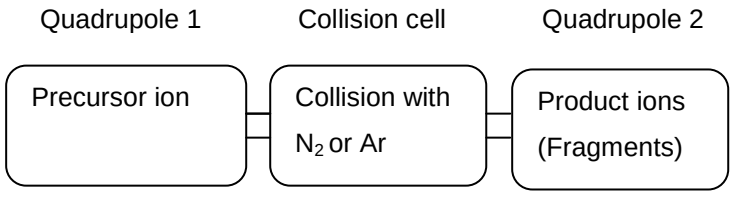


Figure 14: Scheme of a mass analyser in tandem mass spectrometry (Reproduced after Sciex instruction).

The essential parts of an ion trap mass-analyser are three electrodes. The entrance and exit endcap electrodes have a small hole in their centre. The passage of ions into and out of the mass analyser cavity is possible through these holes. The voltages applied to the endcap electrodes are equal in amplitude but are 180 °C out of phase to one another. The trapment, fragmentation and ejection of ions according to their mass-to-charge ratios are possible through various alternating current voltages applied to the ring and endcap electrodes. A radiofrequency voltage is applied to the ring electrode. It produces a three dimensional quadrupole field within the mass analyser cavity. An ionic movement in the axial (towards the endcaps) and radial (from the ring electrode towards the centre) directions is driven. The stream is essential for the ions to remain trapped. The system produces an m/z dependent instability during the ion scan out. The ions are ejected from the mass analyser in the axial direction. When the radiofrequency applied to the endcaps equals the resonance frequency of a trapped ion, the ion gains kinetic energy and is ejected. During the CID in an ion trap, a voltage is applied to the endcap electrodes to fragment precursor ions into product ions. This voltage is not strong enough to eject an ion from the mass analyser but ion motion in the axial direction is enhanced and the ion gains kinetic energy. After many collisions with helium gas in the mass analyser cavity, the ions are energetic enough to dissociate into product ions.

2.2.2.7. Ion detection system

A conversion dynode and a channel electron multiplier are the main part of the ion detection system. A +15 kV potential for negative ion detection or a -15 kV one for positive ion detection is applied to the conversion dynode [82]. When an ion strikes the surface of the conversion dynode, one or more secondary particles are produced. These secondary particles can include positive or negative ions, electrons and neutrons. When positive ions strike a negatively charged conversion dynode, the secondary particles of interest are negative ions and electrons. The result of negative ions are positive secondary particles. The secondary particles are focused by the curved surface of the conversion dynode and are accelerated by a voltage gradient into the electron multiplier [82]. Secondary particles from the conversion dynode strike the inner walls of the electron multiplier with sufficient energy to eject electrons.

The ejected electrons are accelerated further into the cathode, drawn by the increasingly positive gradient. Due to the funnel shape electrons do not travel far. They strike the surface again, thereby causing the emission of more electrons. A cascade of electrons is created finally resulting in a measurable current. The current is proportional to the number of secondary particles striking the cathode [82].

2.2.2.8. Vacuum System

Mass analysis, analyte ionisation and detection take place in a high-vacuum system. This is necessary to avoid a collision of the produced ions with molecules (N_2, O_2) passing from the API stack to the ion detection system at normal pressure. The vacuum system evacuates the region around the API stack, ion optics, mass analyser and ion detection system. High vacuum levels cause reduced sensitivity and reduced lifetime of the electron multiplier. A forepump with a pressure of approximately 1.33 mbar establishes the vacuum necessary for the proper operation of the turbomolecular pump. The vacuum in the API region is usually 1020 mbar and $2.67x10^{-5}$ mbar in the analyser region [82].

2.2.2.9. Adduct formation

Adduct formation frequently occurs in the ionisation process in MS. The molecule ionisation usually results in deprotonated $(M-H)^-$ ions in the negative mode and in protonated $(M+H)^+$ ones in the positive ionisation mode. Parallel to the ionisation of analyte molecules, ionisation with solvent molecules can occur. Adduct formations like $(M+Na)^+$, $(M+K)^+$ or $(M+NH_4)^+$ are reported [83, 84]. Addition of ammonium results in $(M+NH_4)^+$. The first step in fragmentation is the loss of neutral NH_3. The latter can then fragment further. The adduct formation process is not reproducible. Sodium adduct ions are quite stable and yield less fragments. Adduct formation should be included in the validation procedure of a LC-MS method.

2.2.2.10. Retention time

Due to changes in the gradual stationary phase or between different batches of columns with nominally identical packing material, the retention time of a substance is not constant over a period of time. Relative retention time or retention index (RI) has been shown to be an advantageous method to correct this variability [85]. RI is based on the relationship between a substance's retention time and that of a reference compound.

2.2.3. Method validation, GLP and SOP

New analytical methods, to be used in forensic and/or clinical toxicology require careful method development followed by a thorough validation of the final method in order to generate reproducible and reliable data. Reliable analytical data is a prerequisite for correct interpretation of toxicological findings in the evaluation of scientific studies, as well as in daily routine work [86]. The analytical laboratory conducting pharmacology/toxicology and other preclinical studies for regulatory submissions should adhere to FDA's Good Laboratory Practice guidelines (GLP). A set of written standard operating procedures (SOP) should be compiled in the analytical laboratory to ensure a complete system of quality control and assurance. The fundamental parameters of a method validation should include the following:

2.2.3.1. Selectivity

Selectivity is defined as "the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, matrix components, etc." [86]. Method selectivity is established by proof of the lack of response by analysing blank matrices from different lots [86-88]. Signals interfering with the signal of an analyte or an internal standard must be avoided.

2.2.3.2. Calibration model (linearity)

The relationship between the concentration ratios of an analyte in the sample versus the corresponding internal standard must be investigated. Calibrators must cover the whole calibration range and should be matrix based. A minimum of five to eight concentration levels is required as well as a blank and a zero sample [87, 88].

2.2.3.3. Accuracy, precision and recovery

The accuracy of an analytical method compares the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte and should be measured using a minimum of five determinations per concentration. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20% (own definition). The deviation of the mean from the true value serves as the measure of accuracy [89]. The precision of an analytical method is defined as the amount of scatter in the results obtained from multiple analyses of a homogeneous sample. The exact sample and standard preparation procedures that will be used in the final method must be used in the precision study. Instrument precision or injection repeatability is the first type of precision study [90]. With 10 injections of one sample solution the performance of the chromatographic instrument is tested. The second type is repeatability or intra-assay precision [91]. The data is obtained by repeatedly analysing, in one laboratory on one day, aliquots of a homogeneous sample. The inter-assay precision describes the sample aliquots measured on different days. Intermediate precision is the third precision test obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory. Different sources of reagents and multiple lots of columns should also be included in this study [91]. The last type of precision study, reproducibility, is determined by testing homogeneous samples in multiple laboratories, often as part of inter-laboratory cross-over studies [91].

The recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery of the analyte need not to be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with un-extracted standards that represent 100% recovery [89].

2.2.3.4. Range of measurement (LLOQ, LLOD)

The lower limit of quantification (LLOQ) is defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (bias) [87]. The acceptance criteria for both parameters at LLOQ are 20% relative standard deviation for precision and ± 20% for bias [87]. With a signal-to-noise ratio a LLOQ can be estimated as well. It should be ≥ 10. The upper limit of quantification (ULOQ) is the highest calibration standard that can be quantified with fulfilled acceptable precision and accuracy (bias). The limit of detection (LLOD) is defined as the lowest analyte concentration that can be detected. The LLOD is usually estimated at a signal-to-noise ration of 3:1.

2.2.3.5. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. Stability procedures should evaluate the stability of the analytes during sample collection, handling and storage, as well as after going through freeze-thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis [89].

2.2.3.6. Matrix effect

2.2.3.6.1. Matrix effect in mass spectrometry

The suppression or enhancement of analyte ionisation by co-eluting compounds is a well known phenomenon in LC-MS(-MS) analysis. It depends on the sample matrix, the sample preparation procedure, constituents of the mobile phase, the chromatographic separation and the ionisation type [71]. The precision, sensitivity and accuracy of an analytical procedure are affected by the ion suppression or enhancement [92].

Matrix effects are more pronounced with ESI than with APCI [71]. The ionisation process in ESI occurs in the liquid phase. The matrix compounds and molecules of interest stand in competition for access to the droplet surface and subsequent gas-phase emission. The ionisation process can also be affected by a change of the properties of the surface tension and viscosity caused by matrix constituents [71]. Matrix effects also occur in APCI where the ionisation process is taking place in the gas phase. The non-volatile matrix compounds can co-precipitate with the analyte molecules [93].

Matrix effects are caused by the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation and affects the amount of charged ions in the gas phase that reaches the detector.

2.2.3.6.2. Evaluation of matrix effects

a. Evaluation after Matuszewski et al.

A comprehensive strategy for the evaluation of matrix effects was recently published by Matuszewski et al. [94]. Three sets of samples are necessary. The principal approach involves determination of peak areas of analyte in three different sets of

samples. One consists of aqueous standard solutions (set A). For set B blank matrices are supplemented (after extraction) with the same amount of standards as used for set A. Set C consists of extracts of different blank matrices, supplemented with the same amount of standards but added before extraction. Recovery was calculated with the following formula: RE% = C/B x 100. Matrix effects were calculated with the formula: ME% = B/A x 100 and process efficiency with the formula: PE% = C/A x 100. For the calculation the resulting peak areas are needed.

b. Post-column infusion after Bonfiglio et al.

Bonfiglio et al. described another procedure to evaluate matrix effects. A solution of analyte is constantly infused into the eluent from the column via post-column tee connection using a syringe pump. The continuous post-column infusion leads to a constant signal in the detector, unless compounds that elute from the column suppress or enhance ionisation, which would lead to a decreased or increased detector response, respectively [86].

c. Influence of sample preparation and disturbing compounds

Protein precipitation as a sample preparation method is not recommended because of its potential to cause matrix effects. On the one hand it was possible to remove the hydrophilic interfering compounds with SPE but on the other hand the hydrophobic interactions were increased. Matrix effects can be decreased by a reduced injection of matrix constituents. The injection of a smaller sample volume, a diluted sample or by applying more selective extraction techniques can help. Unfortunately, the separation in the liquid chromatographic system is limited by these steps. The use of a co-eluting internal standard like a labelled IS is recommended. The matrix effect on the analyte and on the internal standard is expected to be identical. Unfortunately, not every labelled standard is available and costs are often very expensive. There are compounds known to cause matrix effects like anticoagulants, dosing vehicles (polyethylenglycol, propylenglycol and cremophore), salts, fatty acids, triglycerides and constituents of sampling material (e.g. polymers) [95, 96].

3. Publications

3.1.

Development of a LC-MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of *Cannabis sativa L*. extracts.

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Submitted to: Journal of Chromatography B

Received 13th October 2006; accepted 22nd December 2006

Available online 11th January 2007

Abstract

A novel high-performance liquid chromatographic separation method with tandem-mass spectrometry detection was developed for the simultaneous determination of Δ 9-tetrahydrocannabinol (THC) and its major metabolites 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) as well as the components cannabidiol (CBD) and cannabinol (CBN) in human EDTA-plasma and urine. Run time was 25 minutes. Lower limit of quantification was 0.2 ng/ml. The coefficients of variation of all inter- and intra-assay determinations were between 1.3 and 15.5%. The method was successfully applied to the determination of cannabinoids in human plasma and human urine after administration of Δ9-tetrahydrocannabinol or *Cannabis sativa L.* extracts.

Keywords

Δ9-tetrahydrocannabinol; 11-hydroxy-Δ9-tetrahydrocannabinol; 11-Nor Δ9-tetrahydro-cannabinol-9-carboxylic acid; Cannabidiol; Cannabinol; Cannabinoids; LC-MS/MS; APCI; Human EDTA-plasma; Human urine

1. Introduction

After Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD) is the next most abundant cannabinoid substance that occurs in the blood and urine following ingestion of hashish, marijuana or cannabis. According to recent results [26, 27], the carboxylated precursors used in the biosynthesis of CBD and THC are both directly derived from cannabigerolic acid. The existence of the postulated enzyme CBD-cyclase catalysing the synthesis of THC via CBD has not been experimentally confirmed. The CBD/THC ratio is mainly dependent on the genetic background of the individual plant.

There is some evidence that CBD is devoid of psychotropic actions and may even antagonise the psychotropic effects of THC [4]. This is thought to occur by inhibition of the hydroxylation of THC to 11-OH-THC [1]. Cannabinoids are currently under clinical investigations because of the potential of proven sedative [97], anti-epileptic [98-101], anti-emetic- [102] and anti-inflammatory effects [103]. They may reduce intraocular pressure [104] and may have anxiolytic effects [105]. Psychic symptoms (such as acute and chronic psychosis and depression) have been observed after cannabinoid consumption [106, 107], especially after high doses. CBD does not bind to the known cannabinoid receptors, and its mechanism of action is unknown yet [29].

To characterise the metabolic pattern of THC and its metabolites resulting from ingestion of cannabinoids, we have developed a sensitive and specific analytical method using LC-MS/MS and atmospheric pressure chemical ionisation (APCI). This method was used to characterise the metabolism of cannabinoids based on the source (extracts or pure substances) and route of administration (oral ingestion). Electrospray ionisation (ESI) methods often are more sensitive than APCI methods. However, for the actual method APCI was chosen because of less matrix effects. In addition, the lower limits of detection (LLODs) and lower limits of quantification (LLOQs) were low enough for the determination of THC and its metabolites in the provided study.

Several methods are reported in the literature for the estimation of THC or its metabolites in plasma and urine by gas chromatography/mass spectrometry (GC/MS) after liquid/liquid or solid-phase extraction (SPE) and derivatisation [108-111]. Major disadvantages of these methods are the elaborate sample preparation and the need to use various derivatisation techniques for non-volatile and thermolabile compounds. Other reported chromatographic methods are high-performance liquid chromatography (HPLC) [108] with ultraviolet or electrochemical detection (UV, ED), and gas chromatography (GC) with electron capture, flame ionisation or nitrogen-phosphorous detection (ECD, FID, NPD) [110]. Generally these methods lack either

specificity or sensitivity. Recently, LC/MS or LC-MS/MS using electrospray ionisation and atmospheric pressure chemical ionisation were found to be suitable for the detection of drugs of abuse like THC and its main metabolites [94, 112-120].

The following data show that our method has some distinct advantages, such as a high sensitivity (LLOQ of 0.2 ng/ml in plasma) and the detection of five cannabinoids from two matrices (human plasma and urine), compared to the already reported methods.

The purpose of this paper is to describe a new, sensitive LC-MS/MS method for detection of THC and its metabolites, 11-OH-THC and THC-COOH, as well as CBD and CBN from human EDTA-plasma and urine. This method was used to characterise metabolic patterns of the compounds resulting from pharmacokinetic transformations that occur in humans following ingestion of hashish, marijuana and *Cannabis sativa L*. extracts.

2. Experimental

2.1. Chemicals

THC and metabolite reference material was obtained from Lipomed (Arlesheim, Switzerland). All solvents (acetonitrile, isopropanol 20%, *ortho*-phosphoric acid 85%, ammonia solution 25%, diethylether, ethylacetate, acetic acid 100%, formic acid 98%) and chemicals (ammonium formate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate) in analytical grade were purchased from Merck (Darmstadt, Germany) and Aldrich (Buchs, Switzerland). Fetal bovine serum was obtained from Biochroma (Berlin, Germany) and urine quality controls from Biorad (Reinach, Switzerland). Silanised glass vials (type I plus) were purchased from Schott AG (Muellheim-Huegelheim, Germany) and 6 ml glass test tubes from Gilson

(Mettmenstetten, Switzerland). Solid-phase extraction was carried out on Certify II extraction cartridges from Varian (Zug, Switzerland). De-ionised water was generated with a Milli-Q water purification system from Millipore (Kloten, Switzerland). β-glucuronidase was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The buffer (pH 6.0, 0.1 M) was prepared with a KH_2PO_4 solution (13.61 g/950 ml water), adjusted to the desired pH by appropriate addition of KOH 1 M and filled up with water to one liter. The buffer (pH 9.1, 0.1 M) was prepared with a K_2HPO_4 solution (8.71 g/480 ml water), adjusted to the desired pH by appropriate addition of *ortho*-phosphoric acid 85% and filled up with water to 500 ml.

2.2. Specimens, standard solutions, calibration standards and quality controls

Stock THC and metabolite standard solutions were made in acetonitrile/H_2O (50/50, v/v). (The concentrations were THC, 11-OH-THC, CBD, CBN: 100 mg/l; THC-d3, THC-COOH-d3: 10 mg/l (v/v).) The standards were used to spike the matrix. Calibration standards for assay calibration and determination of the linear measuring range were prepared in bovine serum or human urine by spiking with the needed amount of the standard solutions to obtain the range of 0.2 - 100 ng/ml of THC and its metabolites. EDTA-plasma was taken from healthy male volunteers. After drawing, the blood was gently inverted, centrifuged (10 min at 2000 × *g*) and the supernatant filled in a silanised glass vial. The tubes were sealed and stored at -70 °C.

In order to determine for quality control purposes, the intra- and inter-assay precision and the accuracy, blood quality controls were prepared in blank bovine serum by spiking with THC (3.0, 10.0 and 25.0 ng/ml). The four commercially available urine quality controls contained 0, 37.5, 65.0 and 125.0 ng/ml THC-COOH.

2.3. Extraction procedure

2.3.1. Human EDTA-plasma

The solid-phase extraction procedure was carried out on Varian Bond Elut Certify II cartridges (off-line). Certify II is a mixed mode sorbent with an anion exchange sorbent and retains acidic and neutral drugs (THC and metabolites). The cartridges were conditioned initially with 1 ml acetonitrile and followed by 1 ml 0.1 M phosphate buffer (pH 9.1). 1 ml EDTA-plasma sample acidified with 20 µl *ortho*-phosphoric acid (85%) was loaded onto the cartridges. Cartridges were subsequently washed with 1 ml 40% acetonitrile and dried under vacuum at 45 kPa for two minutes. The compound of interest was eluted with 1 ml acetonitrile:ammonia (NH_4^+) = 98:2 (v/v) into a 6 ml glass test tube and aliquots were evaporated to dryness at 37 °C under nitrogen.

2.3.2. Human urine

First, a hydrolysation of THC-COOH-glucuronide to THC-COOH was done. To 2.5 ml urine 1 ml phosphate buffer (pH 6.0; 0.1 mmol/L) was added, mixed and supplemented with 50 µl β-glucuronidase. After vortexing, the sample was incubated at 50 °C for 3h. The liquid-liquid extraction (LLE) procedure for urine was carried out in glass tubes (off-line). One millilitre phosphate buffer (0.1 M, pH 6.0) and 1 ml of acetic acid 0.1 M were added to 2 ml of the de-glucuronised urine. After vortexing, 4 ml organic phase (diethylether:ethylacetate 50% (v/v)) was added. After mechanical shaking (10 min) and centrifugation (10 min at 2000 × *g*), 3 ml of the organic phase were transferred to a 6 ml glass test tube and then evaporated to dryness at 37 °C under nitrogen.

The extracts were reconstituted in 60 µl of mobile phase (40% mobile phase A, 60% mobile phase B), 10 µl internal standard THC-d3 (EDTA-plasma), respectively, THC-

COOH-d3 for urine analysis (10 mg/l in acetonitrile 50% (v/v)) was added, mixed and 50 µl injected into the LC-MS/MS system (see section 2.4.1).

The internal standard was added in order to overcome possible variations of the concentrations in the LC/detection system. Possible deviations of the extraction rates were overcome by treating the standards in both matrices (EDTA-plasma and urine) in the same way as samples and by calibrating each series of analysis.

2.4. LC-MS/MS

2.4.1. Chromatographic separation and mass spectral detection conditions

The chromatographic system consisted of a Rheos 2000 Micro HPLC pump from Thermo Finnigan (Allschwil, Switzerland) and a Midas Symbiosis Autosampler from Spark (Emmen, Netherlands) equipped with a 100 µl loop. A four-channel degasser was integrated into the Rheos CPS LC system. The LC-MS/MS apparatus was a LCQ Advantage MAX from Thermo Finnigan (Allschwil, Switzerland) equipped with an APCI device operating in the positive detection mode. The chromatographic separation was performed on a Synergi MAX-RP 80A C_{12} column (length 2 mm × 75 mm, i.d. 4 µm) from Brechbuehler (Zuerich, Switzerland). The mobile phase was delivered at a flow rate of 400 µl/min. Each chromatographic run was performed with a binary, linear A/B gradient (Solvent A was 10 mM ammonium formate, pH 3.0; Solvent B was 90% acetonitrile, 10% 10 mM ammonium formate, pH 3.0). The program was as follows: 0 min: 50% B; 1–12.0 min: 50–79% B; 12.01–12.50 min: 79–95% B; 12.51–15.00 min: 95% B; 15.01–25.00 min: B linear from 95 to 50%. All solvents were degassed before usage.

The following atmospheric pressure chemical ionisation inlet conditions were applied. The heated vaporiser was kept at 465 degrees Celsius. Both, the sheath gas and the auxiliary gas were nitrogen set at 60 and 15 relative units, respectively. The capillary

entrance to the ion trap was at an offset of 10 V in the positive mode and was maintained at 220 °C. The corona current was 5 µA. For quantification, the selected ion monitoring mode was used. *Table 1* lists the precursor and product ions and the relative collision energy for each analyte.

Table 1: Precursor and product ions and the relative collision energy for each analyte

Substance	Mass APCI $[M-H]^+$ (*m*/*z*)	Product ions $[M-H]^+$ (*m*/*z*)	Collision energy (%)
CBN	311.2	293.2	34
CBD	315.1	259.2	34
THC-COOH	345.2	327.1	30
11-OH-THC	331.0	313.2	34
THC	315.2	259.1	38

2.5. LC-MS/MS assay validation

2.5.1. Linear measuring range, quantification, lower limit of quantification (LLOQ), lower limit of detection (LLOD), quality controls, precision, accuracy and recovery

2.5.1.1. Linear measuring range

The *linearity* of the measuring range was assessed with standard curves ranging from 0.2 to 100 ng/ml (0.2; 0.3; 0.5; 1.0; 2.0; 3.0; 5.0; 10.0; 25.0; 50.0; 100.0 ng/ml) in bovine serum and human urine and analysed using the described LC-MS/MS method. Standard response curves were generated using a weighted (1/*x*) linear regression model.

2.5.1.2. Quantification

The concentrations of the analytes were calculated by comparing the peak area (%) of an analyte with the corresponding area (%) on the standard curve. System variations were adjusted by comparing the areas (%) of the internal standards. The internal standards were THC-d3 for EDTA-plasma and THC-COOH-d3 for urine.

2.5.1.3. The lower limit of quantification (LLOQ) and detection (LLOD)

Bovine serum and human urine samples spiked with decreasing concentrations of the analytes were analysed in order to determine LLOQs and LLODs. Samples of each concentration were extracted and analysed five times. The LLOD was set at the lowest concentration where the signal of the compound was three standard deviations higher than the background noise. The LLOQs were determined by measuring five samples per concentration for all substances. The LLOQ was defined as the lowest concentration of THC and its metabolites which can still be determined with a precision <10% (CV).

2.5.1.4. Precision

Intra-assay precision was determined by replicate (n = 4) analysis of the QC samples in one run for both fluids.

Inter-assay precision was determined by replicate analysis of the QC samples in several experiments performed on different days. Eight urine- and five EDTA-plasma samples were measured. A comparison with the nominal concentrations of the QC samples was used to assess the accuracy (bias) of the method.

2.5.1.5. Accuracy and recovery

Accuracy was committed not to be >±20% of the nominal concentration.

Recovery was estimated in both matrices by comparing the response of a blank human urine or a blank bovine serum spiked with 100 ng/ml analyte before the extraction step (*n* = 4) with the response obtained when the analyte was added to the corresponding matrix after the extraction procedure (*n* = 4). The deuterated internal standard was added in all samples after the extraction step under both conditions.

2.5.2. Sample stability

The stability of the cannabinoids during storage was monitored in human EDTA-plasma (THC) and human urine samples spiked with the initial concentration of 100 ng/ml for all measured substances. The concentrations in the samples were determined immediately after spiking (control samples, *n* = 3). Aliquots of the same samples were filled into silanised glass vials, closed with chlorbutyl gum stoppers and aluminium seals. The tubes were frozen at -70 ° C. The urine samples were thawed and measured (*n* = 3) after 62 and 133 days, the EDTA-plasma samples after 21 and 41 days.

For the evaluation of the freeze/thaw stability, the spiked sample (same sample material as above) was analysed before (control samples, *n* = 3) and after three freeze/thaw cycles (stability samples, *n* = 3). For each freeze/thaw cycle, the sample was frozen at -70 °C for 24 h, thawed, extracted a nd measured.

2.5.3. Assessment of matrix effects

To assess any possible suppression or enhancement of ionisation due to the sample matrix, four types of experiments were performed. In the first experiment blank samples from the test persons used as negative controls were analysed. The second test was to analyse blank bovine serum. The third trial included the evaluation of the matrix as described by Matuszewski et al. [94]. This assessment includes the recovery and process efficiency. For this test three sets of samples are necessary. Set A consists of aqueous standard solutions (100 ng/ml). For set B blank matrices are supplemented (after extraction) with the same amount of standards as used for set A. Set C consists of extracts of different blank matrices, supplemented with the same amount of standards but added before extraction. Recovery was calculated (for the calculation the resulting peak areas are needed) with the following formula: RE% = C/B × 100. Matrix effects were calculated with the formula: ME% = B/A × 100. Process efficiency was calculated with the formula: PE% = C/A × 100. The difference in analyte concentration not more than 50% was acceptable for all three tests.

The last test provided a continuous post-column infusion [121] of an analyte in a chromatographic run of an extract or a blank matrix. This procedure is based on the post-column infusion of an analyte in a chromatographic run of an extract or a blank matrix. This signal is compared to the signal obtained with the post-column infusion of the same model analyte in a chromatographic run with eluent only. This procedure indicates also critical areas in the chromatogram.

3. Results and discussion

An example of a typical chromatogram of 100 ng/ml of the analytes in bovine serum is shown in *Figure 1*. The retention times were 13.80 ± 0.08 min for THC, 7.38 ± 0.08 min for 11-OH-THC, 7.75 ± 0.09 min for THC-COOH, 11.27 ± 0.08 min for CBD and 13.00 ± 0.15 min for CBN. The total run time for each sample was 25 min. Acceptable retention times were considered to be within a time window of ± 0.5 min.

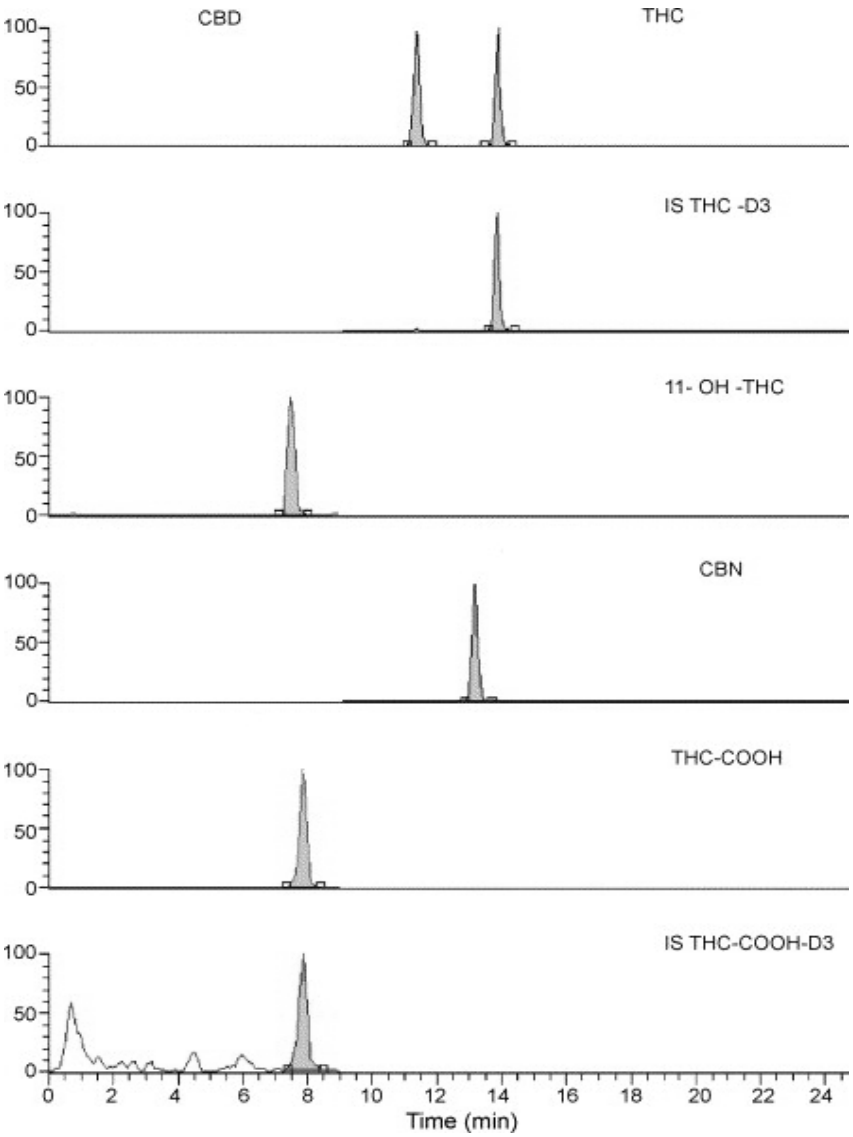

Figure 1: LC-MS/MS chromatogram of 100 ng/ml of the analytes in bovine serum.

3.1. Linearity

The linear measuring range was assessed with calibration curves ranging from 0.2 to 100 ng/ml in bovine serum and human urine. *Figure 2* shows an example of a standard response curve of each analyte in bovine serum generated using a weighted (1/*x*) linear regression model. The correlation coefficients (R^2) in the diagrams were ≥0.994 with one acceptable exception of 0.979.

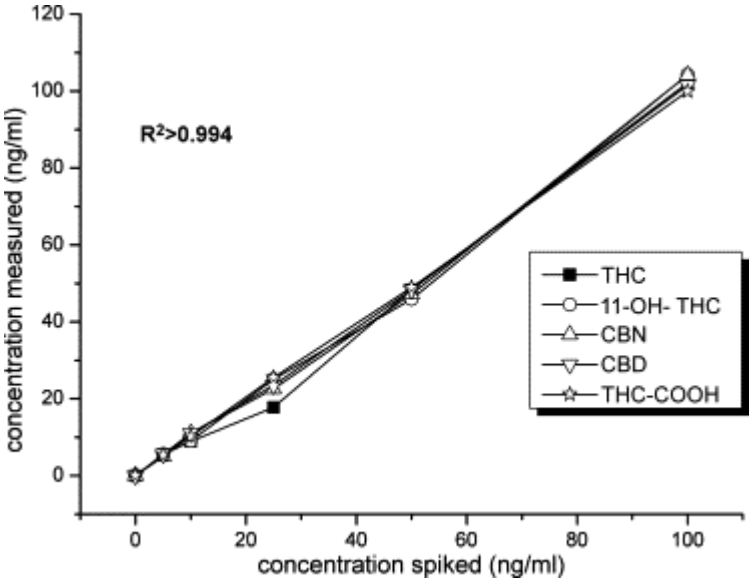

Figure 2: Example of a standard response curve of each analyte in bovine serum.

3.2. The lower limit of quantification (LLOQ) and detection (LLOD)

The limit of quantification in EDTA-plasma was 0.2 ng/ml for CBN, THC, THC-COOH, CBD and 11-OH-THC, in urine 3 ng/ml for CBN, 1 ng/ml for CBD, THC and THC-COOH and 2 ng/ml for 11-OH-THC.

The limit of detection in EDTA-plasma for all substances was 0.1 ng/ml. The LLODs in urine were 0.5 ng/ml for THC, CBD and THC-COOH and 1 ng/ml for CBN and 11-OH-THC.

3.3. Accuracy and intra- and inter-assay precision

Results of the accuracy, intra- and inter-assay precision studies are presented in *Table 2* and *Table 3*. The deviation of accuracy of the intra-assay study in EDTA-plasma (THC) and urine (THC-COOH) did generally not exceed 18% (accepted 20%).

Table 2: Results of the intra-assay precision

	Intra-assay precision					
	Urine (THC-COOH)			EDTA-plasma (THC)		
Number of measurements (*n*)	4	4	4	4	4	4
Mean (ng/ml)	28.5	65.7	132.5	3.0	8.2	24.9
Nominal concentration (ng/ml)	37.5	65.0	125.0	3.0	10.0	25.0
S.D. (ng/ml)	3.0	0.9	1.7	0.2	0.4	1.9
CV (%)	10.5	1.4	1.3	8.8	5.2	7.9
Accuracy (%)	24.0	1.0	6.0	0.0	18.0	0.4

S.D.: Standard deviation; CV: coefficient of variation.

Table 3: Results of the inter-assay precision

	Inter-assay precision (ng/ml)					
	Urine (THC-COOH)			EDTA-plasma (THC)		
Number of measurements (*n*)	8	8	8	5	5	5
Mean (ng/ml)	27.8	64.7	132.6	2.9	8.8	27.0
Nominal concentration (ng/ml)	37.5	65.0	125.0	3.0	10.0	25.0
S.D. (ng/ml)	4.3	4.2	3.9	0.6	1.1	1.6
CV (%)	15.5	6.5	1.9	11.8	12.8	7.9
Accuracy (%)	26.0	0.4	5.6	3.3	12.0	8.0

S.D.: Standard deviation; CV: coefficient of variation.

The deviation in the lowest concentrated urine control sample was 24% (intra) and 26% (inter) which exceeds the acceptable value (20%). This fact was ignored because of possible method differences in the choice of the nominal concentrations and a good precision (with coefficients of variation of 10.5% (intra-assay) and 15.5% (inter-assay)). In both matrices (EDTA-plasma and urine) the intra- and inter-assay precision displayed by the CV was much better than 20% (defined acceptable CV by the authors). CVs were in the range 1.3 - 10.5 (intra-assay) and 1.9 - 15.5 (inter-assay).

3.4. Stability

In urine, the substance stability tests at a temperature of -70 °C in silanised glass vials showed that the concentrations of each cannabinoid with the exception of THC did not decrease more than 20% after 5 months (mean of three measurements). THC concentration showed a decrease of 26%. In EDTA-plasma, the THC concentration decreased steadily during the observation period with a decrease of 30% after 21 days and 43% after 41 days.

3.5. Assessment of matrix effects

Blood and urine samples from the test persons were taken immediately before the administration of the cannabis drug. The samples were used as negative controls and to compare the baseline chromatograms with those after the drug application. No peaks with measurable areas were found in the chromatograms (data not shown).

3.6. Blank bovine serum used as negative controls

Blank bovine serum was measured to compare the chromatograms with the spiked bovine serum samples used for the standard curves. No peaks with measurable areas were found in the blank bovine serum chromatograms (data not shown). *Table 4* shows the recovery, matrix effects and process efficiency of the analytes. No statement about value acceptability was described by Matuszewski et al

Table 4: Recovery, matrix effect and process efficiency

	Recovery (%)		Matrix effects (%)		Process efficiency (%)	
	Bovine serum	Urine	Bovine serum	Urine	Bovine serum	Urine
THC	77.5	78.7	97.6	87.9	70.7	70.3
11-OH-THC	77.6	63.4	117.6	154.2	91.5	142.6
CBD	71.4	70.0	84.2	93.1	60.0	69.5
CBN	47.7	78.1	73.4	85.1	34.7	68.7
THC-COOH	50.0	61.4	172.9	283.8	87.5	173.9

Parameters were determined according to Matuszewski et al. [94].

3.7. Recovery

All studies show enough recovery (%) for each substance in both matrices (range 47.7 - 78.7) in relation to the sensitivity results (LLOD/LLOQ).

3.8. Matrix effects

This study showed the low influence on the qualitative and quantitative determinations, which was confirmed by the test after Matuszewski *(Table 4)*. The range of this study was 73.4 - 283.8% and shows a common effect for several substances in different matrices on the extraction rate over 100% yield compared to aqueous standards.

3.9. Process efficiency

The sensitivity for the determination of the substances is high enough for the analysis in the samples of the provided trial. This is due to overall good process efficiency. One exception was CBN because of a low recovery (47.7%), bad process efficiency (34.7%) and a relatively high LLOD.

3.10. Post-column infusion after Bonfiglio et al. [121]

The post-column infusion (100 µl/min) of the cannabinoids in a chromatographic run of eluent only, indicated no critical areas in the chromatograms compared with those of blank human urine and bovine serum. No critical area around the retention time of THC or the other analytes was detected (data not shown). No change in the ionisation process (enhancement, suppression of the ionisation) of an analyte due to a co-eluting compound was found.

3.11. An example of determination after administration of cannabinoids to human subjects

The described method was applied to determine the pharmacokinetics of THC and its metabolites after administration of either 20 mg synthetic THC (Marinol™) or an extract from *Cannabis sativa L*. (capsule containing 20 mg of cannabinoids) to the same healthy male volunteer. *Figure 3* and *Figure 4* show that the metabolites are detectable for at least 24 h. Except after the intake of THC, no CBD was detectable in the EDTA-plasma.

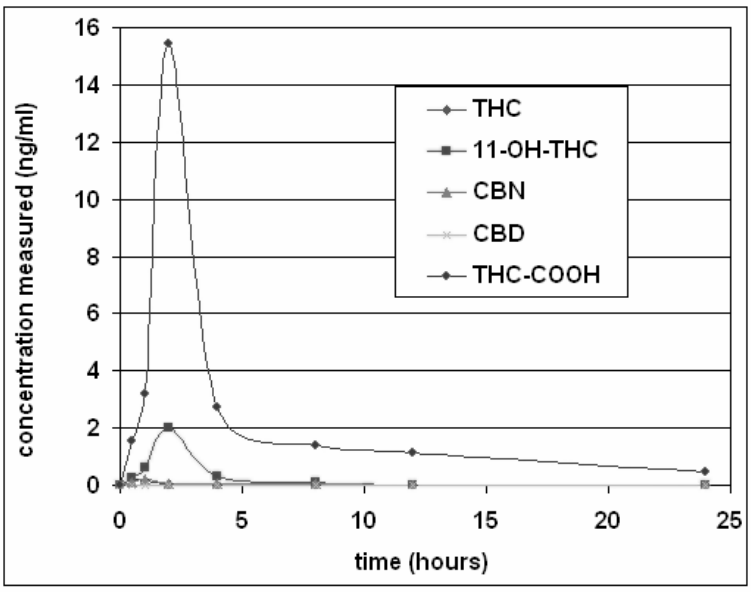

Figure 3: Plasma concentration time curve of THC and its major metabolites as well as CBD and CBN after the intake of 20 mg synthetic THC (Marinol™) in one healthy male subject.

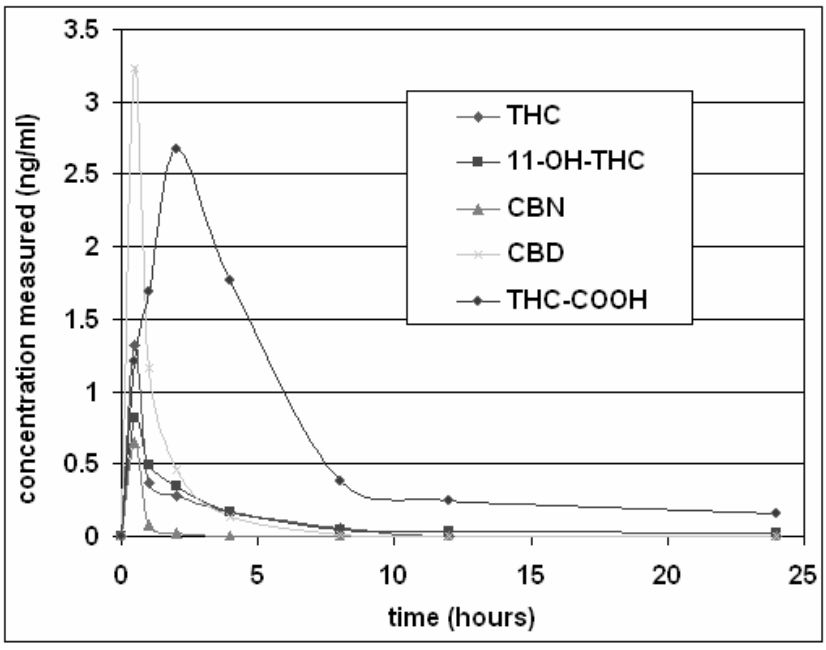

Figure 4: Plasma concentration time curve of THC and its major metabolites as well as CBD and CBN after the intake of a Cannabis sativa L. extract (containing 20 mg of cannabinoids) in one healthy male subject.

The range of the measured cannabinoid concentrations is within 0 - 16.0 ng/ml. The consumption of THC (Marinol™) shows higher concentrations compared to those after the administration of the herbal extract.

3.12. Comparison with other methods

The analysis of plasma and urine samples for cannabinoids by GC/MS is daily routine in forensic toxicology laboratories. Normally, the investigations are limited to THC, 11-OH-THC and THC-COOH and detection limits of 1 ng/ml are sufficient. However, in the present study, CBD and CBN had to be included and a detection limit below 0.5 ng/ml was necessary in order to follow up the concentrations for a sufficiently long time after drug administration.

Only 2 - 2.5 ml plasma per sample were available. Therefore, the method, particularly the extraction, had to be adapted to these requirements before application to the study.

The described LC-MS/MS method allows the simultaneous analysis of five cannabinoids from urine or plasma in the same run over 25 min. There are several other methods published in the literature so far. Most reported methods detect only one or two metabolites from one matrix. Another important issue of cannabinoid analytics, especially for forensic purposes, is a high sensitivity. The described method has a LLOQ of 0.2 ng/ml and is more sensitive than previously described analytical methods used in pharmacokinetic trials. Most of those reported methods show LLOQs >5 ng/ml.

Only one GC/MS method reported likewise the simultaneous analysis in plasma of THC, 11-OH-THC, CBN, CBD and THC-COOH. This method had a slightly lower

sensitivity with a LLOQ of 0.5 - 3.9 ng/ml and had a run time of 13 min [122]. However, this method is more elaborate than ours.

Compared to other reported GC/MS methods [108-111] for estimation of THC or its metabolites, definite advantage of our LC-MS/MS method is a gain of time. There is no need to do an elaborate sample preparation and to use various derivatisation techniques for non-volatile and thermolabile compounds like in GC/MS.

4. Conclusions

We developed and validated a sensitive and selective method for the determination of THC, its major metabolites as well as CBD and CBN in human EDTA-plasma and urine, using high-performance liquid chromatographic separation with tandem-mass spectrometry detection which showed a satisfactory overall analytical performance well suited for applications in medical science. With a LLOQ of 0.2 ng/ml (EDTA-plasma), pharmacokinetic profiles of the drugs and their concentrations could be determined for up to 24 h after a single oral administration of a *Cannabis sativa L*. extract capsule of 20 mg THC_{tot} *(Fig. 3 and Fig. 4)*. The stability tests showed a constant decrease of THC in urine and of all parameters in EDTA-plasma. This decrease does not exceed 10% if the samples are analysed within a short time period. This method is very efficient because it permits the measurement of low concentrations and the simultaneous quantification of five analytes.

3.2.

Sensitivity and specificity of urinary cannabinoid detection with two immunoassays after controlled oral administration of cannabinoids to humans

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Submitted to: Therapeutic Drug Monitoring

Received December 2007; accepted Mai 2008

Abstract

Background: For forensic and clinical toxicological purposes, cannabis consumption is screened using easy to handle immunoassays. The sensitivity and specificity of these immunoassays have not yet been established in samples from volunteers receiving oral synthetic THC or cannabis extracts, using tandem mass spectrometry (LC-MS/MS) as the reference method.

Methods: Urine samples were collected in an open, randomised, single-center, three-periods cross-over study including 18 healthy male volunteers, given either 20 mg synthetic THC (Marinol^{TM}) as a control substance or 5 different types of *Cannabis sativa L.* extracts. The study was performed according to the guidelines set forth in the Declaration of Helsinki at the University Hospital Basel, Switzerland. Only male volunteers were accepted, they had to be cannabis-naïve with a good ability to understand German. The subjects were informed about the aims, methods, anticipated benefits, potential hazards and discomfort the study may entail, as well as the subjects right to abstain from participating in the study and to withdraw their consent at any time. They gave their written informed consent that has been approved by the local State Ethics Committee of the two cantons Basel (EKBB) and were paid for participating. There were a lot of exclusion criteria all of which will be published in an upcoming paper.

All urine samples were collected and frozen at -70 °C until analysis. Urinary 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) excretion was evaluated by two immunoassays (CEDIA, FPIA) using the National Institute on Drug Abuse (NIDA) and a Substance Abuse and Mental Health Service (SAMHSA) set general immunoassay cut-off value of 50 ng/ml as well as an approved LC-MS/MS method [123] with a cut-off value of 15 ng/ml. The sensitivity, specificity and cut-off values were evaluated by a ROC-curve analysis. All urine samples were measured with and without enzymatic hydrolysis to analyse the reactivity in the two immunoassays and the concentrations of both THC metabolites (THC-COOH-

glucuronide, THC-COOH) with LC-MS/MS. At the same time the direct hydrolysation rate by the MS method in the ion source was determined.

Results: The ROC curve analysis indicated that the FPIA test discriminated better than the CEDIA test. The results show that the NIDA set general immunoassay cut-off of 50 ng/ml is possibly not applicable for analysis in samples from persons consuming the *Cannabis sativa L*. extracts orally instead of smoking. It has to be discussed whether a general cut-off value for the two immunoassays is applicable or not. LC-MS/MS is an excellent confirmation method for the continuation of immunoassay results, allowing the detection of many cannabinoids. The enzymatic hydrolysis method showed a nearly 100% transformation of THC-COOH-glucuronide to THC-COOH.

Conclusions: The use of cannabinoid-based therapeutics and continued abuse of oral cannabis require scientific data for accurate interpretation of cannabinoid tests and for establishing a reliable administrative drug-testing policy. Preliminary positive immunoassay results have to be confirmed by a second, more specific analysis method such as GC-MS or LC-MS. For correct quantitative results of the analytes, it is important to include the glucuronides in the analysis. From our data it is not possible to set a general method-independent cut-off value for immunoassays when cannabinoids are consumed orally as extracts.

1. Introduction

Laboratory analyses of cannabinoids or other drugs have forensic, criminal and clinical applications. Urine, blood, saliva and hair are the common matrices for the detection of drugs. The collection of urine is easy. The drugs and their metabolites found in urine can be detected for a longer time frame after drug ingestion compared to blood or saliva [55]. A hydrolysation of the urine before the analysis, to convert drug conjugates (glucuronides) to their free metabolites, is advantageous for several analytical techniques. The rate of false positive or false negative results

should be minimised in an analytical method. To achieve this objective, two steps are necessary. To identify positive specimens a screening step has to be performed. The principle of an immunoassay test is the use of antibodies binding to the drug, but its selectivity is limited by the specificity of the antibodies used in the immunoassay. False positive results can be caused if substances chemically similar to the drug or its metabolites interfere in the test. A sample concentration below the accepted threshold, or a dilution or adulteration of the sample to obscure the presence of a drug, can lead to false negative results. Unfortunately, in a screening test often cross-reacting substances and different cross-reactivities with metabolites often have to be considered [56]. Therefore, preliminary positive immunoassay results have to be confirmed by a second, more specific analysis method such as mass spectrometry (MS) [55, 57].

Urine contains creatinine, a metabolic waste product removed from muscle tissue. It is filtered from the blood by the kidney and excreted without reabsorption in the urine. For measuring drugs excreted into the urine, the drug-to-creatinine ratio is often used to normalise different measurements if they have to be compared [124].

The principal psychoactive component of *Cannabis sativa L.* is ∆9-tetrahydrocannabinol (THC). After intake in humans, 11-hydroxy-∆9-tetrahydrocannabinol (11-OH-THC) is the major psychoactive THC metabolite formed. Due to its pharmacokinetic properties (high concentration) [125-127], the non-psychotropic 11-nor-∆9-tetrahydrocannabinol-carboxylic acid (THC-COOH) is usually accepted as the metabolite for drug testing with immunoassays, disregarding the long half-life of about 30 h. However, this long half-life renders this metabolite questionable for testing in shortly after drug intake. THC-COOH and its glucuronide are the main metabolites in urine.

To assess the sensitivity and specificity of two routinely used semi-quantitative immunoassays (CEDIA: Microgenics, FPIA: Abbott Laboratories) for urinary THC-COOH determination, human urine samples from a study were analysed by these two immunoassays, using a cut-off value of 50 ng/ml and by tandem mass-

spectrometric analysis (LC-MS/MS) with a cut-off value of 15 ng/ml [123]. The 18 healthy male volunteers received either 20 mg synthetic THC (Marinol^{TM}) as a control substance or 5 different types of *Cannabis sativa L.* extracts containing 20 mg THC_{tot}.

The objectives of the present study were to compare the results of these two immunoassays with our quantitative LC-MS/MS method that was used as the gold standard. Specific objectives were a) to discuss the SAMHSA set THC carboxylic acid cut-off for immunoassays in the special samples of this trial, b) to determine the cross-reactivity of the sum of THC-COOH-glucuronide and THC carboxylic acid with both methods, before and after hydrolysis, c) to assess the influence of other metabolites on the immunoassay results, and d) to assess, whether the measurements of THC-COOH only, without considering other cannabis metabolites, as it is the standard in all urine drug of abuse screening procedures, will be valuable too after oral intake of *Cannabis Sativa L*. extracts with lower THC concentrations and possibly different metabolism.

2. Materials and Methods

2.1. Chemicals

THC and metabolite reference material were obtained from Lipomed (Arlesheim, Switzerland). All solvents (acetonitrile, isopropanol 20%, ammonia solution 25%, diethylether, ethylacetate, acetic acid 100%, formic acid 98%) and chemicals (ammonium formate, potassium dihydrogen phosphate) were analytical grade and were purchased from Merck (Darmstadt, Germany) and Aldrich (Buchs, Switzerland). Urine quality control samples were purchased from Biorad (Reinach, Switzerland) for LC-MS/MS. The reagents, quality controls and calibrators for the immunoassays were obtained from Microgenics (Fremont, CA, USA) for CEDIA

immunoassay and from Abbott (Abbott Park, IL, USA) for AxSYM THC-COOH immunoassay. Silanised glass vials (type I plus) were purchased from Schott (Muellheim-Huegelheim, Germany) and 6 ml glass test tubes from Gilson (Mettmenstetten, Switzerland). De-ionised water was generated with a Milli-Q water purification system from Millipore (Kloten, Switzerland). β-glucuronidase was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The buffer (pH 6.0, 0.1 mol/L) was prepared from a KH_2PO_4 solution (13.61 g in 950 ml water), adjusted to the desired pH by appropriate addition of KOH 1 mol/L and adjusted to 1 L with water.

2.2. Specimens, standard solutions, calibration standards and quality controls

Stock THC and metabolite standard solutions were prepared in acetonitrile/H_2O (50/50, v/v). (The concentrations were 100 mg/l for THC, 11-OH-THC, cannabidiol (CBD), cannabinol (CBN), THC-COOH and 10 mg/l (v/v) for THC-d3, THC-COOH-d3). The standards were used to spike a blank urine matrix (Biorad liquicheck urine toxicology control, level 0). Calibration standards for assay calibration and determination of the linear measuring range were prepared in human urine by spiking with the necessary amount of the standard solutions to obtain a linear calibration curve in the range of 0.5 -100 ng/ml of THC and its metabolites.

To determine the performance of the method, the intra- and inter-assay precision and the accuracy were assessed for LC-MS/MS [123]. Four commercially available urine quality controls for THC-COOH were used (Biorad liquicheck urine toxicology controls, level 0, 1, 2, 3). For the FPIA, THC assay quality controls from Abbott (Abbott Park, IL, USA) were used (low and high). The CEDIA THC assay uses the following quality control levels: THC 25 (low, high), THC 50 (low, high) and THC 100 (low, high) from Thermo Fisher (Allschwil, Switzerland). For the analysis of

creatinine, quality controls from Microgenics (MAS Urichem TRAK, levels 1 and 2) were obtained.

2.3. Extraction procedure

First, a hydrolysis of THC-COOH-glucuronide to THC-COOH was performed. To 2.5 ml urine, 1 ml phosphate buffer (pH 6.0; 0.1 mmol/L) was added, mixed and supplemented with 50 µl β-glucuronidase. After vortexing, the sample was incubated at 50 °C for 3h. The liquid–liquid extra ction (LLE) procedure for urine was carried out in glass tubes (off-line). One ml phosphate buffer (0.1 M, pH 6.0) and 1 ml of acetic acid 0.1 mol/L were added to 2 ml of the de-glucuronised urine. After vortexing, 4 ml organic phase (diethylether/ethylacetate 50% (v/v)) was added. After mechanical shaking (10 min) and centrifugation (10 min at 2000 × *g*), 3 ml of the organic phase was transferred to a 6 ml glass test tube and then evaporated to dryness at 37 °C under nitrogen. The extracts were reconstituted in 60 µl of mobile phase (40% mobile phase A, 60% mobile phase B), 10 µl internal standard THC-COOH-d3 (10 mg/l in acetonitrile 50% (v/v)) was added, mixed and 50 µl injected into the LC-MS/MS system.

The internal standard was added in order to overcome possible variations of the concentrations in the extraction step and in the LC/detection system. Possible deviations of the extraction rates were overcome by treating the standards in the same way as samples and by calibrating each series of analyses. For strict scientific reasons the use of a deuterated THC-COOH-glucuronide internal standard would have been preferred. At the moment, there is no commercial THC carbonic acid glucuronide available which is stable enough and we did not have the possibility to produce ourselves. Because of the direct hydrolysis in the LC-MS system we did not investigate the analysis of another glucuronidated internal standard (see 3.1.).

All urine samples were measured with and without enzymatic hydrolysis to compare the total concentration of THC-COOH with the concentration produced by a spontaneous hydrolysis in the ion source of the LC-MS/MS detector.

2.4. Human urine

Human urine was collected from healthy male volunteers at the following time points: 0, 4, 12, 24, 48, 72 h. The samples were sealed and stored at -70 °C in silanised glass vials until measurement. As reported previously [123], the limit of quantification in urine with the LC-MS/MS method was 3 ng/ml for CBN, 1 ng/ml for CBD, THC and THC-COOH and 2 ng/ml for 11-OH-THC. The limit of detection in human urine was 0.5 ng/ml for THC, CBD and THC-COOH and 1 ng/ml for CBN and 11-OH-THC.

2.5. Immunoassays

The CEDIA assay for THC uses recombinant DNA technology to produce an enzyme immunoassay system. The antibodies are monoclonal. The SAMHSA set general cut-off is 50 ng/ml. The accuracy by recovery is guaranteed up to a concentration of 100 ng/ml. The CEDIA Microgenics immunoassay for THC-COOH was from Thermo Fisher (Passau, Germany). Enzymatic creatinine was obtained from Wako (Neuss, Germany). Both assays were performed on a Roche Hitachi 917 system (Indianapolis, IN, USA).

The FPIA THC assay uses a sheep polyclonal antibody. The SAMHSA set general cut-off of this assay is 50 ng/ml. The accuracy by recovery is guaranteed up to a concentration of 135 ng/ml. The FPIA assay was from Abbott Laboratories (Abbott Park, IL, USA) and performed on an AxSYM analyser (Abbott Park, IL, USA). For

both immunoassays only a few cross-reactions with THC metabolites are known and presented in the package insert.

2.6. LC-MS/MS

The LC-MS/MS analysis was performed with a validated high-performance liquid chromatographic method with tandem-mass spectrometric detection, as previously reported [123]. In brief: the chromatographic system consisted of a Rheos 2000 Micro HPLC pump (Thermo Fisher, Allschwil, Switzerland) and a Midas Symbiosis Autosampler from Spark (Emmen, Netherlands) equipped with a 100 µl loop. A four-channel degasser was integrated into the Rheos CPS LC system. The LC-MS/MS apparatus was a LCQ Advantage MAX from Thermo Fisher (Basel, Switzerland) equipped with an APCI device operating in the positive ionisation mode. The chromatographic separation was performed on a Synergi MAX-RP 80A C_{12} column (length 2 mm × 75 mm, i.d. 4 µm) from Brechbuehler (Zuerich, Switzerland). The mobile phase was delivered at a flow rate of 400 µl/min. Each chromatographic run was performed with a binary, linear A/B gradient (Solvent A was 10 mmol/L ammonium formate, pH 3.0. Solvent B was 90% acetonitrile, 10% 10 mmol/L ammonium formate, pH 3.0). The program was as follows: 0 min: 50% B; 1 - 12.0 min: 50 - 79% B; 12.01 - 12.50 min: 79 - 95% B; 12.51 - 15.00 min: 95% B; 15.01 - 25.00 min: B linear from 95 to 50%. All solvents were degassed before use.

The following APCI inlet conditions were applied: The heated vaporiser was kept at 465 °C. Both, the sheath gas and the auxiliary gas were nitrogen set at 60 and 15 relative units, respectively. The capillary entrance to the ion trap was at an offset of 10 V in the positive mode and was maintained at 220 °C. The corona current was 5 µA. For quantification, the selected ion monitoring mode was used.

2.7. Sensitivity, specificity and cut-off value

For the determination of the sensitivity and specificity of the two immunoassay results, LC-MS/MS determinations of cannabinoids in urine after hydrolysis were performed using the chromatographic method as the gold standard. Sensitivity and specificity of the immunoassays without hydrolysis were calculated as:

$$sensitivity = \frac{number\ of\ \ true\ \ positives}{number\ \ of\ \ true\ \ positives + number\ \ of\ \ \ false\ negatives}$$

$$specificity = \frac{number\ of\ \ true\ negatives}{number\ \ of\ \ true\ negatives + number\ \ of\ \ \ false\ \ positives}$$

The cut-off value was determined as the limit for decisions (yes/no) as to whether a result should be interpreted as positive or negative. The recommendations of SAMSHA for cut-off values for cannabinoid analysis are 50 ng/ml (FPIA, CEDIA) and 15 ng/ml (LC-MS/MS). These cut-offs are set after studies indicating a very low probability giving false positive results after passive inhalation of cannabinoids. All these cut-off values are based on experimental work and not by pharmacokinetic studies of drug excretion.

2.8. ROC curve analysis

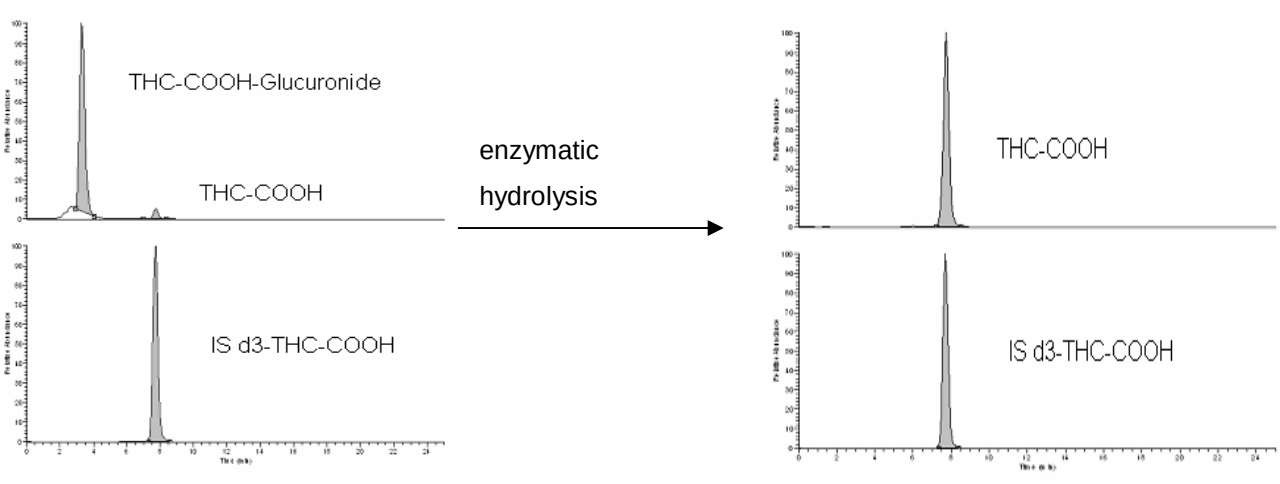
In signal detection theory, a receiver operating characteristic (ROC-curve), is a graphical plot of the sensitivity vs. (1 - specificity) for a binary classifier system as its discrimination threshold is varied. The ROC curve can also be represented equivalently by plotting the fraction of true positives vs. the fraction of false positives [128, 129].

Sensitivity and specificity of the immunoassays were assessed by a ROC analysis. For this analysis each sample was classified by its THC-COOH LC-MS/MS result after hydrolysis as the gold standard. This classification was compared to the classifications of results obtained by two immunoassays (CEDIA, FPIA) using the SAMHSA cut-offs. If the concentration was above the cut-off value of 15 ng/ml (LC-MS/MS), the sample was classified to be positive for THC-COOH.

3. Results and discussion

3.1. Urine samples measured with and without enzymatic hydrolysis

Urine samples from the clinical trial after the intake of oral cannabinoids were measured using the LC-MS/MS, FPIA and CEDIA methods. All samples were stored at -70 ° C before analysis, thawed and measu red with and without hydrolysis. For the LC-MS/MS method the hydrolysis procedure showed a total transformation of the THC-COOH-glucuronide in the detection system confirmed by the nearly 100% agreement of the total concentrations after hydrolysis and the sum of free THC-COOH in the un-hydrolysed samples. *Figure 1* shows an example of an extracted urine sample measured before and after enzymatic hydrolysis by LC-MS/MS. The THC-COOH-glucuronide in contrary to free THC-COOH is nearly not retained in the HPLC separation column and is presumably totally hydrolysed in the vaporisation step at the entrance of the APCI nozzle because of the high temperature of about 500 ° C. Otherwise there would not be any signal with the mass spectrum of THC-COOH (main and daughter ions, *Figure 1*). The two immunoassays showed a nearly 100% cross-reactivity with the glucuronide showing no difference between the results before and after hydrolysis.



THC-COOH-glucuronide:	1021.16 pmol/ml	THC-COOH-glucuronide:	0.00 pmol/ml
THC-COOH:	105.16 pmol/ml	THC-COOH:	1101.30 pmol/ml
Total:	1126.32 pmol/ml	Total:	1101.30 pmol/ml

Figure 1: One urine sample measured with and without enzymatic hydrolysis with LC-MS/MS.

3.2. Sensitivity, specificity and cut-off values

In *Table 1*, the results in agreement, as well as the false positive and false negative sample numbers are listed. Both immunoassays showed false negative and false positive results. The CEDIA test showed more discrepancies than the FPIA test. Nearly no quantitative difference in the number of false positive/negative values was determined between the pure and de-glucuronised samples in all tests.

Table 1: Sensitivity and specificity data for 50 ng/ml (CEDIA/FPIA) and 15 ng/ml (LC-MS/MS) cut-offs.

		LC-MS/MS HYDROLYSED URINE (THC-COOH TOTAL)		LC-MS/MS NATIVE URINE (THC-COOH+THC-COOH-GLUCURONIDE)	
		negative	positive	negative	positive
CEDIA	neg	105	34	105	34
	pos	22	164	27	160
CEDIA hydrolysed	neg	104	57	114	56
	pos	17	146	21	141
FPIA	neg	100	18	102	16
	pos	16	171	22	165
FPIA hydrolysed	neg	102	19	102	18
	pos	14	179	19	172

CEDIA: Cloned enzyme donor immunoassay

FPIA: Fluorescence polarisation immunoassay

Pos: positive (higher than 15ng/ml (LC-MS/MS)/50 ng/ml (Immunoassay))

Neg: negative (lower than 15ng/ml (LC-MS/MS)/50 ng/ml (Immunoassay))

3.3. ROC curve analysis

The following ROC curve shows the discrimination power of the two immunoassays. The ROC analysis in *Figure 2* indicates that the FPIA test discriminates better than the CEDIA test. Tests with lower performance tend towards the 45° diagonal (CEDIA) whereas those with good performance curves move upward and leftward (FPIA). With a probability of 94.7% in the FPIA test, any selected accidental value from the positive group is higher than any selected accidental value from the negative group. CEDIA had a probability of 88.6%. These results show that the SAMHSA set general immunoassay cut-off of 50 ng/ml is possibly not applicable for analysis of samples from persons consuming the *Cannabis sativa L.* extracts orally. At this cut-off the FPIA assay shows a sensitivity of 90.6% and a specificity of 86.2%, whilst the CEDIA assay shows a sensitivity of 82.8% and a specificity of 82.7%. Optimum cut-off values are then evident as being the best combined value for highest sensitivity when traded against specificity, i.e.

the test cut-off related to the top-left point on the graph. In this population any change for a higher sensitivity will decrease the specificity in both assays. This shows that it is impossible to fix a new general cut-off independent of the method. An explanation for these differences between the immunoassays in our study population could be an insufficient stability of the reagents in the CEDIA test. The laboratory of the University Hospital in Basel recognised a decrease of signals in the assays when kits were used shortly before their expiration date.

The high rate of false positive results could be explained by cross-reactivity of the immunoassays with metabolites not approved by the manufacturers of the kits and possible interactions with other substances. The tests are only developed to measure THC-COOH concentrations in the urine samples produced after smoking cannabinoids and not after oral consumption. But there are some other compounds with a similar chemical structure which cross-react with the test. In *Table 2,* cross-reactivity results of the parent compounds and metabolites are given for the CEDIA and FPIA THC assay.

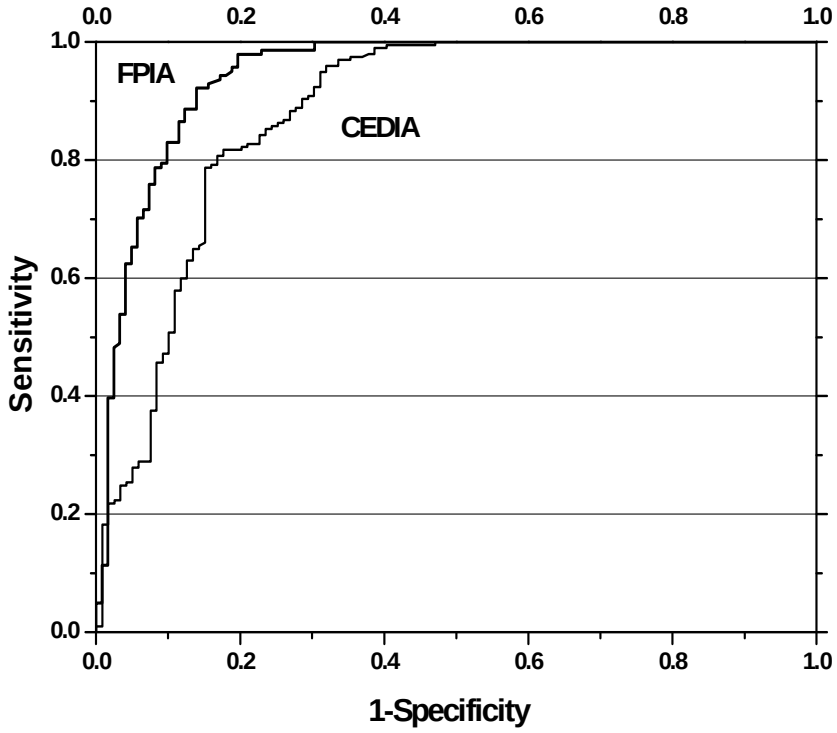


Figure 2: ROC curve of two immunoassays vs. LC-MS/MS with cut-offs 50 ng/ml (FPIA, CEDIA) and 15 ng/ml LC-MS/MS.

Table 2: Cross-reactivity of cannabinoids in the CEDIA and FPIA THC assay (Reproduced after the package insert of both tests)

compound	**tested (ng/ml)**	**% cross reactivity**	**test**
11-nor-Δ9-THC-COOH	50	100	CEDIA
11-nor-Δ8-THC-COOH	40	125	
Δ9-THC	500	10.4	
11-OH-Δ9-THC	125	43	
8β-OH-Δ9-THC	1000	2.8	
8β, 11-di-OH-Δ9-THC	5000	8.4	
1--Δ9-THC-glucuronide	62	78	
Cannabinol	1000	2.9	
Cannabidiol	1000	< 0.1	
11-nor-Δ8-THC-9-carboxylic acid	25		FPIA
11-OH-Δ9-THC	25		
Cannabinol	80		
Other metabolites	-	-	both

4. Conclusions

Our results show that LC-MS/MS is an excellent confirmation method for immunoassays allowing the detection of many cannabinoids.

Our results further suggest that it is important to include the THC-COOH-glucuronide in the urine analysis to get correct quantitative results of the analytes when measured by LC-MS/MS. In the described LC-MS/MS method, the glucuronide is automatically detected together with THC-COOH. The glucuronide is directly hydrolysed at the high tempered (500 °C) vaporisation step in the APCI nozzle in the detector.

We used the described enzymatic hydrolysis method with β-glucuronidase to confirm the total transformation of the THC-COOH-glucuronide to THC-COOH in the detector system and for trials with the immunoassays.

The cut-offs for the sum of urinary excretion products cross-reacting in the immunoassays from Microgenics CEDIA and Abbotts FPIA for the classification of oral cannabinoid intake do not seem to be optimal after intake of oral cannabinoids. Lower cut-off values for the immunoassays might increase the sensitivity but decrease the specificity, however, this has to be confirmed by further studies. From our results we can not recommend a cut-off for immunoassays for sample testing after the intake of cannabis extracts.

Both immunoassays are routinely used and established methods. A possible explanation for the higher than expected rate of false positive and false negative results for the measurement of THC-COOH by both assays could be that they usually detect the analyte in urine samples of patients inhaling the smoke of cannabis and the lower dose of THC in the extracts. The results described in this paper may result from the analysis of urine samples after oral intake of cannabinoids. An interesting aspect is that the immunoassay results (negatives and positives) correspond nearly

100% with the LC-MS/MS results of analysis in the same samples after cannabis smoking. It is possible that additional substances in the oral capsule formulations disturb the immunoassay tests.

The urine samples were derived from subjects receiving cannabis extracts orally instead of smoking THC containing cigarettes. We suppose that the concentrations of metabolites differ strongly depending on the route of application. The amount and appearance of different metabolites may disturb the immunoassay methods.

Another interesting aspect is that many concentrations in the MS positive/immunoassay negative population correspond with each other (47% in the MS positive/FPIA negative group and 70% in the MS positive/CEDIA negative group). Both tests often showed the same concentrations which actually seem to be optimal. The concentrations evaluated with the SAMHSA defined cut-off values, however, lead to a high rate of discrepancy of false negative/positive results.

3.3.

Relative Bioavailabilities of Different Oral Formulations of Extracts of *Cannabis sativa L*.

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To be published in two papers in 2008

Abstract

This review describes an open, randomised, single-center, three-periods cross-over phase I study including 18 healthy, male volunteers, with different, standardised *Cannabis sativa L*. extracts and Marinol™ as control substance. The best *Cannabis sativa L*. extract capsule formulation was evaluated for a possible future implementation as a new, concomitant medicament in cancer, HIV and AIDS therapy instead of a synthetic medication like Marinol™. The study was performed in two parts in parallel. In the first part, the heating-effect on the relative content of cannabinoids was investigated. In the second study part, the effect of *Cannabis sativa L*. extract capsule formulations, containing different concentrations of TPGS, on the bioavailabilities of different active cannabinoids was assessed. The plasma concentrations of the cannabinoids were assessed with an approved LC-MS/MS method. The study endpoints were a) to assess the relative bioavailability of ∆9-tetrahydrocannabinol and its metabolites defined as area under the plasma concentration/time curve from time T = 0 h extrapolated to infinity (AUC(0-∞)), b) to assess the relative tolerability and safety of six different oral formulations of 20 mg ∆9-tetrahydrocannabinol$_{tot}$ (∆9-tetrahydrocannabinol and ∆9-tetrahydrocannabinol acid A), c) to assess the effect of six different oral formulations of 20 mg ∆9-tetrahydrocannabinol$_{tot}$ on psychomotor function defined as simulator assisted evaluation of driving ability, d) to assess repetitive heart rate, blood pressure and a visual analogue scale (VAS) for sedation. The pharmacokinetic of the cannabinoids was highly variable between the subjects. Due to this variability, no statistically significant differences between the AUC of the different forms could be detected, neither in part I nor in part II of the study. There was a significantly different pattern of cannabinoids between the treatments. The extracts have a slightly lower bioavailability of total cannabinoids than Marinol™. However, the pharmacological effects seem to be comparable. TPGS addition leads to a qualitatively different pattern of cannabinoid metabolites. Addition of TPGS increases the bioavailability of cannabinoids up to about 135% compared to extracts without TPGS (=100%) without deteriorating tolerability. It can, therefore, be concluded that the administration of *Cannabis sativa L*. extracts is safe. The pharmacodynamic results are reported in a second, separate publications.

1. Introduction

Worldwide, the use of natural cannabis products for medical purposes is practically not allowed. In contrast, drugs containing synthetic cannabinoids like dronabinol (Marinol^{TM}), a synthetic THC are often exempt from these restrictions. The United States Food and Drug Administration (FDA) approved Marinol^{TM} to treat nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional treatments. Furthermore, the FDA approved Marinol^{TM} to treat appetite loss associated with weight loss in people with acquired immunodeficiency syndrome (AIDS). It was shown in recent studies that THC or cannabis preparations have a promising potential as a releasing factor, in moving disorders and as a pain reducer in patients suffering from multiple sclerosis.

In naturally grown *Cannabis sativa L*., up to 95% of the occurring total cannabinoids (THC_{tot}) are in the form of ∆9-tetrahydrocannabinolic acid A (THCA-A) [25]. By heating to 200 - 210 °C for 5 minutes, THCA-A is quantitatively decarboxylised to phenolic THC [7]. It is usually assumed that conversion to THC during smoking is complete, however, recently THCA-A was detected in blood and urine in samples collected from police controls of drivers suspected for driving under the influence of drugs [24]. The % of THCA-A / THC was 8-18% in these samples. Although THCA-A is described as pharmacologically inactive and devoid of psychotropic effects [7], reports of popular medicinal use of unheated cannabis or cannabis preparations show pharmacological effects often accompanied with a lower rate of adverse effects (anecdotal reports). It also possesses some anti-inflammatory and analgesic effects [8]. Recently, it was shown that unheated cannabis extract was able to inhibit tumor necrosis factor alpha in macrophage culture and peripheral marcrophages after LPS stimulation [9].

Although cannabidiol (CBD), a cannabinoid occurring in the natural grown cannabis plant, is devoid of psychotropic effects (e.g. euphoria, effects on cognition and behavior) it may have some beneficial effects (such as sedating, anticonvulsant, anti-inflammatory, and neuroprotective effects [29, 130-133]).

Plant extracts may be superior to administration of synthetic THC for treating medical diseases because of a well-balanced combination of active substances which can be found in the natural cannabis plant. Therefore, in the present study, the pharmacokinetics of five different *Cannabis sativa L*. extracts are compared with those after the oral administration of synthetic THC. Natural plant extracts of *Cannabis sativa L*. contain a large amount of THCA-A, which is transformed to THC by heating. Because of the potentially beneficial effect of THCA-A, four unheated cannabis extracts and only one heated extract were used in the clinical trial.

There are two problems of oral administrations of synthetic THC or *Cannabis sativa L.* extracts: the low oral bioavailability and large intra- and intersubject variability in the pharmacokinetics and pharmacodynamics. The low enteral absorption of cannabinoids may be a result of gastrointestinal efflux pumps in the intestinal mucosa (such as P-glycoprotein). Substrates of P-glycoprotein are very efficiently transported back from the enterocytes into the gut lumen immediately after absorption. These substrates usually show a limited and highly variable oral absorption. It was recently shown that THC and other cannabinoids are interacting with P-glycoprotein and with BCRP (another intestinal efflux pump) [134-137]. Therefore, it was a secondary objective of the present study to investigate, whether inhibition of P-glycoprotein function could enhance enteral absorption of cannabinoids and possibly decrease the pharmacokinetic variability. For this purpose, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS, Eastman, USA) was used. TPGS has been used as a solubilising agent and absorption enhancer in different oral drug formulations (e.g. cyclosporine) [12, 138-141]. However, up to now, the mechanism of the absorption enhancing effect is not yet clear. Besides improving solubility of lipophilic compounds, it is also a potent inhibitor of P-glycoprotein [12, 138, 141, 142].

Four different unheated *Cannabis sativa L*. extracts have been studied: Without and with 0.5, 5.0 and 7.5% TPGS to assess dose-dependent effects of TPGS.

2. Materials and methods

2.1. Subjects and study design

An open, randomised, single center, three-periods cross-over clinical phase I study with different *Cannabis sativa L*. extracts and Marinol™, a synthetic THC medication was performed to evaluate the best *Cannabis sativa L*. extract capsule formulation for a possible future implementation as a new, concomitant medication in cancer, HIV and AIDS treatment.

The study was performed according to the guidelines set forth in the Declaration of Helsinki at the University Hospital in Basel, Switzerland. The clinical trial was performed in two parts in parallel. Both parts were performed in nine healthy (interview to the past medical history; measurements of blood pressure, heart rate, temperature, height, weight and ECG; blood and urine baseline assessments), cannabis-naïve, male volunteers with a good ability to understand German, each. The subjects were informed about the aims, methods, anticipated benefits, potential hazards and the discomfort the study may entail, as well as the subject's right to abstain from participating in the study and to withdraw their consent at any time. They gave their written informed consent that has been approved by the local State Ethics Committee of the two cantons Basel (EKBB) and were paid for participating. Exclusion criteria were history or indication of drug abuse (including alcohol and drugs; a urinary screening for cannabinoids and opiates was assessed before each session), known or suspected hypersensibility to cannabinoids, presence of significant cardiovascular, hepatic, endocrine or renal diseases, smoking, subjects unable or unwilling to comply fully with the protocol and participating in any other clinical study. Drop outs were replaced. A post-study examination of the subjects was assessed at the end of the clinical trial. Drug administration was given to the subjects at 8 a.m. after a fasting period of at least twelve hours. Four hours after start of the administration, a standardised liquid meal was ingested (Ensure 500ml containing a caloric content of 2090 kJ) and after seven hours a standardised snack (roll with a slice of ham or cheese, an apple and 0.3 l orange juice) was given. The subjects

were allowed to drink ad libitum mineral water. The study protocol, the CRFs, the Investigator's Brochure, and the informed consent form were submitted to the local State Ethics Committee of both cantons of Basel (EKBB) for review and approval. An exceptional permit for scientific use of THC, cannabinoids and Marinol™ was obtained by the Federal Office for Public Health. The study was notified to the Swiss health agency (Swissmedic). This study was carried out in accordance with the recommendations of the Declaration of Helsinki adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964 and finally amended by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000.

Each subject received either 20 mg THC (dronabinol, Marinol™ soft gelatine capsules; Unimed Pharmaceuticals, Inc., Marietta, GA, USA, through DiaMo Narcotics Ltd., Thun, Switzerland), or one of five different encapsuled *Cannabis sativa L*. extract formulations (chapter 2.2.). Plant extracts were manufactured by Frutarom (Wädenswil, Switzerland). Galenical formulation was done by the Institute for Hospital Pharmacy, University Hospital Basel according to GMP regulations. All capsule formulations were standardised containing 20 mg THC_{tot}. The blinding of the test medications was performed by two investigators by enclosing the test medication capsules in another, dark-coloured glass flask named with the letters A-F depending on the medication content.

In the first study part, the effect of heating on the relative content of cannabinoids was investigated. Data were compared to the commercial formulation Marinol™. In the second study part, the effect of different formulations with TPGS on the bioavailabilities of different active cannabinoids was investigated. The study endpoints were a) to assess the relative bioavailabilities of THC and its metabolites assessed as area under the plasma concentration/time curve from time T = 0 h extrapolated to infinity (AUC(0-∞)), b) to assess the relative tolerability and safety of six different oral formulations of 20 mg THC_{tot} (THC and THCA-A), c) to assess the effect of six different oral formulations of 20 mg THC_{tot} on psychomotor function assessed as simulator assisted evaluation of driving ability, d) to assess repetitive heart rate, blood pressure and a visual analogue scale (VAS) for sedation.

The wash-out phases between the consecutive treatments were at least two weeks depending on the outcome of the urinary screening (fluorescence polarisation immunoassay (FPIA); cloned enzyme donor immunoassay (CEDIA)) for THC-COOH. If a test was positive for THC-COOH (SAMHSA set general immunoassay cut-off of 50 ng/ml) after two weeks, it was repeated seven days later and then in weekly intervals until the test was negative for the THC-COOH.

Blood (5 - 10 ml) was collected in all sessions through a cubital vein catheter of the forearm at baseline, 0.5, 1, 2, 4, 8, 12, 24 h and additionally at 48 and 72 h in the second study phase post drug intake.

The blood samples were gently inverted, centrifuged (10 min at 2000 × *g*) and the supernatant (EDTA-plasma) filled in silanised glass vials. The vials were sealed and instantly deep-frozen at -70 °C until analysis. EDTA-plasma concentrations of THC and its metabolites 11-OH-THC and THC-COOH as well as cannabidiol (CBD) and cannabinol (CBN) were measured with an approved LC-MS/MS method [123] after an off-line solid-phase extraction.

In this paper version only the pharmacokinetic calculations of the study are described, evaluated and discussed. The pharmacodynamic results are reported in another, separate publication.

2.2. Study Medication

All subjects received each of the treatments in a double-blind manner according to a randomised cross-over design. The different treatments are described in *Table 1*. The study dose was chosen, since it is used as a standard dose in clinical studies with THC and cannabinoids.

Table 1: Study medication

Part A	
Medication A	20 mg ∆9-THC (Marinol™, reference medication).
Medication B	Cannabis extract from herba cannabis (heated), corresponding to 20 mg ∆9-THC_{tot} and 20-30 mg CBD_{tot}.
Medication C	Cannabis extract from herba cannabis (unheated), corresponding to 20 mg ∆9-THC_{tot} and 20-30 mg CBD_{tot}.
Part B	
Medication D	Cannabis extract from unheated herba cannabis (containing 20 mg ∆9-THC_{tot} and 20-30 mg CBD_{tot}) + 0.5 % TPGS.
Medication E	Cannabis extract from unheated herba cannabis (containing 20 mg ∆9-THC_{tot} and 20-30 mg CBD_{tot}) + 5 % TPGS.
Medication F	Cannabis extract from unheated herba cannabis (containing 20 mg ∆9-THC_{tot} and 20-30 mg CBD_{tot}) + 7.5 % TPGS.

2.3. Summary of Demographic Characteristics of Subjects and Disposition of subjects

Nineteen subjects entered the study and received at least one administration of a study drug (ten for Part I and nine for Part II). One subject (no. 1) discontinued his participation due to mild to moderate adverse effects on his second administration

day, where he had received Marinol (Form A). He experienced mild paresthesia, warm feeling, conjunctional injection, vertigo, visual disturbances, abdominal discomfort, dry mouth, tremor and paleness as well as moderate short-lasting anxiety. Since, the symptoms were in the vast majority of mild severity, this subject was replaced. The other 18 subjects completed the study.

Demographic characteristics of the subjects are listed in *Table 2*.

Table 2: Summary of demographic characteristics of subjects

		Age (years)	**Weight (kg)**	**Height (cm)**	**BMI (kg/cm^2)**
N		19	17	19	17
Mean		26.8	75.5	177.8	23.9
Median		25	76	178	24.1
Std. Deviation		4.76	7.77	4.31	2.59
Std. Error		1.09	1.88	0.96	0.63
95% confidence interval for mean	Lower bound	21	64	167	18.7
	Upper bound	40	95	188	29.3
Minimum		26.8	64	177.8	
Maximum		25	95	178	

2.4. Chemicals

THC and metabolite reference material was obtained from Lipomed (Arlesheim, Switzerland). All solvents (acetonitrile, isopropanol 20%, *ortho*-phosphoric acid 85%, ammonia solution 25%, formic acid 98%) and chemicals (ammonium formate, dipotassium hydrogen phosphate) in analytical grade were purchased from Merck (Darmstadt, Germany) and Aldrich (Buchs, Switzerland). Fetal bovine serum was obtained from Biochroma (Berlin, Germany). Silanised glass vials (type I plus) were purchased from Schott (Muellheim-Huegelheim, Germany) and 6 ml glass test tubes from Gilson (Mettmenstetten, Switzerland). Solid-phase extraction was

carried out on Certify II extraction cartridges from Varian (Zug, Switzerland). De-ionised water was generated with a Milli-Q water purification system from Millipore (Kloten, Switzerland). The buffer (pH 9.1, 0.1 M) was prepared with a K_2HPO_4 solution (8.71 g/480 ml water), adjusted to the desired pH by appropriate addition of *ortho*-phosphoric acid 85% and filled up with water to 500 ml.

2.5. Specimens, standard solutions, calibration standards and quality controls

Stock THC and metabolite standard solutions were made in acetonitrile/H_2O (50/50, v/v). (The concentrations were THC, 11-OH-THC, CBD, CBN: 100 mg/l; THC-d3, THC-COOH-d3: 10 mg/l (v/v).) The standards were used to spike the matrix. Calibration standards for assay calibration and determination of the linear measuring range were prepared in bovine serum by spiking with the needed amount of the standard solutions to obtain the range of 0.2 - 100 ng/ml of THC and its metabolites. EDTA-plasma was taken from healthy male volunteers. In order to determine for quality control purposes, the intra- and inter-assay precision and the accuracy, blood quality controls were prepared in blank bovine serum by spiking with THC (3.0, 10.0 and 25.0 ng/ml).

2.6. Extraction procedure of human EDTA-plasma

The solid-phase extraction procedure was carried out on Varian Bond Elut Certify II cartridges (off-line). Certify II is a mixed mode sorbent with an anion exchange sorbent and retains acidic and neutral drugs (THC and metabolites). The cartridges were conditioned initially with 1 ml acetonitrile and followed by 1 ml 0.1 M phosphate buffer (pH 9.1). 1 ml EDTA-plasma sample acidified with 20 µl *ortho*-phosphoric acid (85%) was loaded onto the cartridges. Cartridges were subsequently washed with 1 ml 40% acetonitrile and dried under vacuum at 45 kPa for 2 min. The compound of

interest was eluted with 1 ml acetonitrile:ammonia (NH_4^+) = 98:2 (v/v) into a 6 ml glass test tube and aliquots were evaporated to dryness at 37 °C under nitrogen.

The extracts were reconstituted in 60 µl of mobile phase (40% mobile phase A, 60% mobile phase B), 10 µl internal standard THC-d3 (10 mg/l in acetonitrile 50% (v/v)) was added, mixed and 50 µl of the mixture injected into the LC-MS/MS system.

The internal standard was added in order to overcome possible variations of the concentrations in the LC/detection system. Possible deviations of the extraction rates were overcome by treating the standard in EDTA-plasma in the same way as samples and by calibration each series of analysis.

2.7. LC-MS/MS

The LC-MS/MS analysis was performed with a validated high-performance liquid chromatographic separation method with tandem-mass spectrometry, as previously reported [123]. In brief: the chromatographic system consisted of a Rheos 2000 Micro HPLC pump (Thermo Fisher, Allschwil, Switzerland) and a Midas Symbiosis Autosampler from Spark (Emmen, Netherlands) equipped with a 100 µl loop. A four-channel degasser was integrated into the Rheos CPS LC system. The LC-MS/MS apparatus was a LCQ Advantage MAX from Thermo Fisher (Basel, Switzerland) equipped with an APCI device operating in the positive detection mode. The chromatographic separation was performed on a Synergi MAX-RP 80A C_{12} column (length 2 mm × 75 mm, i.d. 4 µm) from Brechbuehler (Zuerich, Switzerland). The mobile phase was delivered at a flow rate of 400 µl/min. Each chromatographic run was performed with a binary, linear A/B gradient (Solvent A was 10 mM ammonium formate, pH 3.0. Solvent B was 90% acetonitrile, 10% 10 mM ammonium formate, pH 3.0). The program was as follows: 0 min: 50% B; 1 - 12.0 min: 50 - 79% B; 12.01 - 12.50 min: 79 - 95% B; 12.51 - 15.00 min: 95% B;

15.01 - 25.00 min: B linear from 95 to 50%. All solvents were degassed before usage.

The following APCI inlet conditions were applied: The heated vaporiser was kept at 465 °C. Both, the sheath gas and the auxiliary gas were nitrogen set at 60 and 15 relative units, respectively. The capillary entrance to the ion trap was at an offset of 10 V in the positive mode and was maintained at 220 °C. The corona current was 5 µA. For quantification, the selected ion monitoring mode was used.

2.8. Study analytes and their Lower Limits of Quantification (LLOQ) and Detection (LLOD)

Plasma concentrations of THC and its metabolites 11-OH-THC and THC-COOH as well as cannabidiol and cannabinol were measured with this approved LC-MS/MS method. The LLOQ in EDTA-plasma was 0.2 ng/ml, the LLOD 0.1 ng/ml for all substances.

2.9. Pharmacokinetic Parameters

The following pharmacokinetic parameters have been assessed: The maximum plasma concentration (C_{max}) and the time of its occurrence (T_{max}) have been determined by inspection of raw data. The area under the plasma concentration / time curve (AUC) has been determined by linear trapezoidal rule. If appropriate, plasma profiles were to be evaluated by model dependent analysis using non-linear regression (WinNonlin version 5.0; copyright © Pharsight Corporation 2006). Otherwise, non-parametric analysis using WinNonlin had to be applied. The following parameters have been determined: Area under the plasma concentration / time curve over the first 24 hours after drug administration, the maximum plasma concentration (C_{max}) and the time of its occurrence (T_{max}). The concentrations of the analytes were calculated by comparing the peak area (%) of an analyte with the corresponding area

(%) on the standard curve. System variations were adjusted by comparing the area (%) of the internal standard THC-d3.

2.10. Statistical and analysis plan

Descriptive analysis of all parameters has been performed. For all medications, analysis of variance and subsequent Tukey multicomparison test (normally distributed data) or Friedman test with subsequent multiple Wilcoxon signed ranks test with Bonferoni's correction was used for data not normally distributed, as appropriate.

All statistical analyses have been performed with the SPSS for Windows software (version 14.0). All comparisons have been performed as two-tailed analyses. The level of significance was p= 0.05.

3. Results and Discussion

3.1. Pharmacokinetic evaluation

Mean pharmacokinetic data are displayed in *Figure 1* (1a-1f) and *Figure 2* (2a-2f). The summary of the pharmacokinetic data is given in *Table 3* and *5*. Data are given in molar concentrations to allow an estimation of absorption independent of different molecular weights of THC metabolites.

3.1.1. Plasma levels Part I (heated/unheated extracts, Marinol™)

Although there were some slight differences in the pharmacokinetic parameters AUC, C_{max} and $T_{max,}$ values of THC showed no statistically significant differences. This was due to the high inter-subject variability.

AUC and C_{max} values of 11-OH-THC showed no statistically significant differences. However, after administration of the unheated extract significantly (P = 0.042) shorter T_{max} values were observed (1.00 ± 0.14 (1.0) hours) than after administration of Marinol™ (1.67 ± 0.17 (2.0) hours).

AUC and C_{max} values of THC-COOH showed no statistically significant differences. However, after administration of the heated and unheated extract significantly (P = 0.05) longer T_{max} values were observed (2.89 ± 0.35 (1.0) hours and 2.11 ± 0.26 (2.0) hours, respectively) than after administration of Marinol™ (1.78 ± 0.32 (22.0) hours).

As expected, no CBD plasma concentrations could be detected after administration of the synthetic THC (Marinol™). Although after administration of the unheated extract the AUC of CBD was about 2-fold higher (7.67 ± 2.06 (4.63) pmol×h/ml) than after administration of the heated extract (3.68 ± 1.34 (2.53) pmol×h/ml), this difference was not statistically significant. C_{max} values were significantly different between the treatments (P = 0.002): after administration of the unheated extract, C_{max} amounted to 3.95 ± 0.92 (3.06) pmol/ml) and after administration of the heated extract to 0.94 ± 0.22 (0.87) pmol/ml. Although T_{max} values were longer for the unheated than heated extract, this difference was not statistically significant.

For CBN, no significant differences could be detected for AUC, C_{max} and T_{max} between the treatments.

Although mean values indicated large difference in AUC values of overall absorption (as expressed as the AUC (0-24h) of the sum of THC, 11-OH-THC, THC-COOH and

CBN), median values were comparable between the treatments: Form A= 149.13 ± 44.24 (99.98) pmol×h/ml, Form B= 181.15 ± 90.54 (90.57) pmol×h/ml and Form C= 62.53 ± 14.04 (60.36) pmol×h/ml. Hence, no statistical differences could be detected. Likewise, no statistically significant differences could be detected for C_{max} values, although, values were about 2-fold higher after administration of Marinol™ than after administration of both extracts. T_{max} values were significantly higher after administration of the heated extract (2.67 ± 0.33 (2.0) h) than after administration of Marinol™ (1.44 ± 0.18 (1.0) h; P = 0.005) or the unheated extract (1.22 ± 0.21 (1.0) h; P = 0.001).

A summary of the pharmacokinetic data (study part I) is presented in *Table 3*. Mean ± SEM plasma concentration curves of the cannabinoids are presented in *Figure 1.*

Table 3: Summary of pharmacokinetic data: Study part I (Means ± SEM (Median))

Study part I	**Marinol 20 mg**	**Heated extract**	**Unheated extract**
(1) Δ^9THC			
AUC(0-24) (pmol×h/ml)	8.43 ± 4.23 (4.58)	3.48 ± 0.84 (2.84)	9.75 ± 2.95 (6.59)
Cmax (pmol/ml)	3.26 ± 1.74 (1.53)	1.33 ± 0.42 (0.8)	3.24 ± 0.83 (2.26)
Tmax (h)	1.06 ± 0.19 (1.0)	0.78 ± 0.09 (1.0)	1.17 ± 0.22 (1.0)
(2) 11-OH-THC			
AUC(0-24) (pmol×h/ml)	9.51 ± 2.07 (6.86)	10.61 ± 3.83 (7.24)	7.52 ± 2.15 (7.27)
Cmax (pmol/ml)	2.99 ± 0.65 (2.53)	2.22 ± 0.69 (1.51)	1.72 ± 0.41 (1.5)
Tmax (h)	1.67 ± 0.17 (2.0)	1.44 ± 0.23 (2.0)	1.00 ± 0.14 (1.0)
(3) THC-COOH			
AUC(0-24) (pmol×h/ml)	121.94 ± 39.91 (84.32)	157.80 ± 85.16 (70.68)	39.03 ± 10.44 (45.14)
Cmax (pmol/ml)	20.71 ± 5.47 (22.11)	16.88 ± 7.34 (10.04)	5.62 ± 1.06 (6.62)
Tmax (h)	1.78 ± 0.32 (2.0)	2.89 ± 0.35 (2.0)	2.11 ± 0.26 (2.0)
(4) CBN			
AUC(0-24) (pmol×h/ml)	10.66 ± 4.70 (7.14)	9.25 ± 1.91 (8.41)	6.23 ± 2.23 (3.77)
Cmax (pmol/ml)	2.05 ± 0.78 (1.19)	1.94 ± 0.40 (1.82)	1.74 ± 0.31 (1.88)
Tmax (h)	1.06 ± 0.19 (1.0)	0.94 ± 0.15 (1.0)	1.00 ± 0.14 (1.0)
(5) CBD			
AUC(0-24) (pmol×h/ml)	0.00 ± 0.00 (0.0)	3.68 ± 1.34 (2.53)	7.67 ± 2.06 (4.63)
Cmax (pmol/ml)	0.00 ± 0.00 (0.0)	0.94 ± 0.22 (0.87)	3.95 ± 0.92 (3.06)
Tmax (h)	NA	0.83 ± 0.17 (0.5)	1.17 ± 0.39 (1.0)
6) Sum [(1) – (4)]			
AUC(0-24) (pmol×h/ml)	149.13 ± 44.24 (99.98)	181.15 ± 90.54 (90.57)	62.53 ± 14.04 (60.36)
Cmax (pmol/ml)	26.90 ± 6.53 (27.47)	19.73 ± 8.03 (12.29)	10.47 ± 1.86 (12.29)
Tmax (h)	1.44 ± 0.18 (1.0)	2.67 ± 0.33 (2.0)	1.22 ± 0.21 (1.0)
Bioavailability (%)	100	345.7 ± 180.5 (83.3)	57.4 ± 12.6 (60.4)

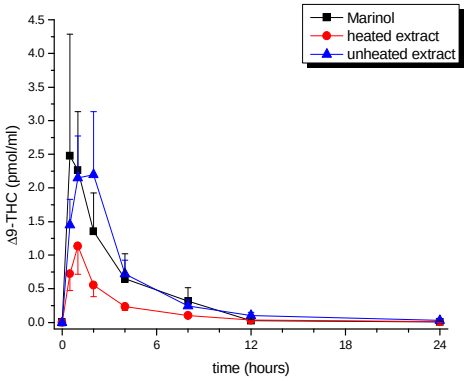


Figure 1a

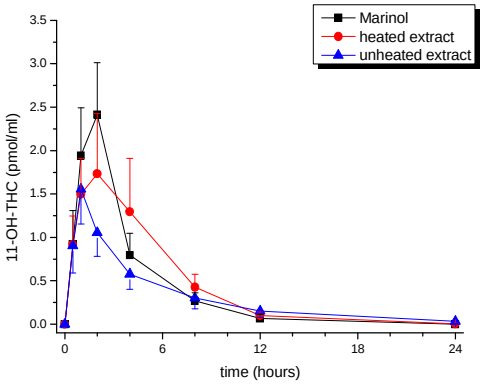
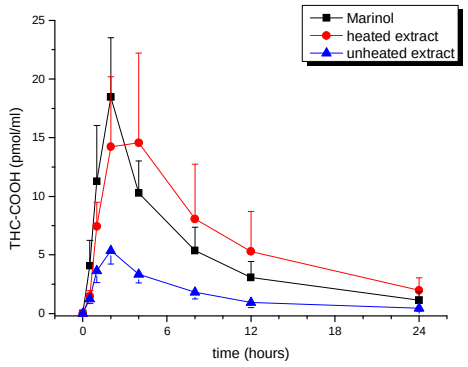


Figure 1b



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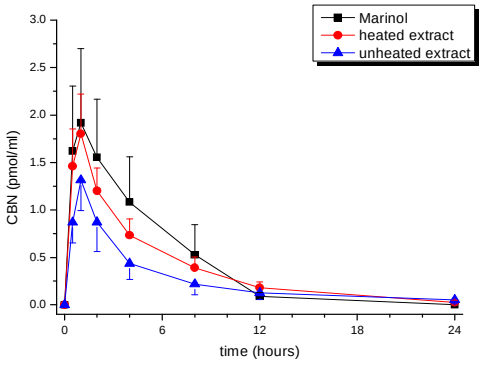

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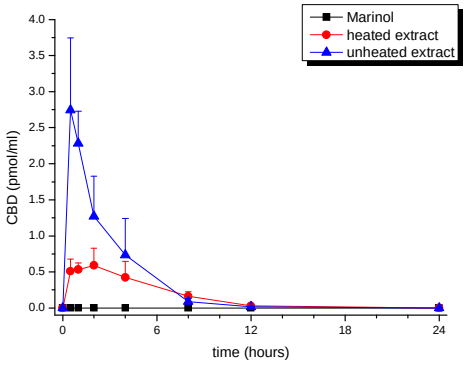

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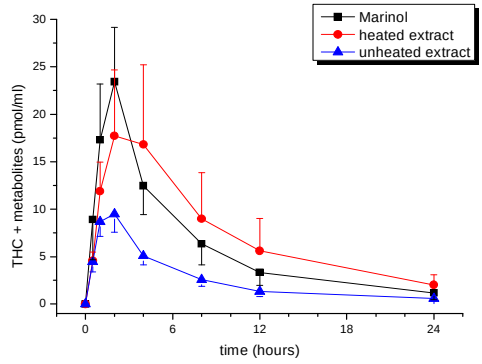

Figure 1f

Figure 1: 1a-1e: Mean ± SEM plasma concentrations of THC, 11-OH-THC, THC-COOH, CBD, CBN (pmol/ml) in study part I. 1f: Mean ± SEM of total concentrations THC and metabolites (11-OH-THC, THC-COOH, CBN) (pmol/ml) in study part I

After administration of cannabis extracts, a different metabolic pattern was detected (*Table 4*):

Table 4: Percentage of total AUC (0-24h) of different metabolites in study part I

Form	THC	11-OH-THC	THC-COOH	CBD	CBN
Form A Marinol™	5.45 ± 1.04 (5.13)	9.70 ± 2.03 (8.79)	76.44 ± 3.50 (80.83)	0.0 ± 0.0 (0.0)	8.41 ± 2.66 (6.98)
Form B Heated extract	3.39 ± 1.05 (2.46)	7.53 ± 1.54 (6.14)	77.02 ± 4.08 (77.16)	3.02 ± 1.09 (1.50)	9.04 ± 2.43 (7.68)
Form C Unheated extract	15.82 ± 3.32 (11.37)	10.42 ± 1.69 (12.30)	48.55 ± 6.82 (55.34)	14.85 ± 4.40 (12.56)	10.35 ± 3.00 (5.67)
	A: P = 0.005 **B: P = 0.001**		**A: P = 0.002** **B: P = 0.001**	**A: P = 0.001** **B: P = 0.01**	

After administration of Form C (unheated extract), the highest proportion of THC AUC of all cannabinoids were observed (15.82 ± 3.32 (11.37) %). This proportion was significantly higher than after administration of Form A (Marinol™; 5.45 ± 1.04 (5.13) %, P = 0.005) and after administration of Form B (heated extract; 3.39 ± 1.05 (2.46); P = 0.001). The proportion of 11-OH-THC and CBN was virtually unchanged between the different treatments. The largest differences were seen for the proportion of THC-COOH: the unheated extract showed the lowest proportion (48.55 ± 6.82 (55.34) %), which was significantly (P = 0.001) lower than that after administration of the heated extract (77.02 ± 4.08 (77.16) %) and also lower (P = 0.002) than after administration of Marinol™ (76.44 ± 3.50 (80.83) %).

After administration of the synthetic Marinol™, no plasma concentrations of CBD could be detected. This was expected, since THC is not converted to CBD *in vivo* and is found only in cannabis plants. Heating of extracts decreased the proportion of CBD significantly (P = 0.01) from 14.85 ± 4.40 (12.56) % for the unheated to 3.02 ± 1.09 (1.50) % for the heated extract.

3.1.2. Plasma levels Part II (0.5, 5, 7.5 % TPGS)

The highest values of THC AUC (0-24h) were observed after administration of Form E (unheated extract containing 5% TPGS). These values amounted to 9.73 ± 1.53 (8.93) pmol×h/ml and were statistically significant greater (P = 0.04) than after administration of Form F (unheated extract containing 7.5% TPGS): 4.96 ± 1.02 (4.61) pmol×h/ml. After administration of Form D (unheated extract containing 0.5% TPGS), AUC was 6.02 ± 1.28 (4.06) pmol×h/ml, which was not significantly different from the AUC value after administration of Form E. After administration of Form E, C_{max} values were significant higher (2.83 ± 0.39 (2.87) pmol/ml) than after administration of Form D (1.66 ± 0.33 (1.26) pmol/ml; P = 0.039) and Form F (1.45 ± 0.21 (1.58) pmol/ml; P = 0.014). Maximum plasma concentrations were most rapidly observed after administration of Form E (0.89 ± 0.16 (1.0) h) than after administration or Form E (0.94 ± 0.15 (1.0) h) and finally Form D (1.33 ± 0.39 (1.0) h). However, these differences were not statistically significant different.

The values of 11-OH-THC AUC (0-24h), C_{max} and T_{max} were not statistically significant different between the treatments. AUC values amounted to 6.77 ± 2.28 (4.07), 6.30 ± 1.56 (4.87) and 6.14 ± 1.44 (6.68) pmol×h/ml after administrations of Forms D, E and F, respectively. C_{max} values amounted to 1.37 ± 0.26 (1.61), 2.13 ± 0.69 (1.22) and 1.69 ± 0.40 (1.58) pmol/ml after administrations of Forms D, E and F, respectively. T_{max} values amounted to 2.00 ± 0.43 (2.0), 1.50 ± 0.20 (2.0) and 1.50 ± 0.20 (2.0) h after administrations of Forms D, E and F, respectively.

The values of THC-COOH AUC (0-24h), C_{max} and T_{max} were likewise not statistically significant different between the treatments. However, as indicated by the median values there was a trend of higher AUC and C_{max} values and shorter T_{max} values after administration of Form E. AUC values amounted to 61.90 ± 18.99 (35.22), 57.60 ± 11.66 (49.63) and 53.94 ± 10.03 (45.5) pmol×h/ml after administrations of Forms D, E and F, respectively. C_{max} values amounted to 8.21 ± 2.15 (6.05), 10.32 ± 2.40 (8.06) and 8.26 ± 1.35 (7.84) pmol/ml after administrations of Forms D, E and F,

respectively. T_{max} values amounted to 2.89 ± 0.68 (2.0), 1.56 ± 0.18 (2.0) and 2.11 ± 0.26 (2.0) h after administrations of Forms D, E and F, respectively.

The values of CBN AUC (0-24h), C_{max} and T_{max} were likewise not statistically significant different between the treatments. AUC values amounted to 10.17 ± 1.31 (10.8), 11.21 ± 2.28 (9.23) and 11.55 ± 2.03 (11.32) pmol×h/ml after administration of Form D, E and F, respectively. C_{max} values amounted to 1.59 ± 0.19 (1.64), 1.95 ± 0.28 (2.05) and 1.66 ± 0.29 (1.52) pmol/ml after administrations of Forms D, E and F, respectively. T_{max} values amounted to 2.11 ± 0.39 (2.0), 2.33 ± 0.44 (2.0) and 2.11 ± 0.39 (2.0) h after administrations of Forms D, E and F, respectively.

The highest values of CBD AUC (0-24h) and C_{max} were observed after administration of Form D (6.11 ± 0.95 (7.87) pmol×h/ml and 3.68 ± 1.03 (2.49) pmol/ml, respectively). These values gradually decreased with increasing TPGS content: over 4.85 ± 0.46 (5.25) pmol×h/ml (AUC) and 3.20 ± 0.36 (2.92) pmol/ml (C_{max}) after administration of Form E to 3.34 ± 0.68 (2.65) pmol×h/ml (AUC) and 2.30 ± 0.36 (1.75) pmol/ml (C_{max}) after administration of Form F. For AUC the difference between AUC values after administration of Form D and E was statistically significant (P = 0.033). T_{max} were likewise not statistically significant different between the treatments. AUC values amounted to 6.11 ± 0.95 (7.87), 4.85 ± 0.46 (5.25) and 3.34 ± 0.68 (2.65) pmol×h/ml after administrations of Forms D, E and F, respectively. C_{max} values amounted to 3.68 ± 1.03 (2.49), 3.20 ± 0.36 (2.92) and 2.30 ± 0.36 (1.75) pmol/ml after administrations of Forms D, E and F, respectively. T_{max} values amounted to 1.17 ± 0.36 (1.0), 0.78 ± 0.09 (1.0) and 0.72 ± 0.09 (0.5) h after administration of Form D, E and F, respectively. T_{max} values showed a trend to decrease with high TPGS content; however, this was not statistically significant.

No significant differences between the treatments were observed for AUC (0-24h), C_{max} and T_{max} of total cannabinoids (sum of THC, 11-OH-THC, THC-COOH and CBN). AUC values amounted to 84.36 ± 20.84 (58.29), 84.84 ± 12.13 (79.12) and 76.59 ± 10.34 (58.29) pmol×h/ml after administration of Form D, E and F,

respectively. C_{max} values amounted to 10.86 ± 2.46 (9.18), 15.54 ± 2.60 (14.32) and 11.60 ± 1.49 (9.18) pmol/ml after administration of Form D, E and F, respectively. T_{max} values amounted to 2.89 ± 0.68 (2.0), 1.56 ± 0.18 (2.0) and 2.11 ± 0.26 (2.0) h after administrations of Forms D, E and F, respectively.

When mean values of the administrations of different unheated extracts were compared for total cannabinoids (estimate of total oral bioavailability), TPGS addition resulted in an increased relative bioavailability (versus administration of unheated extract without TPGS (=100%) in study part I) of 134.91%, 135.88% and 122.49% after administration of Forms D, E and F, respectively.

A summary of the pharmacokinetic data (study part II) is presented in *Table 5*. Mean ± SEM plasma concentration curves of the cannabinoids are presented in *Figure 2.*

Table 5: Summary of pharmacokinetic data: Part II (Means ± SEM (Median)

Study part II	**Form D** **Unheated extract, 0.5 % TPGS**	**Form E** **Unheated extract, 5.0 % TPGS**	**Form F** **Unheated extract, 7.5 % TPGS**
(1) Δ9-THC			
AUC(0-24) (pmol×h/ml)	6.02 ± 1.28 (4.06)	9.73 ± 1.53 (8.93) vs. Form F: P = 0.04	4.96 ± 1.02 (4.61)
Cmax (pmol/ml)	1.66 ± 0.33 (1.26)	2.83 ± 0.39 (2.87) vs. Form D: P = 0.039 vs. From F: P = 0.014	1.45 ± 0.21 (1.58)
Tmax (h)	1.33 ± 0.39 (1.0)	0.89 ± 0.16 (1.0)	0.94 ± 0.15 (1.0)
(2) 11-OH-THC			
AUC(0-24) (pmol×h/ml)	6.77 ± 2.28 (4.07)	6.30 ± 1.56 (4.87)	6.14 ± 1.44 (6.68)
Cmax (pmol/ml)	1.37 ± 0.26 (1.61)	2.13 ± 0.69 (1.22)	1.69 ± 0.40 (1.58)
Tmax (h)	2.00 ± 0.43 (2.0)	1.50 ± 0.20 (2.0)	1.50 ± 0.20 (2.0)
(3) THC-COOH			
AUC(0-24) (pmol×h/ml)	61.90 ± 18.99 (35.22)	57.60 ± 11.66 (49.63)	53.94 ± 10.03 (45.5)
Cmax (pmol/ml)	8.21 ± 2.15 (6.05)	10.32 ± 2.40 (8.06)	8.26 ± 1.35 (7.84)
Tmax (h)	2.89 ± 0.68 (2.0)	1.56 ± 0.18 (2.0)	2.11 ± 0.26 (2.0)
(4) CBN			
AUC(0-24) (pmol×h/ml)	10.17 ± 1.31 (10.8)	11.21 ± 2.28 (9.23)	11.55 ± 2.03 (11.32)
Cmax (pmol/ml)	1.59 ± 0.19 (1.64)	1.95 ± 0.28 (2.05)	1.66 ± 0.29 (1.52)
Tmax (h)	2.11 ± 0.39 (2.0)	2.33 ± 0.44 (2.0)	2.11 ± 0.39 (2.0)
(5) CBD			
AUC(0-24) (pmol×h/ml)	6.11 ± 0.95 (7.87)	4.85 ± 0.46 (5.25)	3.34 ± 0.68 (2.65)
Cmax (pmol/ml)	3.68 ± 1.03 (2.49)	3.20 ± 0.36 (2.92)	2.30 ± 0.36 (1.75)
Tmax (h)	1.17 ± 0.36 (1.0)	0.78 ± 0.09 (1.0)	0.72 ± 0.09 (0.5)
6) Sum [(1) – (4)]			
AUC(0-24) (pmol×h/ml)	84.36 ± 20.84 (58.29)	84.84 ± 12.13 (79.12)	76.59 ± 10.34 (58.29)
Cmax (pmol/ml)	10.86 ± 2.46 (9.18)	15.54 ± 2.60 (14.32)	11.60 ± 1.49 (9.18)
Tmax (h)	2.89 ± 0.68 (2.0)	1.56 ± 0.18 (2.0)	2.11 ± 0.26 (2.0)
Ratio to unheated extract study I			
AUC(0-24) (%)	**134.91**	**135.88**	**122.49**

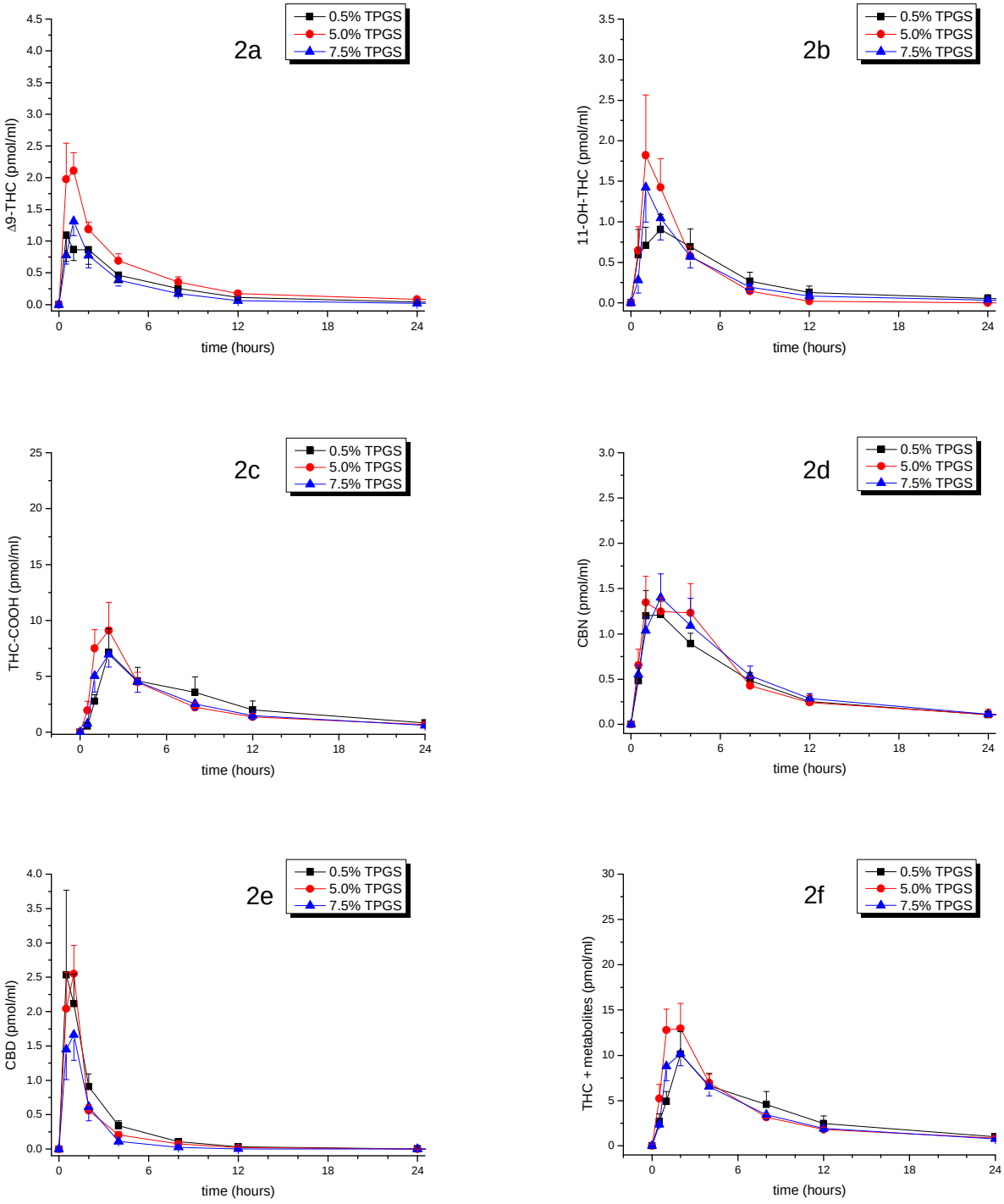

Figure 2: 2a-e: Mean ± SEM plasma concentrations of THC, 11-OH THC, THC-COOH, CBN, CBD (pmol/ml) in study part II. 2f: Mean ± SEM plasma concentration of total THC and metabolites (11-OH THC, THC-COOH, CBN) (pmol/ml) in study part II

Besides a bioavailability enhancing effect, addition of TPGS leads to a qualitatively different pattern of cannabinoid metabolites (*Table 6*).

Table 6: Percentage of total AUC (0-24h) of different metabolites of unheated extracts (Parts I and II)

Form	THC	11-OH-THC	THC-COOH	CBD	CBN
Form C 0% TPGS	15.82 ± 3.32 (11.37)	10.42 ± 1.69 (12.30)	48.55 ± 6.82 (55.34)	14.85 ± 4.40 (12.56)	10.35 ± 3.00 (5.67)
Form D 0.5% TPGS	8.67 ± 2.31 (5.59)	6.92 ± 1.09 (6.37)	61.18 ± 4.54 (55.18)	8.61 ± 1.82 (5.54)	14.62 ± 2.94 (12.84)
Form E 5.0% TPGS	11.78 ± 1.86 (11.19)	6.76 ± 1.13 (6.10)	60.97 ± 3.75 (60.60)	5.94 ± 5.32 (0.78)	14.55 ± 3.22 (13.67)
				C: P = 0.065	
Form F 7.5% TPGS	6.85 ± 1.78 (5.06)	8.07 ± 2.03 (5.93)	64.91 ± 3.45 (65.93)	4.28 ± 0.60 (4.77)	15.89 ± 2.41 (15.98)
	C: P = 0.057		**C: P = 0.098**	**C: P = 0.021**	

With the exception of 11-OH-THC, all other cannabinoids showed systematic changes of their proportion with increasing TPGS content of unheated cannabis extracts when compared to the results of the unheated extract without TPGS used in study part I: THC proportion decreased from 15.82 ± 3.32 (11.37) % (Form C) over 8.67 ± 2.31 (5.59) % (Form D) and 11.78 ± 1.86 (11.19) % (Form E) to finally 6.85 ± 1.78 (5.06) % (Form F). The latter proportion was borderline significantly lower (P = 0.057) than that of Form C. THC-COOH proportion increased from 48.55 ± 6.82 (55.34) % (Form C) over 61.18 ± 4.54 (55.18) % (Form D) and 60.97 ± 3.75 (60.60) % (Form E) to finally 64.91 ± 3.45 (65.93) % (Form F). The latter proportion was borderline significantly higher (P = 0.098) than that of Form C.

CBD proportion decreased from 14.85 ± 4.40 (12.56) % (Form C) over 8.61 ± 1.82 (5.54) % (Form D) and 5.94 ± 5.32 (0.78) % (Form E) (P = 0.065) to finally 4.28 ± 0.60 (4.77) % (Form F). The latter proportion was borderline significantly lower (P = 0.021) than that of Form C.

There was a trend of increasing proportions of CBN with increasing TPGS content: CBN proportion increased from 10.35 ± 3.00 (5.67) % (Form C) over 14.62 ± 2.94 (12.84) % (Form D) and 14.55 ± 3.22 (13.67) % (Form E) (P = 0.065) to finally 15.89 ± 2.41 (15.98) % (Form F). However, none of these changes reached statistical significance.

4. Discussion and overall conclusion

The study was performed in two parts: In the first part, the relative bioavailabilities of a heated and an unheated *Cannabis sativa* L. extract (containing 20 mg THC_{tot} and 20 - 30 mg CBD_{tot}) was compared to 20 mg synthetic THC. In the second study part, the effect of the addition of different amounts of TPGS to the cannabis extracts on the bioavailabilities of cannabinoids has been investigated.

The pharmacokinetics of cannabinoid metabolites showed a high inter-subject variability. The median relative oral bioavailability of the heated and unheated extract (versus Marinol^{TM}) was 83.3% and 60.4%. The metabolic pattern of cannabinoid metabolites after administration of Marinol^{TM} and the heated and unheated *Cannabis sativa L.* extract was qualitatively different with a significantly higher relative bioavailability of THC and CBD and a significantly lower relative bioavailability of THC-COOH. This could be the result of changes in the absorption of cannabinoid metabolites, in changes in metabolic activity or elimination processes. With this experimental design, the cause(s) of the change(s) could not be identified.

Addition of different amounts of TPGS resulted in an increase in relative bioavailability of the sum of cannabinoid metabolites (THC + 11-OH-THC + THC-COOH + CBN) to 122.5% (7.5% TPGS), 134.9% (0.5% TPGS) and 135.9% (5% TPGS) compared with the AUC of the unheated extract (=100%) in study part I. However, the weakness of this assessment is, that the AUC of the unheated extract

without addition of TPGS was determined in part I of the study, hence in a different subject population.

There was an indication that addition of TPGS resulted in a dose-dependent effect on the metabolic pattern of cannabinoid metabolites. Since TPGS is known to interact with efflux pumps (such as p-glycoprotein [12, 138, 141, 142]) it may also effect the enteral absorption of some of the cannabinoids. Whether it effects also metabolic reactions of cannabinoid is not known.

It can, therefore, be concluded that the administration of *Cannabis sativa L*. extracts is safe. These extracts have a slightly lower bioavailability of total cannabinoids than Marinol^{TM}. However, the pharmacological effects seem to be comparable. Addition of TPGS increases the bioavailability of cannabinoids up to about 35% compared to extracts without TPGS without deteriorating tolerability.

4. Conclusions and outlook

Since the United States FDA approved Marinol™ (a synthetic THC medication) to treat nausea and vomiting associated with cancer chemotherapy and to treat appetite loss associated with weight reduction in people with AIDS, the interest in additional, possible clinical indications of cannabis preparations increased. A cannabis extract formulation is supposed to have less adverse side effects than a synthetic one because of a well-balanced combination of active substances which can be found in the natural cannabis plant.

Therefore, a clinical trial with different *Cannabis sativa L*. extracts and Marinol™ was performed to analyse the pharmacokinetics and pharmacodynamics of the cannabinoids and to evaluate the best *Cannabis sativa L*. extract capsule formulation in this clinical phase I study for a possible future implementation as a new, concomitant medicament in cancer, HIV and AIDS therapies.

First, a sensitive LC-MS/MS method was developed for the simultaneous detection and quantification of the main cannabinoids THC, 11-OH-THC, THC-COOH, CBD and CBN in human plasma and urine. Optimal conditions for the analysis method, such as extraction procedure, matrices, column, quality controls, wavelength, mobile phases, run time, optimal separation (gradient, retention times), temperature, voltages, vacuum and internal standards, resulting in the best sensitivity and selectivity, were developed in preliminary experiments.

The validation of the method was performed according to the FDA Good Laboratory Practice guidelines, containing linear measuring range, quantification, lower limit of quantification (LLOQ), lower limit of detection (LLOD), quality controls, precision, accuracy, recovery, stability and matrix effects.

In conclusion, the described high-performance liquid chromatographic separation method with tandem-mass spectrometry detection showed a satisfactory overall

analytical performance well suited for applications in medical science. The combination of SPE/LLE, LC and APCI-MS/MS represents an attractive alternative to the well-established technique of GC-MS.

Second, the sensitivity and specificity of two immunoassays (CEDIA, FPIA) were established in urinary samples from volunteers receiving oral synthetic THC or *Cannabis sativa L*. extracts. Urinary THC-COOH excretion was evaluated by the immunoassays with a cut-off value of 50 ng/ml as well as the described LC-MS/MS method (gold standard) with a cut-off value of 15 ng/ml. It was demonstrated that LC-MS/MS is an excellent confirmation method for immunoassays allowing the qualitative and quantitative detection of many cannabinoids. The ROC analysis indicated that the FPIA test discriminates better between users and non-users than the CEDIA test. The results of both immunoassays show that the SAMHSA set general immunoassay cut-off of 50 ng/ml is possibly not applicable for analysis of samples from persons consuming the *Cannabis sativa L*. extracts orally instead of smoking. It has to be discussed whether a lower cut-off value would be advantageous. It is supposed that metabolite concentrations differ strongly depending on the route of application. The amount and appearance of different metabolites may disturb the immunoassay methods.

The glucuronide hydrolysation procedure showed a total transformation of the THC-COOH-glucuronides to THC-COOH confirmed by the nearly 100% agreement of the concentrations in the different samples analysed with the two immunoassays and the LC-MS/MS comparisons. The glucuronide is automatically detected together with THC-COOH and it is direct de-glucuronated in the APCI unit of the detector.

In conclusion, the use of cannabinoid-based therapeutics and continued abuse of oral cannabis products require scientific data for accurate interpretation of cannabinoid tests and for establishing a reliable administrative drug-testing policy. Preliminary positive immunoassay results have to be confirmed by a second, more

specific analysis method like GC-MS or LC-MS. To get correct quantitative results of the analytes, it is important to include the glucuronides in the analysis.

Third, a clinical trial was performed. In the first study part, the relative bioavailabilities of a heated and an unheated *Cannabis sativa L*. extract was compared to Marinol™. In the second study part, the effect of the addition of different amounts of TPGS to the cannabis extracts on the bioavailabilities of cannabinoids has been investigated.

The pharmacokinetics of the cannabinoids was highly variable between the subjects. Due to this variability, no statistically significant differences between the AUC of the different forms could be detected, neither in part I nor in part II of the study.

Addition of different amounts of TPGS resulted in an increase in relative bioavailability of the sum of cannabinoid metabolites (THC + 11-OH-THC + THC-COOH + CBN) to 122.5% (7.5% TPGS), 134.9% (0.5% TPGS) and 135.9% (5% TPGS) compared with the AUC of the unheated extract (=100%) in study part I. The administration of cannabis extracts as well as the addition of TPGS leads to a qualitatively different pattern of cannabinoid metabolites. After administration of the unheated extract, a significantly higher proportion of THC AUC and a significantly lower THC-COOH AUC of all cannabinoids were observed compared to the heated extract or Marinol™. After administration of the synthetic Marinol™, no plasma concentrations of CBD could be detected. This was expected, since THC is not converted to CBD *in vivo* and CBD is found only in cannabis plants. Heating of extracts decreased the proportion of CBD significantly.

It can be concluded that the administration of *Cannabis sativa L*. extracts is safe. The extracts have a slightly lower bioavailability of total cannabinoids than Marinol™. However, the pharmacological effects seem to be comparable. The future approach will address further research. The 5% *Cannabis sativa L.* extract capsule formulation showed a marginal higher increase in relative bioavailability than the 0.5% one. However, the 0.5% TPGS formulation contains more CBD than the 5% one. The

importance of a high CBD concentration (less adverse effects) has to be discussed for the development of a new medication. Further clinical studies with the 5% or 0.5% TPGS *Cannabis sativa L.* extract capsule formulations may be helpful. The study should be placebo controlled and later tested in the future patient group.

For a better understanding of cannabis consumption and the cannabis metabolism in the human body, the detection of further cannabinoids like THCA-A would be useful. THCA-A may prove an interesting field for the interpretation of cannabis consumption habits (single/frequent intake). If THCA-A shows a shorter elimination half-life time and a lower partition coefficient than THC it is important for the estimation of the elapsed time between the last intake and blood sampling. Therefore, THCA-A might be detectable only a very short time after cannabis consumption. The detection of THCA-A is a field of trace analysis. Therefore, a sensitive method with a low LLOQ is necessary for future investigations.

Future cannabinoid detection for criminal and forensic purposes requires analyses methods in additional matrices like hair or saliva. It can be difficult to detect the used drugs (active products at low dosages, chemical instability). The drug can be quickly eliminated from body fluids or in case of long delay between the alleged crime and clinical examination, collection of blood or urine can be of little value. The non-invasive collection of a saliva sample, which is relatively easy to perform and can be achieved under close supervision, is one of the most important benefits in a driving under the influence situation. Moreover, the presence of THC in oral fluid is a better indication of recent use than when the drug is detected in urine. The probability is higher that the subject is experiencing pharmacological effects at the time of sampling. Due to a long delay between an alleged crime and the clinical examination, hair testing can be very useful but depending on the hair colour of a human being drug detection is possible for months after crime.

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6. Curriculum vitae

Particulars

Name	Grauwiler Sandra Béatrice
Date of birth	8th May 1980
Place of birth	Binningen (BL), Switzerland
Nationality	Swiss
Address	Unterm Stallen 17
	4104 Oberwil (BL), Switzerland
	0041 (0)78 652 94 45
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Profession	Pharmacist (phil II)

Languages

Swiss-German	Native languages
German	
English	Very good skills
French	Adequate skills
Italian	Adequate skills

Education

2005-2008	PhD thesis at the Clinical Chemical Laboratory, Toxicology Section, University Hospital Basel (Basel, Switzerland), entitled: "Development and application of a LC-MS/MS method for the analysis of plasma bioavailabilities of different cannabinoids after the administration of *Cannabis sativa L*. extracts and Marinol™."
	Supervised by Professor Jürgen Drewe and André Scholer, PhD.
2004	Swiss Federal Diploma in Pharmacy (M. Sc.), University of Basel (Basel, Switzerland). Mark (mean): 5.5 (of 6.0).
	Diploma thesis at the Institute of Pharmaceutical Technology, University of Basel (Basel, Switzerland), entitled: "Formulierungsintegrierte, komplexe Mikroemulsionssysteme als transdermale Penetrationsenhancer des hydrophilen Modellwirkstoffes Nicotinsäure". Mark: 5.5 (of 6.0).
	Directed by Georgios Imanidis, PhD and Heiko Nalenz, PhD.
	Amedis award for the diploma thesis, 2004 (3. Rang).
2000-2004	Studies of pharmacy (phil II) at the Faculty of Natural Sciences, University of Basel (Basel, Switzerland).
	Graduation (Staatsexamen) in 2004.
1991-1999	High School (Gymnasium) Kohlenberg/Leonhard in Basel, Switzerland, Graduation Matura Typus D in 1999.
1987-1991	Elementary School in Basel, Switzerland

Personal management

2005-2008 Assisting in undergraduate practical classes (pharmacy students, laboratory school), clinical chemistry, chromatography (HPLC, LC-MS (MS)).

3/2007-7/2007 Supervision of diploma thesis of pharmacy students:

- Noëlle Kuonen: “Diagnostic with carbohydrate deficient transferrin (CDT)”
- Stephanie Mahler: “Detection of isoforms of transferrin with capillary electrophoresis in serum, liquor and nose secret”

2005-2008 Training and supervising of visiting pharmacy students from the University of Albany (USA).

Practical work experience

3/2005-6/2005 Replacement as pharmacist at Amavita Pharmacy Stadthausgasse (Basel, Switzerland)

9/2003-10/2003 Replacement as pharmacist at Breite Pharmacy (Basel, Switzerland)

9/2002-9/2004 Replacement as pharmacist at Stern Pharmacy (Basel, Switzerland)

9/2001-9/2002 Practical year at Stern Pharmacy (Basel, Switzerland)

Certifications 5/2005-4/2008

2005/2007	Education in taking blood and vaccination at the University Hospital Basel (Basel, Switzerland)
2007	Certification as a proof-leader for the supervision of GLP studies
2005-2007	Writing of Standard Operating Procedures (SOP)
2008	Temporary replacement (February/March 2008) of André Scholer, PhD, head of the clinical chemistry laboratory, in the area of toxicology including the evaluation and approval/disapproval of analytical results of clinical patients in the toxicology laboratory at the University Hospital Basel (Basel, Switzerland).

Lectures

7.12.2006	„Cannabis als Medikament“ presented in the WIND (Weiterbildung, Information, Diskussion) at the University Hospital Basel (Basel, Switzerland)
22.-24.8.2007	Labmed Deutschschweiz, Höhere Fach- und Führungsausbildung, Chromatographiekurs
2005-2008	Teaching of pharmacy students and at the laboratory school in clinical chemistry and chromatography (HPLC, LC-MS(MS))

1/2008 „Analysis of the urine metabolic pattern after oral intake of *Cannabis sativa L*. extracts or Marinol™ in humans“. Presented in the Forschungsseminar at the University Hospital Basel (Basel, Switzerland)

Publications

2006 Grauwiler SB, Scholer A, Drewe J. Development and application of a LC-MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of *Cannabis sativa L*. extracts.

J Chromatogr B Analyt Technol Biomed Life Sci. 2007 May 1;850 (1-2):515-22.

2007/2008 Grauwiler SB, Drewe J, Scholer A. Sensitivity and specificity of urinary cannabinoid detection with two immunoassays after controlled oral administration of cannabinoids to humans. Submitted to Therapeutic Drug Monitoring.

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2008 Grauwiler SB, Scholer A, Drewe J. Relative Bioavailabilities of Different Oral Formulations of Extracts of *Cannabis sativa L*.

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Oral sessions and posters presented at international congresses

9/9-14/9 2007 Oral session:

Analysis of the urine metabolic pattern after oral intake of *Cannabis sativa L*. extracts or Marinol™ in humans.

Presented at IATDMCT in Nice, France

21/9-23/9 2005 Poster:

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Presented at the SGKC in Yverdon-les-Bains, Switzerland

26/8-1/9 2006 Poster:

“Analysis of THC, 11-OH-THC, THC-COOH, CBD and CBN by liquid chromatography-tandem mass spectrometry in human EDTA-plasma and urine after small doses of THC and cannabis extracts”.

Presented at TIAFT in Ljubljana, Slovenia

9/9-14/9 2007 Poster:

“Urinary detection of THC carbonic acid with LC-MS/MS and two immunoassays”.

Presented at IATDMCT in Nice, France

During my studies I followed courses of the following lecturers:

P. Oelhafen, G.-R. Plattner, W. Schaffner, A. Zuberbühler, P. Strazewski, H.-J. Müller, M. Meyer, A. Hädener, P. Huber, A. Scholer, R. Leu Marseiler, U. Séquin, U. Spornitz, T.-W. Guentert, G. Imanidis, H. Leuenberger, S. Krähenbühl, J. Drewe, K. Hersberger, J. Huwyler, B. Ernst, M. Lampert, G. Folkers, A. Regeniter, K. Berger-Büter, K. Beier, P. Hauser, E. Constable, A. Fahr, A. Vedani, H. Durrer, P. Erb, C. Moroni, J. Meyer.

Grant

Senglet-foundation for “sponsorship of pharmaceutical procreation in Basel”, 2005-2008.

Basel, 8th April 2008, _____

Sandra Béatrice Grauwiler