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Characterization of the metabolic profile of novel psychoactive substances by a combination of *in vitro* and *in vivo* studies and chromatographic-spectrometric techniques

PhD Program in Chemistry

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Outline of the study

This PhD work is focused on those Novel Psychoactive Substances (NPS) which could exert effects as doping agent in sport competitions. Analysis were carried out at "Laboratorio Antidoping FMSI" of Rome part of laboratories around the world accredited by WADA to conduct human doping control sample analyses.

Different substances were selected thorough the years of study, among those NPS with structures and action similar to other compounds already prohibited in sport competitions. The substances of interest were selected according to University of Ferrara department of morphology, surgery and experimental medicine, section of legal medicine, a collaborative centre of Italian Early Warning System (IEWS) and University Cattolica of Rome. This collaboration led to the establishment of a multicentric collaborative group for the IEWS. The aim was to introduce these selected NPS as recognized doping agents searched by laboratories routine drug test of World Antidoping Agency (WADA), or forensic toxicology laboratories. With this aim specific compounds were selected and their potential effects were evaluated through *in vivo* behavioural studies employing murine model as a model of human behaviour and metabolism. The substances were administered to mice groups and behavioral studies were carried out at University of Ferrara to establish potential stimulant effects, which configure substances as stimulant compound in-competition. When a substance has showed typical effects as a doping agent, metabolism studies were carried out by our laboratory employing human liver microsomes or CYPs isoform as a model of human oxidative metabolism, and samples were analysed through liquid chromatography mass spectrometry techniques.

The selected substances are: Methiopropamine, 4,4' Dimethylaminorex, Pyrovalerone, Methedrone, γ-valerolactone, ADB-CHMICA, CUMYL-THPINACA.

All of these substances show potential stimulant effects known in literature or proved by behavioral studies provided by University of Ferrara. They were selected in accordance with IEWS alert on their abuse in Italy and their potential implication with fatal intoxication. For selected substances (Methiopropamine, GVL, 4,4' DMAR) tissue damage were also estimated by collaboration with University Cattolica of Rome.

For all the substances *in vitro* metabolism studies were carried out to in order to investigate metabolic pathways reactions and select the most suitable markers of intake.

Methiopropamine, 4,4'-dimethylaminorex, and γ-valerolactone are substances of interest in forensic analysis as potential hazardous novel abused recreational drugs with unknown metabolism and /or excretion. The *in vivo* metabolism of these compounds was therefore studied employing mice as metabolism model. The matrix selected was urine as the elected matrix for doping and toxicological analysis. Collected data from the three unit were linked and proposed to IEWS with the aim to bring up the unknowledge on these substances and to propose the introduction of fundamental data on their toxicology, effects and metabolism on EWS international database.

Part I

Chapter I

General Introduction

1 General introduction

1.1 The World Anti-Doping Agency (WADA)

The origin of the word "doping" is still unclear but appeared for the first time in an English dictionary in 1889, where the word was used to describe a remedy containing opium commonly used to increase racehorse's performances [1]. Doping could be defined as the attempt to enhance the athlete's performances in sport by illegal administration of pharmaceuticals or application of prohibited methods [2]. Today, violation of doping rules were described in the World Anti-Doping Code (WADC), where "doping" is defined as the occurrence of one or more of the anti-doping rules violations described from the article 2.1 to the article 2.8 of the Code, that are: "(i) presence of a prohibited substance or its metabolites or markers in an athlete sample; (ii) the use or attempted use by athlete of a prohibited substance or a prohibited method; (iii) refusing or falling without compelling justification to submit to sample collection after notification as authorized in applicable anti-doping rules, or otherwise evading sample collection; (iv) violation of applicable requirements regarding athlete availability of out-of-competition testing, including failure to file required whereabouts information and missed tests which are declared based on rules which comply with the international standard for testing; (v) tampering or attempting to tamper, with any part of doping control; (vi) possession of prohibited substances and prohibited methods; (vii) trafficking or attempted trafficking in any prohibited substance or prohibited method, and (viii) administration or attempted administration to any athlete of any prohibited method or prohibited substance"[3].

In 1998, one of the biggest scandals in the history of sports led to the creation of the World Anti-Doping Agency (WADA). Right before the beginning of 1998 Tour de France, a large number of prohibited substances were found by French Custom police inside the car of the long-time masseur for the former Festina road-racing team, Willy Voet and several top teams and athletes were also implicated in this kind of illicit tasks [4–6].

Subsequently a World Conference on Doping was held in February 1999 in Lausanne. The need for an independent international agency for doping became clear, which led to the establishment of the WADA on November of the same year. Its main mission was to coordinate and promote the development of international standardized antidoping rules, facilitate the coordination between sport organization and governmental authorities, ongoing with doping control research, and promote doping prevention activities [7,8].

In 2003, WADA adopted the World Anti-Doping Code (WADC) that became the first worldwide accepted document providing a set of harmonized antidoping policies, rules and regulations within sport organizations and public authorities. The current version of WADA Code[3] is under review with the aim to improve several aspects of anti-doping rules (2021 code review process).

Nowadays, the WADA Programme is articulated in three levels that are:

- The WADA Code [3]
- The International Standards [9] (Prohibited List, Testing and Investigation Laboratories, Therapeutic Use Exemption, Protection on Privacy and Personal Information)
- Technical Documents

Following the adoption of the first WADA Code in 2003, WADA, at least once a year, updates the Prohibited List of Substances and Methods in sport.

The current version of the prohibited List is reported below [10]

Table 1. The Prohibited List 2019, class of substances of interest in this PhD thesis are in bold.

PROHIBITED SUBSTANCES
Prohibited in- and out-of-competition
S0. Non-approved substances S1. Anabolic agents S2. Peptide hormone, growth factors, related substances and mimetics S4. Hormone and metabolic modulators S5. Diuretics and masking agents
Prohibited substance in-competition
S6. Stimulants **S7. Narcotics** **S8. Cannabinoids** S9. Glucocorticoids
PROHIBITED METHODS
M1. Manipulation of blood and blood components M2. Chemical and physical manipulation M3. Gene and cell doping
SUBSTANCE PROHIBITED IN PARTICULAR SPORTS
P1. Beta-blockers

1.2 New challenge of antidoping analysis and NPS

In the last fifteen years, from the introduction of the WADA code to date, there were very important milestones in the fight against doping. In 2004 at Summer Olympic Games, a reliable test for hGH was introduced [11], in 2008 it was developed the first method for determination of insulins [12], in 2009 it was introduced the Athlete Biological Passport (ABP) [13] in 2011 the Section S0 of " Non-Approved Substances" was inserted in the WADA Prohibited List, in 2014 and 2015 respectively the gas Xenon and the stabilizes of Hypoxia-inducible factors (HIF) were inserted in the Prohibited List and in Summer Olympic Game of Rio de Janeiro in 2016 the first test for Gene Doping was done[14].

Nowadays anti-doping community faces new challenges with NPS which are the same encountered by forensic and toxicology laboratories. These substances are constantly developing with new compound or classes introduced, with almost unknown structures, effects or metabolism for each compound which configure as an emerging global problem [15]. The identification of prohibited substances through spectrometric techniques requires the previous knowledge of the chemical structure of the substance and its mass spectrum, none of which are known for the NPS recently introduced in the market.

NPS could be detected into different biological matrices as not only drug itself but also as phase I and phase II metabolites. NPS are mainly analysed through MS techniques [16], which is a preferred method of analysis in doping control, or forensic analysis, due to its versatility and the possibility to establish the nature and structure of the detected substance. However, NPS could be detected with different spectroscopy techniques like Raman, IR or NMR[17–21]. These techniques are helpful for those new compounds where the structures are unknown and a characterization is required with information more detailed than MS spectra fragmentation,

becoming a support to MS techniques for the unique identification of the substances structures [17]. Strategies of NPS analysis are based on the characterization of their chemical structures and effects[22], on how NPS could be metabolized into human and how are could be found in biological matrices[23–26]. An important tool for the identification of NPS in biological matrices came from *in vivo* studies [16,27]. However, the metabolism of NPS is unknown and cannot be established by doing excretion studies in humans, since NPS are not approved drugs, and human metabolism could only come from intoxication cases (e.g. emergency room)[28,29]. Therefore, the use of *in vitro* model and *in vivo* studies through animal model (e.g. mouse) is mandatory for a metabolism study as close as possible to human [24,30]. Here a background on NPS classification and impact in doping is provided.

2 NPS

"According to the United Nations, new psychoactive substances are defined as substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat".

The term "new" does not necessarily refer to new compounds as several NPS were first synthesized more than 40 years ago, but to substances that have recently become available on the market [15].Joint Action on New Synthetic Drugs were established by Joint Action 97/396/JHA and that had been in operation since 1997,and it was strengthened in 2005 by Council Decision 2005/387/JHA. As of 23 November 2018, it operates under Regulation (EC) 1920/2006 (as amended by Regulation (EU) 2017/2101). It provides a network for the reporting of new substances called Early Warning System (EWS), which provide a risk assessment on NPS [31], an annual report on commonly abuse drug and NPS recently introduced in Europe and relative risk assessment [32,33].

The EWS is composed of a multiagency and multidisciplinary network, which includes the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 30 national early warning systems (28 EU Member States, Turkey, and Norway), Europol and its law enforcement networks, the European Medicines Agency (EMA), the European Commission, and other partners.

The EMCDDA, in cooperation with Europol, is responsible for linking and communicate the information reported by the network in order to provide initial report on an NPS that may pose health or social risks at EU. That annual draw up a report on commonly abuse drug and NPS recently introduced in Europe and relative risk assessment. By the end of 2018, the EMCDDA was monitoring more than 730 NPS, 55 of which were detected for the first time in Europe in 2018 (Fig. I.1) [32,33].

Regulating organization for NPS include not only EMCDDA, also United Nations Office on Drugs and Crime (UNODC), WADA, World Health Organization (WHO), Food and Drug Administration (FDA) [34–36] . At global level the UNODC established in 2013 a system for collecting information on NPS, similar to the EWS, called UNODC/NPS Early Warning Advisory, part of The Global SMART (Synthetics Monitoring: Analyses, Reporting and Trends) Programme " to monitor the emergence of NPS, analyse the market trends associated with NPS, provide information on legal instruments countries have used to control these substances, further support drug testing laboratories with analytical methodologies to identify NPS and support the formulation of effective measures to mitigate this problem at the international level".[43]

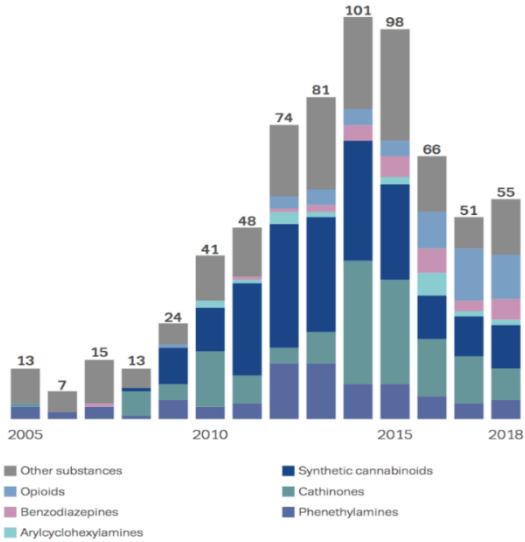

Figure I.1. Number and categories of new psychoactive substances notified to the EU Early Warning System for the first time, 2005-18. [32]

In Italy, these drugs are included in four specific tables of decree DPR 309/90, see figure 2.

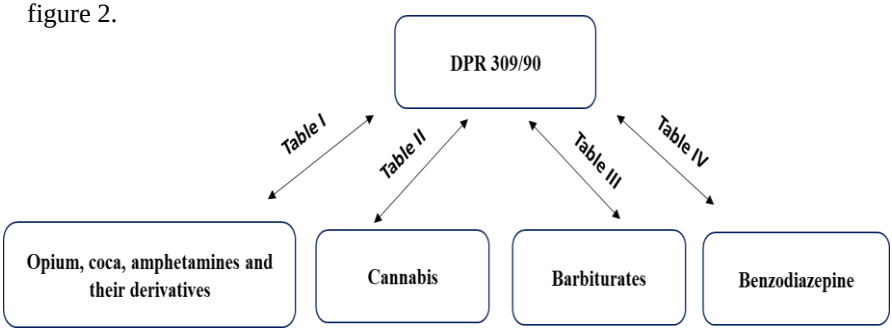

Figure I.2. Classification of illicit substances into DPR 309/90 in Italy.

2.1 NPS classification

NPS represent a large family of substances which include different classes such as synthetic cannabinoids , synthetic cathinone, piperazines, benzofurans, synthetic opioids, synthetic amphetamines etc [36–38]. These substances can be classified both for their structure or their activity. A unique classification of NPS is complex, however, according to Zawilska and Wojcieszak six classes of principal NPS can be defined, based on their pharmacological activity [39].

They are:

Table I.2. Class of NPS where compound of interest in this PhD thesis are in bold.

Class of NPS
Psychostimulants
Narcotics /hypnotics
Synthetic cannabinoids
Psychedelic compounds
Dissociatives compounds
Synthetic opioids

The structures and mechanism of action for those class of substances that are mainly linked with this PhD thesis are described in Chapter II of Part I.

2.2 NPS in doping

Psychoactive substances (PS) are one of the most commonly abused substances into sport competition [40], and they have a prominent place into the Prohibited List since 2004 [41]. In recent years the constant growth of NPS phenomena especially affect young athletes that represent the predominant group in sports[42,43]. Nowadays, NPS can be easily purchased through the Internet and in many cases could be found in dietary supplements, without properly indication of their presence on the packaging. Therefore, the risk of inadvertent doping with supplements is very high for athletes who usually employed dietary supplements. [41,43].

All PS and NPS substances are prohibited only in competition due to their short activity which increases sports performance during the competition.

Principal categories of PS prohibited in sports include also NPS and could be dived into Stimulant, Narcotics and Cannabinoids. These classes of substances are the core of this PhD thesis. Methiopropamine, Methedrone, Pyrovalerone and 4,4 DMAR are classifiable as stimulants, GVL as ascribable to narcotics and it shows also proactive effects at low dosages. Whereas CUMYL-THPINACA, ADB-CHMICA are classified as synthetic cannabinoids and they different effects including stimulant and narcotic effects useful for doping purpose.

-*Stimulants*

The US Anti-Doping Agency defines a stimulant as "An agent, especially a chemical agent such as caffeine, that temporarily arouses or accelerates physiological or organic activity"[44].

All substances with stimulant effects that could be improve sports performance are prohibited and collocated in S6 section of WADA prohibited list (Figure 3A-B), except for those included in the Monitoring Program[45].

To the date of writing, the substances included in the Monitoring Program as potential stimulants are bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol and synephrine.

PROHIBITED SUBSTANCES

S6 STIMULANTS

All stimulants, including all optical isomers, e.g. d- and l- where relevant, are prohibited.

Stimulants include:

a: Non-Specified Stimulants:

Adrafinil;
Amfepramone;
Amfetamine;
Amfetaminil;
Amiphenazole;
Benfluorex;
Benzylpiperazine;
Bromantan;
Clobenzorex;
Cocaine;
Cropropamide;
Crotetamide;
Fencamine;
Fenetylline;
Fenfluramine;
Fenproporex;
Fonturacetam [4- phenylpiracetam (carphedon)];
Furfenorex;
Lisdexamfetamine;
Mefenorex;
Mephentermine;
Mesocarb;
Metamfetamine(d-);
p-methylamfetamine;
Modafinil;
Norfenfluramine;
Phendimetrazine;
Phentermine;
Prenylamine;
Prolintane.

A stimulant not expressly listed in this section is a Specified Substance.

b: Specified Stimulants.

Including, but not limited to:

3-Methylhexan-2-amine (1,2- dimethylpentylamine);
4-Methylhexan-2-amine (methylhexaneamine);
4-Methylpentan-2-amine (1,3- dimethylbutylamine);
5-Methylhexan-2-amine (1,4- dimethylpentylamine);
Benzfetamine;
Cathine**;
Cathinone and its analogues, e.g. mephedrone, methedrone, and α - pyrrolidinovalerophenone;
Dimetamfetamine;
Ephedrine***;
Epinephrine**** (adrenaline);
Etamivan;
Etilamfetamine;
Etilefrine;
Famprofazone;
Fenbutrazate;
Fencamfamin;
Heptaminol;
Hydroxyamfetamine (parahydroxyamphetamine);
Isomethepteне;
Levmetamfetamine;
Meclofenoxate;
Methylenedioxymethamphetamine;
Methylephedrine***;
Methylphenidate;
Nikethamide;
Norfenefrine;
Octopamine;
Oxilofrine (methylsynephrine);
Pemoline;
Pentetrazol;
Phenethylamine and its derivatives;
Phenmetrazine;
Phenpromethamine;
Propylhexedrine;
Pseudoephedrine*****;

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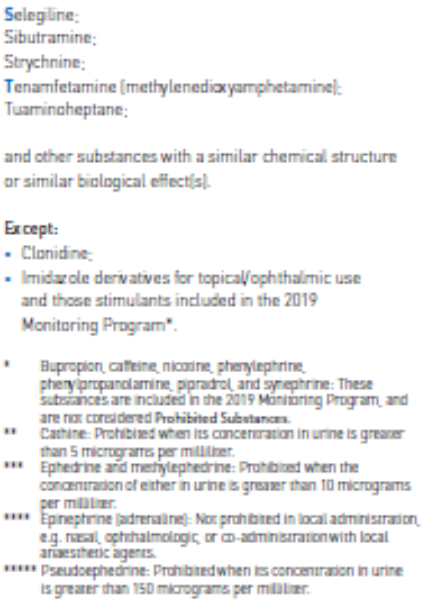
Figure I.3A. S6 section of WADA prohibited list 2019 [10].



Selegiline;
Sibutramine;
Strychnine;
Tenamfetamine (methylenedioxyamphetamine);
Tuaminoheptane;

and other substances with a similar chemical structure or similar biological effect(s).

Except:

- Clonidine;
- Imidazole derivatives for topical/ophthalmic use and those stimulants included in the 2019 Monitoring Program*.

* Bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine: These substances are included in the 2019 Monitoring Program, and are not considered **Prohibited Substances**.
** Cathine: Prohibited when its concentration in urine is greater than 5 micrograms per milliliter.
*** Ephedrine and methylephedrine: Prohibited when the concentration of either in urine is greater than 10 micrograms per milliliter.
**** Epinephrine (adrenaline): Not prohibited in local administration, e.g. nasal, ophthalmologic, or co-administration with local anaesthetic agents.
***** Pseudoephedrine: Prohibited when its concentration in urine is greater than 150 micrograms per milliliter.

Figure I.3B. S6 section of WADA prohibited list 2019 [10].

Stimulants mainly include amphetamine and cathinone derivatives as well as approved drugs (e.g. ethylphenidate, modafinil, phentermine, pseudoephedrine). NPS stimulants are derivatives of phenethylamine or cathinone easily available on internet or included in dietary supplements.

Stimulants were manufactured usually as tablets or powder (e.g for synthetic cathinone sell as “bath salt”) with characteristic logos (Figure 4), not necessary illegal at the first time they appeared and sold.

They were often marketed through criminal networks as ‘Ecstasy’, a term which initially meant MDMA or one of its homologues[31].

Figure I.4. Stimulant sold as club drugs or party drugs, (photo free available on-line)[*1]

Stimulants are divided into the Prohibited List in “specified” and “non-specified” since 2009. This classification is based on different criteria including “risk to health, general use as medications, legitimate market availability, controlled status and historical abuse in sports” [41] .

Specified stimulants are generally drugs that are more susceptible to accidental intake because they also include medical drugs or are sold as supplements where different NPS are added and not named or not properly indicated on the labels. This division is linked to the severity of sanctions and the period of ineligibility for the athlete (2 years for specified or 4 years for non-specified).[1]

[*1] https://storage.googleapis.com/stateless-bhekisisa-website/wordpress-uploads/2019/03/7ce8daa1-00-psychedelic-therapy-mdma-and-mental-health-disorders.jpeg

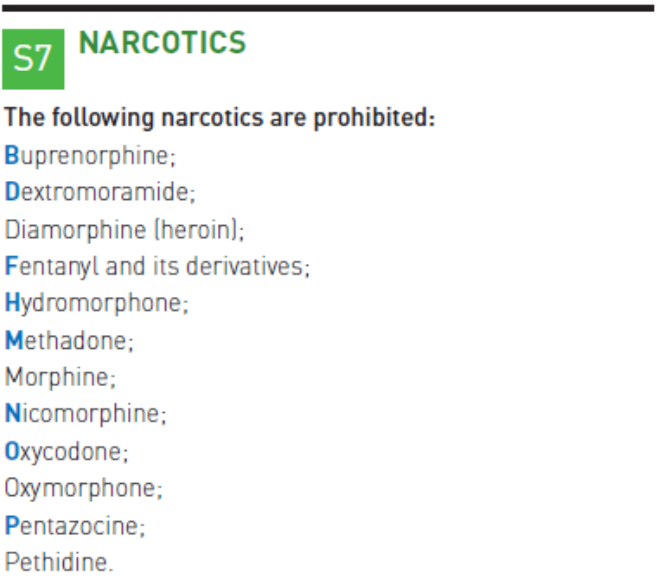
As WADA established "the Prohibited List may identify specified substances, which are particularly susceptible to unintentional anti-doping rule violations because of their general availability in medicinal products or are less likely to be successfully abused as doping agents…Athlete can establish that the use of such a specified substance was not intended to enhance sport performance"[46]. Therefore, athlete's sanction could be reduced.

The Section S6 of the WADA prohibited List is an open list that mean only exemplificative substances are direct included into the list. Substances with similar chemical structures and biological effects will be prohibited despite not explicitly indicated in the list. The open list concept is useful for those substances like NPS where newly substances with similar structure are frequently introduced into the market.

When a class became consistent, it is included by name in the Prohibited List (e.g. cathinone and analogues 2014, phenethylamines and analogues 2015)[41]. Methiopropamine and 4,4' DMAR are referable to phenethylamines class, whereas Pyrovalerone and Methedrone are part of cathinone and analogues.

- *Narcotics*

Narcotics are included in the section S7 of the WADA Prohibited list which is a closed list, mean only drugs included by name are prohibited, except for fentanyl derivatives (Figure 5). Fentanyl derivatives are also ones of the first designer drugs synthesised and sold on illicit drug market (e.g. α-methylfentanyl and 3-methylfentanyl)[31]. Therefore, an abused NPS which is recognizable as abused in sports needs to be introduced by name in the list after it was included in the WADA Monitoring Program to follow the real abuse of it among athletes. At the date of writing codeine, hydrocodone and tramadol are included into monitoring programme as suitable substances prohibited in-competition[45].



S7 NARCOTICS

The following narcotics are prohibited:

Buprenorphine;
Dextromoramide;
Diamorphine (heroin);
Fentanyl and its derivatives;
Hydromorphone;
Methadone;
Morphine;
Nicomorphine;
Oxycodone;
Oxymorphone;
Pentazocine;
Pethidine.

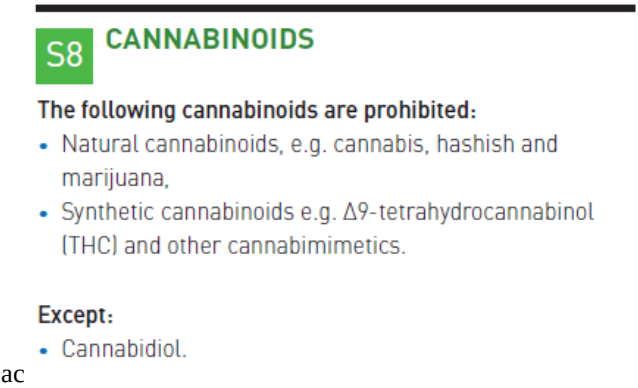
Figure I.5. S7 section of WADA prohibited list 2019 [10].

Few new substances have been introduced in the list of Narcotics (e.g. nicomorphine in 2017)[41]. However, the recent trend on GABA analogue substances, especially GHB [47], could be of impact in doping control and will be lead to the introduction of GHB and analogues into WADA Narcotics section (e.g. 1,4 butanediole or GBL[48], as well as GVL investigated into this PhD thesis). Originally, GHB was reported to be employed by competitive body builders in the 1980s for its effects in acutely facilitating slow-wave sleep, and increase on growth hormone level [31], action confirmed in different studies as well as seen for other GABA analogues [49–52]. These substances show also proactive behaviour, sexual desire[53,54] and, more important, stimulant effects at low doses [55].

Around the 1980s/early 1990s GHB was listed in Schedule IV of the United Nations Convention on Psychotropic Substances. Due to its effects GHB is sold also as "liquid ecstasy"

-*Cannabinoids*

Cannabinoids are introduced in prohibited list at the section S8. Where they are named as natural cannabinoids from *Cannabis sativa* and their products (e.g. hashish, marijuana) and synthetic cannabinoids (SCs) with cannabimimetics action (Figure 6).


S8 CANNABINOIDS

The following cannabinoids are prohibited:

- Natural cannabinoids, e.g. cannabis, hashish and marijuana,
- Synthetic cannabinoids e.g. Δ9-tetrahydrocannabinol (THC) and other cannabimimetics.

Except:

- Cannabidiol.

ac

Figure I.6. S8 section of WADA prohibited list 2019 [10].

S8 section is an open list that mean all substances which show cannabimimetics effects are intended as prohibited in-competition by WADA.

Currently WADA will commission a target study to perform metabolism studies and to develop properly detection method if a SC consume became preponderant [41] in order to set properly analytical strategies to find their intake for doping purpose.

Natural Cannabinoids were introduced in Prohibited List in 2004, followed by the introduction of first synthetic cannabinoids in 2010 because of the growing prevalence of Spice and K2 [41], first identified in smoking mixtures around 2008 [31]. These are the commercial name for mixtures of JWH018, JWH073 or HU-210.

Soon after their introduction into the market they have been abused by young athletes [56], in particular JWH018 and JWH073 were commonly found in urine specimen of athletes, Heltsley et.al. (2012)[42] found that 4.5% of urine samples collected from 5.956 athletes, analysed by high-performance liquid chromatography–tandem mass spectrometry, are positive to JWH018 and JWH073 metabolites.

SCs are sold generally as smokable powder or for oral intake with various logos and without their proper name (Figure 7), labelled as: "non for human consumption, lab tested or verified the absence of prohibited substances". Declaration of "not approval by FDA" is also common reported.

Figure I.7. Different packaging for synthetic cannabinoids, (adapted from different photo free available on-line)[*2]

[*2] https://www.health.ny.gov/professionals/narcotic/synthetic_cannabinoids/images/synthetic_cannabinoids_600x338.jpg%2F%2Fwww.health.ny.gov

Nowadays SCs are one of the most diffused and abused class of NPS. with a high potential and often unknown risks associated with their consumption [57].

Furthermore, SCs are divided into various subclasses with different structures which significantly complicated their detectability through common forensic toxicological screening. In addition, as for other NPS, reference materials for parent drug aren't available for at least 6-24 months after new substances were discovered [31], and also producer of these materials facing new challenge to speed up production processes [58]. Forensic analysis and doping control where cannabinoids are included, use urine as matrix and the metabolism of these illegal NPS was unknown.

Therefore, *in vitro* metabolic investigations, became mandatory to the identification and characterization of metabolites [59] and also to their enzymatic synthesis[41] especially for SCs where parent drug often is not detectable.

- *Not prohibited psychoactive substances*

Not all the psychoactive substances are included in the WADA Prohibited List. Indeed, a psychoactive substance (include NPS) must have specific action that allows is consumption for doping purpose. In particular a substance or method must meet at least two of the following criteria: (1) enhances the performance and poses a health risk, (2) enhances the performance and violates the spirit of sport, or (3) poses a health risk and violates the spirit of sport [60]. This means that some classic hallucinogens (e.g. LSD, psilocybin) and sedatives/hypnotics (e.g. benzodiazepines barbiturates) are not prohibited in sports due to their action as sedatives and depressants of locomotory activity, mean they match with only the third criteria. As the author of this thesis consideration, this is probably why substances like GHB are not included in WADA list because of its most known activity as a rape and depressant drug compared to its underestimated enhance activity effects at low dosage. Furthermore GHB was found in seizure in 2004 by Guardia Civil in Spain with different stimulants, anabolic and masking agents suggesting its possible employ as a doping agent [61].

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Part I

Chapter II

Background

1 Drugs: metabolism, excretion and action

For toxicological and forensic analysis, the understanding of how a substance can be excreted in different biological matrices and how long it will be excreted is mandatory. Metabolism of drugs involves different tissues and organs, liver as the most important organ, but also the kidneys, intestines, lungs, brain, nasal epithelium, and skin can be involved [1]. The metabolism of xenobiotics is often divided into two phases: modification and conjugation, followed by the excretion. These two phases are commonly known as respectively phase I and phase II metabolism. The metabolic pathways of different substances mainly involve oxidative phase I reactions mediated by different cytochromes isoforms (CYPs) which are the major enzymes responsible for drug metabolism, accounting for about 75% of the total metabolism. [2].

CYP450 are a family of enzymes containing as well as for other CYP a heme core (Figure 1) as a cofactor that function as monooxygenases [3],[4]. directly involved in metabolism [5], which play a major role in phase I oxidation reactions [6].

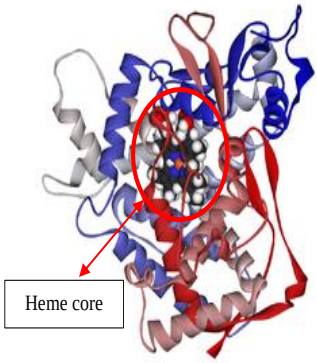

Figure II.1. Representation of cytochrome P450 oxidases

Indeed, in mammals these proteins oxidize different endogenous substances and xenobiotics, leading to an increase in hydrophilicity of substances that induce their urinary excretion as a common mechanism of body defence [7].

Humans have 57 genes and more than 59 pseudogenes divided among 18 families of cytochrome P450 genes and 43 subfamilies [8].

The most common isoforms of CYPs involved in drug metabolism are CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1, where the first number is the family (1,2,3 etc.), the letter (A, B, C etc.) is the subfamily and the last number is gene identifier (1,2,3 etc .), see Figure 2 for the scheme.

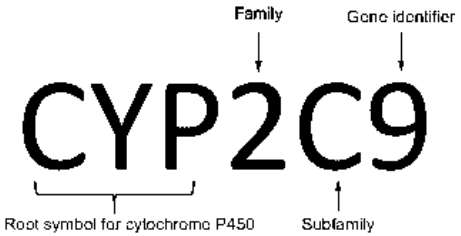

Figure II.2. scheme of nomenclature of different CYP450 into human.

-Phase I reactions

The most common phase I reactions act to incorporate an atom of oxygen into nonactivated hydrocarbons, which could result in either the introduction of hydroxyl groups or N-, O- and S-dealkylation of substrates [9]. These reactions include aryl-hydroxylation, alkyl-hydroxylation, deamination, *O*-dealkylation, *N*-dealkylation, *N*-oxidation, *S*-oxidation.

-Phase II reactions

Phase II enzymes catalysed reactions of conjugation with charged species such as glutathione, sulphate, glycine, or glucuronic acid. Products of conjugation reactions have an increased molecular weight and tend to be less active than their substrates. The addition of these groups produces more polar metabolites that cannot diffuse across membranes, and may, therefore, be actively transported. These reactions are catalysed by a large group of different transferases, which can metabolise the phase I compounds or hydrophobic compounds which contains nucleophilic or electrophilic groups. The most representative phase II enzymes are uridine 5'-diphospho-glucuronosyltransferase (UGT), glutathione S-transferase (GST), Methyl-transferase (MT), N-acetyl transferase (NAT), and sulfotransferase (ST) [10]. A summary scheme of phase I and phase II reaction is reported below in Figure 3.

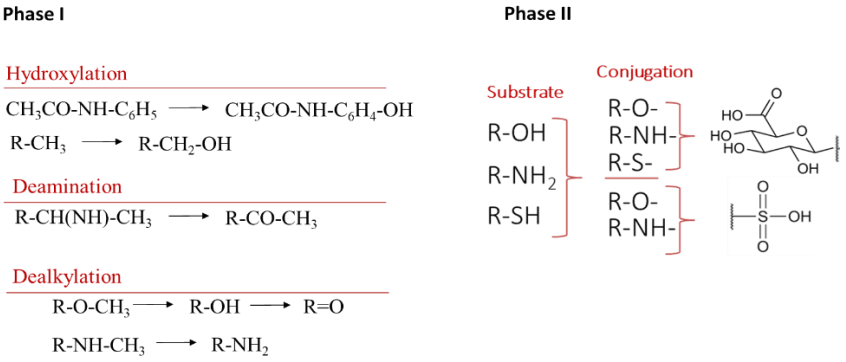

Figure II.3. scheme of common phase I and phase II reactions

1.1. Drug Excretion and biological matrices

Drug excretion is the removal of drugs from the body, as metabolite(s), after phase I and phase II metabolism, or the excretion of the unchanged drug (parent). There are different routes of excretion, including urine, bile, sweat, saliva, tears, milk, and stool, where the principal way of excretion is urine. The most important excretory organs are the kidneys and liver. Renal excretion plays an important role in eliminating unchanged drugs or their metabolites into urine, it depends on glomerular filtration, active tubular secretion, and passive tubular absorption. The main characteristics of compounds excreted in urine is that they are polarized and water-soluble.

Lipophilic drugs are not removed by the kidneys and require hepatic metabolism (e.g., phase I and phase II biotransformation reactions) to increase their water solubility for possible urinary excretion. Drugs that entering the hepatic circulation may also enter the bile and be excreted into the duodenum and small intestines.

Drugs with a molecular weight over 300 daltons and with lipophilic groups are more likely to be excreted in bile [11].

The unchanged drugs and phase I and phase II metabolites could be found into biological matrices after drug intake. A scheme of principal matrices employed in toxicological analysis is proposed in Figure 4.

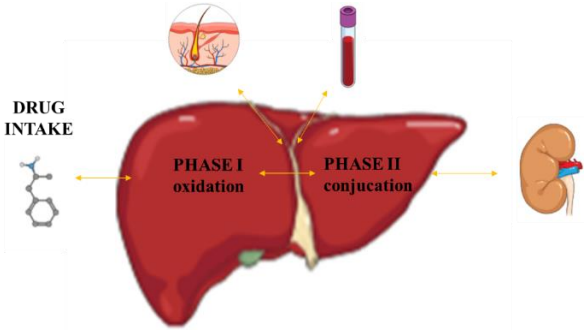

Figure II.4. scheme of metabolism and distribution into principal biological matrices employed.

In forensic toxicology, the most common matrices of excretion are urine and saliva as well as blood and hair that are not considerable as routes of excretion. However, they are both important biological matrices in forensic analysis [12–14]. Hair, urine and saliva are considered non-invasive matrices and they are employed in different toxicological analysis related to drug of abuse discovery [12,15].

Hair are useful for discovery old intake of substances as well as in control of patients with story of drug addiction [16]. Saliva is becoming an important tool in fast drug screening [17]. At the data of writing only urine and blood (whole blood plasma and serum) are employed in doping control, therefore a detailed explanation of the use of other biological matrices in forensic analysis is beyond the scope of this text.

Urine is the matrix of choice for the majority of doping control analysis due to the non-invasive sampling and to the possibility to link excretion data with time of intake. Urine is employed for discovery of all synthetic stimulants, cannabinoids and narcotics (field of study of this thesis), where blood is employed only for identification of "hematic doping" (e.g. determination of: Grow Hormone, erythropoietin, blood transfusion etc.). Furthermore, urine shows long storage stability (especially at -80° C) proved for a variety of substances, easy sampling and managing and also sample treatment. The low volume used for anti-doping analysis allow to employ different aliquot of samples for different drug screening or repetition and confirmation of a sample.

However, possible alternatives in doping analysis were proposed as dried blood spots, dried plasma spots, oral fluid, exhaled breath, and hair [18] and could became useful.

1.2 Models for metabolism studies

Drug metabolism studies are required for the evaluation of drugs of abuse excretion in biological matrices and how they could be identified and how long they are excreted. Different approaches are employed for metabolism studies which include different *in vitro* or *in vivo* models. *In vitro* models include: human liver microsomes (HLM), supersome, liver cytosol, S9 fraction, cell lines or transgenic cell lines, hepatocytes, liver slices, isolated perfused liver [19]. *In vivo* models employ different animals depending on the purpose of studies. For drug metabolism studies, the most employed models are murine, zebra fish and rats. In this PhD work, the focus is on human liver microsomes and murine model.

-Human liver microsomes

Human liver microsomes (HLM) are subcellular fractions from the endoplasmic reticulum of hepatic cells, which contain different metabolic enzymes like cytochrome P450s, flavin monooxygenases, carboxyl esterase and epoxide hydrolase[20]. They are obtained by homogenisation and centrifugation of liver hepatocytes and also from liver slices or liver cell lines [19]. HLM were used to study phase I oxidation or phase II conjugation, depending on cofactor or enzymes added to the mixture. However, HLM metabolism studies show inter-individual variation on their activity, for this reason HLM preparation employed pooled microsomes from donors by different ethnises and gender [21], with the aim to reduce as possible variability. Individual CYPs are also employed to identify specific CYP isoforms involved into metabolic pathways of xenobiotics[22], as a tool to estimate and predict possible drug-drug interaction.

In this PhD work, metabolism studies were carried out performing incubations in the presence of HLM and selected drugs at controlled pH by phosphate buffer and specific cofactors as NADPH generating system (NADP + glucose-6-phosphate dehydrogenase), phase II reaction were, instead, evaluated by in vivo studies. The obtained mixtures were incubated at different time points (10, 25, 40, 60 min, etc.) in a shaking system at a controlled temperature of 37 °C with the aim to simulate biological conditions [23,24]. All data of donors of the enzymatic pool employed are free available at the site of the manufacturer, related to specific product code, no ethical approval is requested for they employ in this type of study. The employed HLM are tested for relevant pathogens. Donors health conditions do not influence the quality of in vitro studies but give the necessary diversity between subjects ensuring a more representative result possible. The reaction is terminated by adding, cold acetonitrile, to precipitate proteins and to stop the metabolic reactions. Incubation medium were subsequently extracted and analysed with properly procedures. See Figure 5 for scheme of generic protocol for *in vitro* studies.

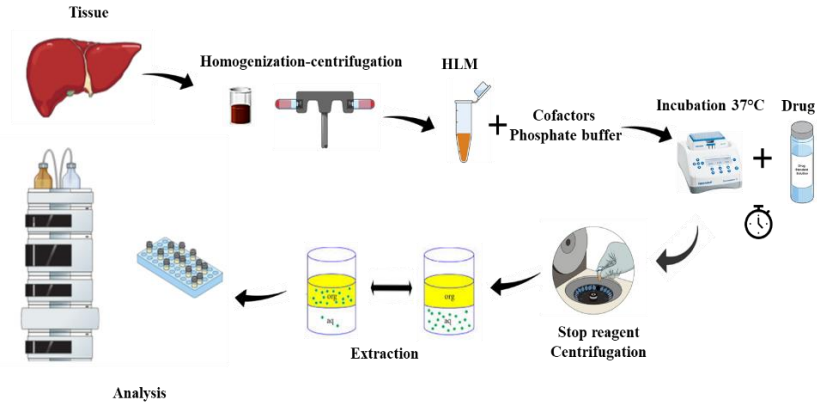

Figure II.5. scheme of generic protocol for in vitro studies with human liver microsomes

The use of HLM shows different advantages as long storage (at −80 °C until use), facility of use and good fit with human *in vivo* data. However, the presence of high levels of CYPs and the absence of other enzymes (e.g NAT, ST, GST) or cytosolic cofactors represent a discrepancy from *in vivo* data. This leads to the use of *in vivo* metabolic studies to understand of how drugs were metabolized and excreted.

-Murine model

Mice are a largely employed animal model for the study of genetic, physiology, diseases and drug metabolism both for abuse and pharmaceutical drugs[25]. Mice present different advantages in drug metabolism studies which includes reduced size and easy reproduction cycle, low husbandry and maintenance costs compared to other mammalians. Furthermore, they present counterparts for all human genes[26]. In particular, mouse has 36 orthologous pair of CYPs with similar function of humans CYP 450 isoforms [27], it configures mice as a human fit model for metabolism studies in forensic toxicology, which involve CYP 450 isoforms oxidative reaction [2]. Different studies show good similarities between human and mice CYPs isoforms (e.g. CYP1A2 or CYP3A subfamily [28,29]).

However, despite these advantages and similarities, there are markable interspecies variations in the expression, activities, and inducibility of CYP enzymes between human and mice, especially for CYP2A subfamily. Although these differences are a disadvantage for metabolism studies, mice are the most valuable animal model for drug metabolism research [25,26].

In this PhD work, all the *in vivo* metabolism studies were carried out employing Male ICR (CD-1®) mice, 25-30 gr, were purchased from Harlan Italy (S. Pietro al Natisone, Italy) with a food diet based on Diet 4RF25 GLP purchased from Mucedola (Settimo Milanese, Milan, Italy). Behavioural studies were performed from department of morphology, surgery and experimental medicine, section of legal medicine, University of Ferrara, Italy.

For the collection of urine and blood samples for metabolism studies, mice were group-housed (8-10 mice per cage; a floor area per animal of 80 cm^2; a minimum enclosure height of 12 cm) in a colony room under a constant temperature (23-24 °C) and humidity (45-55%).

Tap water was available ad libitum during the entire time the animals spent in their home cages. Daylight cycle was artificially maintained (dark between 7 p.m.–7 a.m.). The experiments were performed during the light phase, with only one mouse used per experiment.

Different groups of mice were administered with the selected drugs of abuse and urine and blood samples were collected at different time points and analysed, with the aim to estimate excretion of substances or their affection on biological parameters (i.e., steroidal profile or IGF-1). For the phase II metabolism studies, enzymatic mixture was added to perform the conversion of phase II into phase I metabolites and subsequently extracted with the most appropriate procedure and analysed, (Figure 6).

Figure II 6. scheme of generic protocol for in vitro studies with human liver microsomes

The experimental protocol followed was in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of the Directive 86/609/EEC, and was approved by the **Italian Ministry of Health** (license n. 335/2016-PR) and the **Ethics Committee** of the University of Ferrara.
Moreover, adequate measures were taken to minimize the number of animals used and any subsequent discomfort. This applies to **all** *in vivo* studies conducted in this PhD thesis, although not stated in the individual chapters

1.3 Psychostimulants

These compounds are derivatives of different common drugs of abuse which include amphetamine and cathinone derivatives. The most abused NPS of this class are ascribable to phenethylamine derivatives, which include also amphetamine and cathinone substituted. Phenethylamine can be substituted in different positions (Figure 7) that are aromatic substitute (R2-6), alkyl-substituted (R α,β) and N-substitute(R^N).

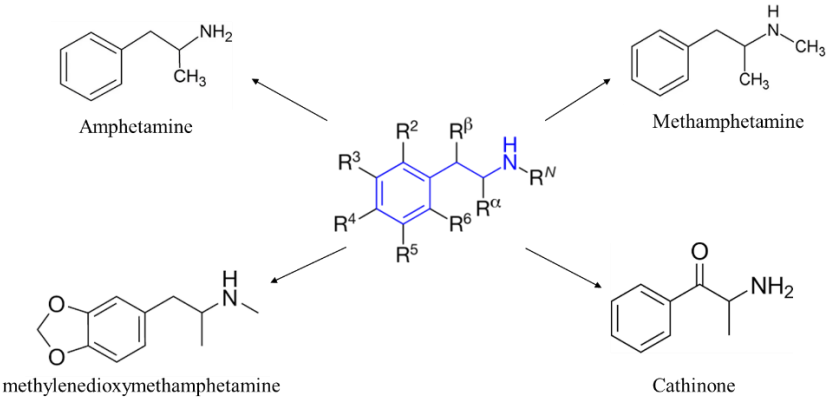

Figure II.7. Structure of phenethylamine derivatives with position of substitution (R=H for phenethylamine) and common derivatives: amphetamine, methamphetamine (METH), Cathinone (Kat), Methylenedioxymethamphetamine (MDMA or ecstasy).

-*Mechanism of action*

Stimulants generally act on the monoaminergic systems: adrenergic (sympathetic, transmitter noradrenaline), dopaminergic (transmitter dopamine) and serotonergic (serotonin, 5-HT). However, many agents act to mimic more than one of these monoamines, and they are call monoaminomimietic action stimulants which include activities as block of re-uptake or, indirect release of neurotransmitters, direct activation of monoaminergic receptors.

Phenethylamine could present stimulant and also hallucinogenic effects (e.g 25-NBOme derivatives) due to their activity on serotonin receptors (e.g. 5-HT2A receptor for 25C-NBOme, a dangerous 25-NBOme derivatives [30]) and also inhibiting action on monoamines reuptake. Phenethylamine derivatives also increase synaptic levels of monoamines by inhibiting their reuptake. In details, they exert effects by altering the use of monoamines as neuronal signals in the brain, primarily in catecholamine neurons [31,32].

The concentrations of the main neurotransmitters involved in signalling function, were increased by acute action of this class of substances in particular dopamine (DA), serotonin (5-HT), and norepinephrine (NE) were significant increased [33–37]. The proactivity effects of substances like amphetamine derivatives, which are included in phenethylamine class, is due to enhanced dopaminergic activity in the mesolimbic pathway [38]. The euphoric and locomotor-stimulating effects of these derivatives are dependent by the level of synaptic dopamine and norepinephrine concentrations in the striatum [39]. There is no mechanism of action or biological target that is common to all members of this subclass. However, most of the phenethylamines that act on DA transporter display the following mechanism. The mechanism is divided into three steps: first, phenethylamine is a substrate for the DA transporter (DAT) that competitively inhibits DA uptake; second, it facilitates the movement of DA out of vesicles and into the synaptic space; and third, it promotes DAT-mediated reverse-transport of DA into the synaptic cleft independently of action-potential-induced vesicular release. Here described below the phenomena of dopamine release from vesicle that increase the DA level into synaptic space after the intake of amphetamine (Figure 8).

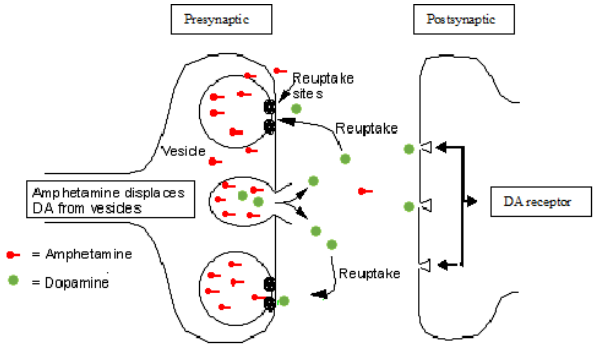

Figure II.8. Release of Dopamine from vesicle into presynaptic neuron that increase dopamine level into synaptic space. Amphetamine also inhibit dopamine reuptake.

-*Stimulant metabolism and detectability*

Phenethylamines present different phase I metabolic pathways which include, aryl-hydroxylation into different positions especially in para- position of aryl ring; alkyl-hydroxylation especially in β position from amino group and subsequently oxidised of hydroxy group, converted into a carboxy group.

N-demethylation for those derivatives which present a R^N substitute as well as deamination with conversion of amino group into carboxy one. This is verifiable especially for amphetamine derivatives [40] or cathinone derivatives [41].

The common metabolic pathways for phase II metabolism include glucorono- and sulfo- conjugate forms. However, stimulant phenethylamines derivatives were found in urine commonly as the parent compound or its demethylated metabolite as the principal markers of intake (e.g. amphetamine and methamphetamine) and could be found in urine over 36 hours after the intake[42].

A common cathinone derivative, Mephedrone, metabolic pathways are reported below in Figure 9 as an example of typical pathway of stimulants.

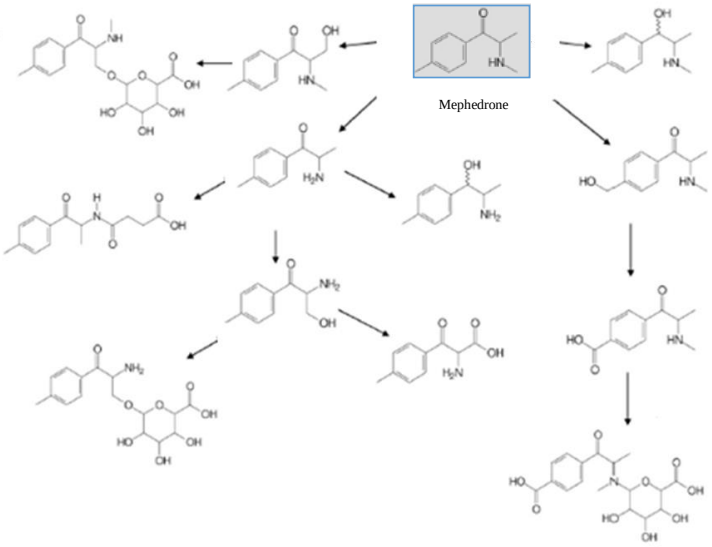

Figure II.9. Scheme of metabolic pathways of Mephedrone adapted from Pozo et.al. [51]

1.4 Narcotics

Narcotics are a non-homogeneous class of substances, not attributable to specific individual structures. The main narcotics employed were opioids derivatives [43] (e.g. methadone, morphine oxycodone). Opiates are alkaloid compounds naturally found in the opium poppy plant *Papaver somniferum*.

The psychoactive compounds found in the opium plant includes morphine, codeine, and thebaine. Opioids are substances classified for their action of bind to opioid receptors in the brain (including antagonists) [44].

In this PhD work the focus is on GVL, a substance which is attributable to a particular class of narcotics called generally GABA analogues or more properly GABA-A/B/C receptor agonists [45]. This class of substances is a newly class of narcotics which include both pharmaceutical and abuse drugs with effects on human similar to other narcotics. However, GABA analogues could show stimulant effects at low dosage an induced proactive behaviour [46].

- *GABA analogues*

Gamma-aminobutyric acid (GABA) is an amino acid and neurotransmitter, that is a significant component of the free amino acid pool. GABA has an amino group on the γ-carbon and it exists in an unbound form. Due to its carboxy group it is highly soluble in water and it can assume several conformations in solution, including a cyclic structure [47] as well as possible for other GABA analogues. GABA is zwitterionic at physiological pH values (pK values of 4.03 and 10.56). GABA is also responsible for the regulation of muscle tone [48], therefore GABA analogues could be of interest in doping field. One of the most representative compounds of this class is GHB that is an endogenous substance found in human body where is a neurotransmitter [49]. GHB is also known as sodium oxybate, sodium 4-hydroxybutyrate, and 4-hydroxy-n-butyric acid [50].

GHB is employed for treatment of alcohol withdrawal [51] as well as for the treatment of narcolepsy [52]. Different GHB analogues were available and sold on illicit market with the aim to elude law restriction on GHB, they are GBL, 1,4 butanediol and other minor GABA analogues [53].

They are employed at low dosage as recreational and proactive drugs, involved into many cases of acute toxicity [53-56] as well as "drug facilitated sexual assault" at higher dose [57-59]

-Mechanism of action

The mechanism of action of GABA analogues is similar among different compounds of this class. However, there are some important differences in how they interact with GABA receptor and, therefore, exert their effects [60]. GHB interferes with GABAergic neurotransmission and it interacts with its own receptor for GHB. It is considered as a weak agonist of GABA-B receptor [61]. GHB is synthesized from GABA in GABAergic neurons. GHB exerts most of its effects through binding to the GABA-B receptor complex but also with its GHB receptor [62,63]. At doses employed for therapeutic purpose, GHB activates GABA-B receptors, which are primarily responsible for its sedative effects.

The activation of both GABA and GHB receptors is responsible for the addictive profile of GHB and its effect on dopamine release [54]. GHB act on dopamine level in a dose dependent way. At low doses GHB stimulates dopamine release, where at higher doses it inhibits dopamine release through its agonist interaction with GABA(B) receptor. Furthermore, at higher dose the first inhibit of dopamine release is followed by a dopamine release through interaction with GHB receptor [63]. Therefore, GHB presents both stimulant and depressant effects.

The effects of GHB occurs 15-30 min after ingestion and lasts for up to 1-6 hours depending on the dose [54].

Here proposed below the principal compounds of GABA analogues class (Figure 10). Where hydroxyvaleric-acid (GHV) is the open ring active form of GVL, as well as for GHB and γ-butirrolactone (GBL). The lactone forms are spontaneously converted into acid forms by human lactonase. 1,4 BD is 1,4 butanediol which is converted into GHB.

GHV and GHB interacts with both GHB and GABA-B receptor where GABA interact only with its own receptor (i.e A, B and C isoforms).

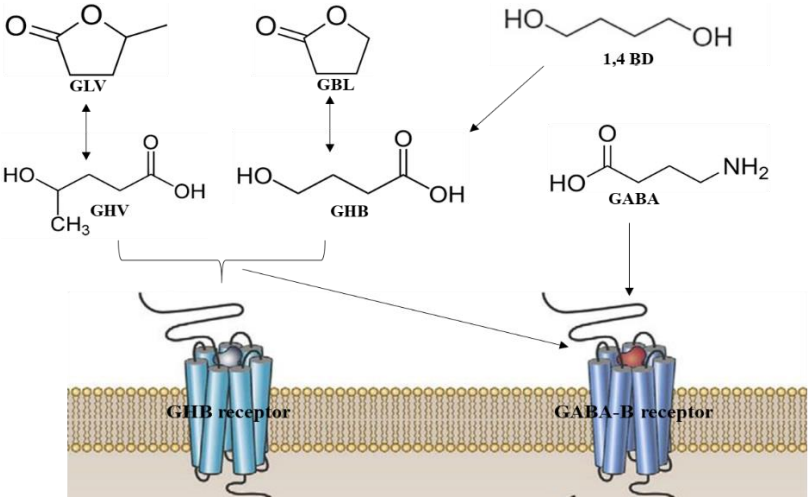

Figure II.10. Principal analogues of GABA and their action on GHB and GABA-B receptor

-Narcotics metabolism and detectability

In view of the similarities between GHB/GBL and GHV/GVL, the metabolism and excretion profile of GHB in biological matrices is described among the GABA analogues.

GHB undergoes to hepatic metabolism after intake in a percentage above 95-98%. As an endogenous substance related to GABA its biosynthesis and metabolism are closely linked.

In details, GHB is oxidised by a specific dehydrogenase (4-hydroxybutyrate dehydrogenase) which convert GHB into succinic semialdehyde and next into succinic acid, an intermediate of the Krebs cycle [54]. The small fraction of 2-5 % of GHB not converted by this metabolic pathway is excreted unchanged or conjugated. Endogenous and exogenous GHB are also excreted as GHB glucuronide or GHB sulphate [64-66]. The detectability of exogenous GHB into biological fluids is linked to the endogenous levels. Trace of GHB are present in body fluids and tissues from healthy subjects. Concentration of GHB in blood sample ranged from 0.17 mg/L to 1.51 mg/L [67]. The range for GHB in urine ranged from 0.34 mg/L to 5.7 mg/L [67-69]. These results led to the introduction of specific cut-off in blood (5mg/l) and urine (10 mg/L) to discriminate endogenous level by an intake[70]. The detectability of GHB into blood range from 2 to 4 hours by the intake with T_{max} from whole blood and serum analysis respectively of 24.6 and 23.8 min.

Detectability in urine ranged from 4 to 6 hours with a T_{max} occurred at 1 h [71].

Here reported a resume scheme of GHB metabolism and excretion, Figure 11.

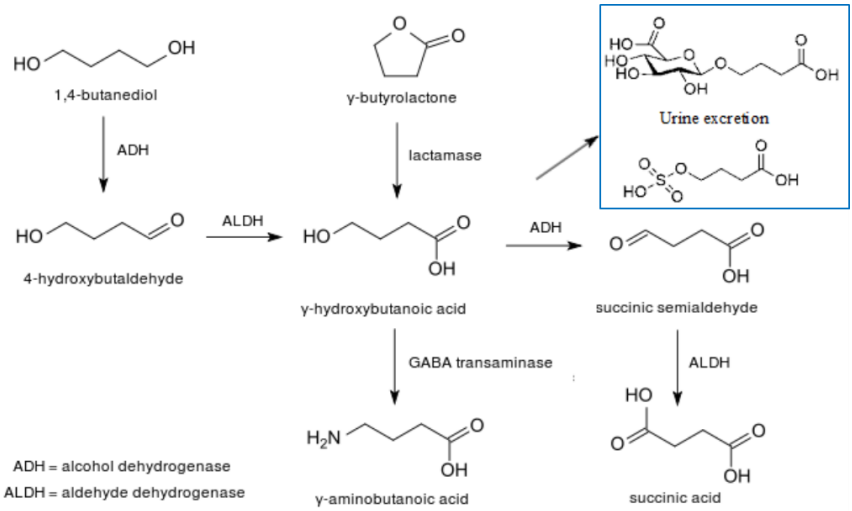

Figure II.11. Metabolism of GHB and urinary excretion of conjugates GHB.

1.5 Synthetic Cannabinoids

Cannabinoids are a group of active terpenophenols derived from 2-substituted 5-amyl-resorcinol, which are found in *Cannabis sativa* (i.e Δ-9- tetrahydrocannabinol, Δ-8-tetrahydrocannabinol cannabinol, and cannabidiol) [72,73], (Figure 12) as well as their synthetic analogues [74].

SCs exert their action through the binding of human cannabinoid receptors. Endocannabinoids are intercellular messengers, with role similar to the monoamine neurotransmitters on dopamine, that act on human cannabinoid receptors [75].

These receptors are dived into two subtypes as CB1 and CB2 both coupled to G-protein.

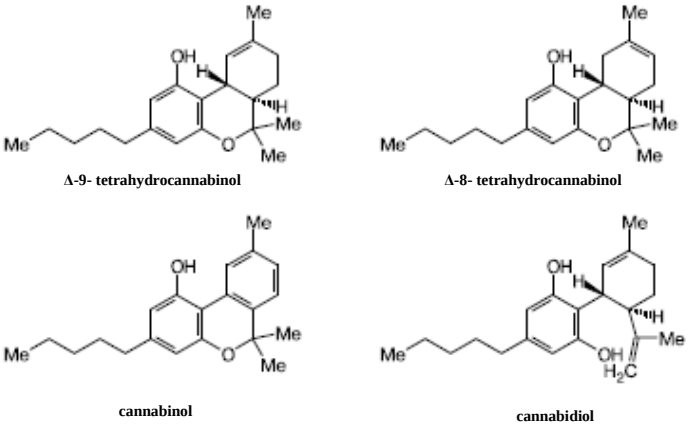

Figure II.12. Structures of principal cannabinoids in Cannabis Sativa

SCs could be divided into different subclasses. There are seven major categories: classical cannabinoids, non-classical cannabinoids, hybrid cannabinoids, carbonyl-indoles, carbonyl-indazoles, eicosanoids and miscellaneous cannabinoids [76]. However, the structural classification of SCs is constantly evolving, which makes their detection in human fluid samples particularly difficult [77].

-Mechanism of action

SCs exert their effects with higher affinity for CB1 and CB2 cannabinoid receptors than natural cannabinoids from *Cannabis sativa*. This action induces long-lasting effects and contribute to different side effects following their intake. Biotransformation of SCs can produce active metabolites, which may extend and intensify CB1 receptors activation [78].

Furthermore, SCs interfere with other receptors where act as inhibitors of the nicotinic acetylcholine receptor and agonists of the ionotropic glutamate receptor, modulate glycine and 5-HT, and inhibit monoamine oxidase activity, which exposes the subject to an enhanced risk of serotonin syndrome, like is observable for amphetamines [79]. Symptoms of serotonin syndrome include important side effects, which are: high body temperature, agitation, increased reflexes, tremor, sweating, dilated pupils, and diarrhea. Body temperature can increase to greater than 41.1 °C (106.0 °F). Complications may include seizures and extensive muscle breakdown [80]

-Synthetic cannabinoids metabolism and detectability

In view of the wide variety of SCs it is not possible to define specific metabolic pathways for all subclasses. However, SCs are commonly characterized by an extensive phase I metabolism with multiple mono-, di-and tri- hydroxylation on aryl or alkyl chain, as well as formation of carboxy group from hydroxy group [81,82]. For indazoles and indoles (like CUMYL THPINACA and ADB CHMICA) the major metabolic pathways were alkyl and indazole/indoles hydroxylation, terminal amide hydrolysis or N-dealkylation, subsequent glucurono- conjugations, and dehydrogenation[80,81], carboxyl acid formation is also possible for SCs [82-85].

Usually parent compound was not detected in biological fluids due to the extensive metabolism as proved by different studies on SCs excretion profile [85-88]. The detection time of SCs metabolites could be very long compared to other drugs of abuse.

Metabolites of JWH-018 and JWH-073 were detected in urine for 2–3 days with maximum concentrations at 3–16.5 h after smoking a single dose and are detectable in urine for about 2–3 weeks after ending a chronic use [89]. Furthermore, urine specimens could be positive for carboxy-metabolites for more than 3-6 weeks after ingestion of SCs [90].

Here it is proposed the typical metabolic pathways of a SC based on an indole ring structure, Figure 13.

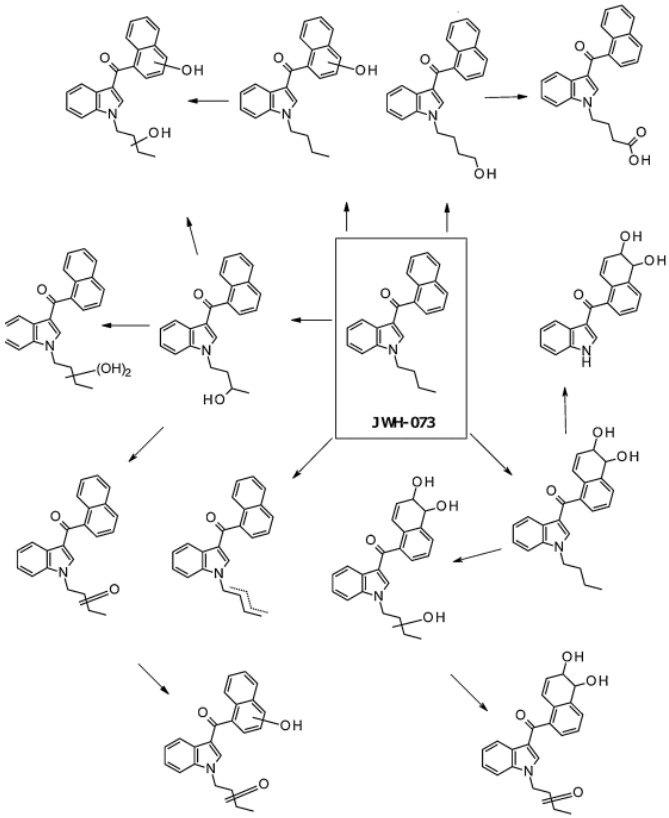

Figure II.13. Structures of metabolic pathways of JWH-073 adapted from Watanabe et.al 2016 [91]

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Part II

Chapter III

Evaluation of the *in vitro* and *in vivo* metabolic profile of Methiopropamine

Adapted from:

C. Camuto, S. Pellegrini, F. De Giorgio, X. de la Torre ,M. Marti, M. Mazzarino, F. Botrè. "*In vitro metabolic study of 2-methiopropamine and urinary excretion profile in mice following intraperitoneal administration: a liquid chromatography-tandem mass spectrometry investigation*". Submitted paper

Abstract

We have considered the urinary excretion profile of 2-methiopropamine, a thiophene ring-based structural analogue of methamphetamine with similar stimulant effects, with the aim of selecting the most appropriate marker(s) of intake that may be useful in forensic analysis. For this purpose, *in vitro* studies were preliminarily performed on human liver microsomes for tracing the phase I metabolic pathways of 2-methiopropamine, pre-selecting the best candidates as potential target analytes and designing the optimal experimental strategy. *In vivo* studies were then conducted on mice, following the intraperitoneal administration of a 10-mg/kg dose. Urine samples were collected every 3 h from 0 to 9 hours and, subsequently, from 24 to 36 hours after the administration, stored at - 80 ° C until analysis, and analysed by a newly-developed procedure based on liquid/liquid extraction, followed by instrumental analysis by liquid chromatography-tandem mass spectrometry. Our results show that 0–9 h after administration, methiopropamine is extensively oxidised mainly to nor-methiopropamine, two hydroxy (i.e., hydroxy-aryl-methiopropamine and hydroxy-alkyl-methiopropamine), and oxo-methiopropamine phase I metabolites. All the phase I metabolites underwent phase II metabolism with the formation of nor-hydroxy-methiopropamine only in phase II, confirmed by the results obtained following enzymatic hydrolysis with β-glucuronidase and arylsulfatase. In the time interval 24–36 h after administration, only unchanged methiopropamine and nor-methiopropamine were identified, suggesting that these markers have the highest diagnostic value. The method was validated for these two principal markers, proving to be fit for the purpose of anti-doping, toxicological, and forensic analysis.

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1 Introduction

The continuous increase in the synthesis of new psychoactive substances (NPSs) has drastically changed the drug scene and consumption among drug users [1,2]. Among synthetic stimulants, amphetamine derivatives are, perhaps, the most abused compounds, primarily for their stimulant and hallucinogenic properties [3], but also owing to the fact that their detection could be problematic in routine drug tests [4-5]. Although stimulant drugs have been traditionally widely abused for doping purposes, the use of synthetic amphetamine derivatives was not very widespread in the first decade of the 2000s [6]. However, the continuous increase in the synthesis of NPS is becoming a potential issue for the anti-doping community.

This study was focused on 2-methiopropamine(1-[thiophen-2-yl]-2-methylaminopropane, MPA), most commonly known as a synthetic methamphetamine analogue in which the benzene ring is bioisosterically replaced by a thiophene ring [7]. Similar to methamphetamine, the presence of the N-methyl group in MPA increases its lipophilicity, thus enhancing its ability to cross the blood-brain barrier and, consequently, its activity and toxicity [8]. Despite being a relatively 'old' compound (i.e., it was first synthesised in 1942 by Blicke and Burckhalter), MPA was first detected as a recreational drug in Finland in January 2011 [7, 9-10]. Its diffusion has been confirmed by subsequent research in the United Kingdom and supported by the analysis of anonymous urine samples collected from street urinals in London [11]. MPA is easily available by itself or mixed with other substances, known as 'Slush Eric' [7], 'Blow' [12], and 'Synthacaine' (a mixture of methiopropamine and 2-aminoindane) at a low cost on various websites [10]. At low doses, MPA is a functional stimulant that creates euphoria, stimulation, and alertness similar to methamphetamine (METH) [14- 15].

Briefly, similar to METH and other psychostimulant drugs, MPA inhibits norepinephrine (Ki ~ 313 nM) and dopamine (Ki ~ 577 nM) reuptake and, to a lesser extent, serotonin (Ki > 10.000 nM) reuptake, showing a greater affinity for the noradrenaline transporters (NET) [16], further confirming its possible use as a recreational stimulant substance, as well as in sport doping. The most common routes of administering MPA are oral, intranasal, and inhalation (e.g., smoking); the most commonly reported adverse effects are vasoconstriction, insomnia, nausea and vomiting, skin irritation, increased heart rate, increased sweating, dizziness, decreased energy, difficulty in urinating, and chest pain [17- 18]. In our recent studies, we have investigated the histological changes in CD-1 male mice following the chronic administration of MPA. Notably, we demonstrated that the mice chronically treated with MPA evidenced myocardial damage and gastrointestinal ischemia, with ischemic-necrotic lesions of variable extent. Acute MPA administration significantly increased their heart rate and promoted vasoconstriction, which were associated with the sudden death of a subset of animals (40% of all chronically treated mice) [19].

Numerous cases of non-fatal intoxication have been reported in the literature [20-21], with some cases, reporting death owing to the intake of MPA; indeed, MPA was detected in 10 deaths in 2013, 27 deaths in 2014, 23 deaths in 2015, and 2 deaths in 2016 [20]. A case of isolated MPA intake has been reported [7].

Despite its toxicological properties and growing diffusion, only a few countries have enacted legislation covering the abuse of MPA: Florida and Ohio in the United States (US), Belarus, China, Denmark, Estonia, Germany, Hungary, Portugal, Slovenia, Sweden, Turkey, and Switzerland. MPA is under a temporary drug classification in the United Kingdom [22, 9].

Previous studies have outlined the phase I and phase II metabolism of MPA in both human and animal models, such as rats [23, 20, 9]. Nonetheless, to the best of our knowledge, no information is yet available on the urinary excretion windows of MPA and its metabolites. The aim of the present study was to select the most appropriate marker(s) of MPA intake by investigating both its metabolism on *in vitro* model systems, and its excretion profile in urine through the murine model, followed up to 36 h after the intraperitoneal administration of MPA.

2 Experimental

2.1 Chemicals

MPA and nor-MPA, also known as thiopropamine, were purchased from Chebios (Rome, Italy); methamphetamine (used as an internal standard) was purchased from Sigma-Aldrich (Milan, Italy). All the reagents (i.e., formic acid, acetic acid, ammonium formate, ammonium acetate, sodium phosphate, sodium chloride, sodium hydrogen phosphate, potassium carbonate, potassium hydrogen carbonate, acetonitrile, absolute ethanol, methanol, dimethylsulfoxide [DMSO] diethyl ether, ethylacetate, and *tert*-butyl methyl-ether) were of analytical grade and provided by Sigma-Aldrich (Milan, Italy). Milli-Q (Millipore Italia, Vimodrone, Milan, Italy) ultra-purified water was used in the study; the enzyme mixture β-glucuronidase/arylsulfatase (from *Helix pomatia*), used for the enzymatic hydrolysis of both the glucurono and sulphate-conjugates, was purchased from Roche (Monza, Italy).

The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian male and female donors of different ages), and all the reagents used for the *in vitro* metabolism experiments (sodium phosphate buffer and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milan, Italy).

2.2 Animals and dose

Male ICR (CD-1®) mice for the *in vivo* studies, a group of five mice was selected, and each mouse was administered a single dose of 10 mg/kg MPA at 9:00 am, and urine samples were collected at different time. A control group of five mice was also selected for urine blank samples.

2.3 Protocol for the *in vitro* studies

All incubation conditions for MPA were optimised (proteins and substrate concentrations, buffer and solvent types, and incubation times), starting from the protocols already published and used by our group [24-25]. Different solvents (methanol, DMSO and acetonitrile), pH values (5.0, 7.4, and 9.0), concentrations of MPA and HLM (0.1, 0.2, 0.5 and 1.0 mg/mL), and incubation times (1, 2, 4, 8, 12, and 24 h) were evaluated with phosphate buffer. The final incubation medium also contained 2 μL of a MPA standard solution (1 mg/mL), 3.3 mM magnesium chloride, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/mL glucose-6-phosphate dehydrogenase in a total volume of 250 μL. Samples were pre-warmed at 37 °C for 5 min, and the phase I reactions were started with the addition of HLM. After incubation at 37 °C, 250 μL of ice-cold acetonitrile was added to stop the phase I reactions. The samples were then transferred to an ice bath for further precipitation of the proteins in the assay medium. The precipitate was subsequently separated from the supernatant by centrifugation at 14.000 rpm at room temperature for 10 min. Each set of assays also included a negative control sample that contained all reaction mixture components excluding the enzymatic proteins to monitor the potential non-enzymatic reactions**.** Each incubation was processed in triplicate.

2.4 Protocol for the *in vivo* studies

-Sample collection

Urine samples were collected every 3 h for 0–9 and 24–36 h after the injection of MPA with the aim of defining the best marker(s) of intake across different periods of time after administration. If not immediately processed and assayed, urine samples were stocked at -80°C after collection and until the analysis. Urine blank samples were also collected in the same hour range from the control mice group.

-Samples pre-treatment

Sample preparation was optimised using the sample obtained after incubation of MPA with HLM, and testing under different conditions (i.e., pH, buffer, and solvent of extraction). The following protocol was selected for the *in vivo* samples as most effective for the extraction of MPA and its metabolites. First, 200 µL of a urine sample were added to 50 µL of the standard solution of methamphetamine (final concentration of 250 ng/mL). The sample was then buffered with 100 µL of a 2 M carbonate buffer (pH 9) and added to 2 mL of *tert*-butyl methyl-ether. After 20 min of gentle stirring, the samples were centrifugated at 3000 rpm for 2 min and transferred to an ice bath for 5 min. The organic layer was then collected and evaporated until dry under nitrogen flow at room temperature. The final residue was dissolved in 50 µL of the mobile phase and then analysed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The aqueous layer was stored for later use in the phase II metabolism studies. Subsequently, the aqueous layer was added to 200 µL of the 2-M acetate buffer (pH 5), 50 µL of the standard solution of methamphetamine (final concentration of 100 ng/mL), and 20 µL of β-glucuronidase/arylsulfatase for the hydrolysis of the glucorono and sulphate conjugates metabolites.

The phase II metabolites were converted into phase I metabolites after the enzymatic hydrolysis by incubation at 55°C for 1.5h, and subsequently as analysed as phase I metabolites. The samples were then added to 300 µL of the carbonate buffer 2M following the same procedure described above after buffer addition.

2.5 Instrumental conditions

Samples were analysed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO C18 column (15 cm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass spectrometer (Sciex) with an ESI source, and operated in the positive-ion mode. Detailed analyses were carried out at constant flow rate of 250 µL/min using mobile-phase ultra purified water, 0.1% formic acid (A), and acetonitrile 0.1% formic acid (B). The selected gradient program started at 2% of B and increased to 30% of B in 10 min, after 3 min up to 60% B in 5 min, and then after 4 min to 100% B.

Multiple reaction monitoring (MRM) was used as the acquisition mode for detection (see Table 1 for the ion transitions selected). For the MRM collision-induced dissociation (CID), nitrogen was used as the collision gas at 5.8 mPa, obtained from a dedicated Parker-Balston nitrogen generator system (model 75-A74) with 99.5% gas purity (CPS Analitica Milan, Italy). The mass spectrometric parameters (declustering and needle voltages, gas pressure, source temperature, collision cell exit potential, and collision energy) were optimised by infusing the standard solution of methiopropamine at a concentration of 10 µg/mL.

All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by Analyst software version 1.6.1

2.6 Method Validation

The method was qualitatively validated for two principal metabolites, MPA and nor-MPA, as defined by sensitivity, limit of detection (LOD), recovery, and repeatability. Results are in accordance with WADA guidelines for minimum required performance level for non-threshold substances identified in urine. For non-threshold substances quantification of analytes and relative parameters (e.g LOQ, linearity) are not required by WADA.

-*Selectivity*

Selectivity was assessed by analysing blank mouse urine samples, following the same protocol of positive samples to verify the presence of compounds that may interfere with the specific precursor and product ions set into the method.

In view of the potential application pf the method also on human urine, selectivity was also evaluated by analysing the blank human urine samples from twenty volunteers.

-*LOD*

LOD was evaluated for the two principal metabolites. Blank urine samples were spiked with MPA and nor-MPA, starting at a concentration of 500 ng/mL with progressive dilution. LOD was defined with an S/N value major and close to S/N=3, evaluating the transition of lower intensity among the three principal ones: 58 *m/z* for MPA and 125 *m/z* for nor-MPA.

-*Recovery*

Recovery was evaluated with eight urine samples spiked with MPA and nor-MPA at a concentration of three times LOD for both compounds. The normality of the data distribution was evaluated by a Shapiro Wilk test to apply the Dixon test for the identification of outliers. Variance, confidence intervals, and coefficient of variation (CV) were also evaluated.

-Repeatability

Repeatability was evaluated using twenty blank urine samples, spiked with a concentration of three times LOD for both MPA and nor-MPA and calculating the ratio between the substances and internal standard. Ten samples were analysed on the first day and the other ten after two days to evaluate the repeatability of analysis with the same operator.

Statistical parameters were defined following the UNICHIM directives [26] in accordance with WADA technical documents for non-threshold substances. The normality of the data distributions was evaluated via Shapiro-Wilk tests (formulas [27] to conduct statistical tests that require a normal distribution, such as the Dixon test for outliers [28], homogeneity variance test [29], and ANOVA [30]. Variance, confidence intervals, and CVs were also calculated.

The variance homogeneity of the two data distributions of MPA and nor-MPA for the two days of analysis was evaluated with two different variance homogeneity tests: Cochran's C variance test and the minimum variance test [29]. Variance homogeneity allows the application of a one-way ANOVA test for repeatability.

3 Results and discussion

3.1 Instrumental conditions

The instrumental parameters were optimised by infusing the standard MPA dissolved in the mobile phase at a concentration of 10 µg/mL. The best ionisation conditions for MPA $[M+H]^+$ ions at *m/z* 156 were obtained using a curtain gas pressure of 25 psi, a source temperature of 500 °C, an ion source gas 1 (auxiliary gas) pressure of 35 psi, an ion source gas 2 (nebuliser gas) pressure of 40 psi, a declustering voltage of 80 V, and a needle voltage of 5,500 V. Different collision energies were evaluated to select the characteristic fragmentation pattern of MPA and the possible product ions of its metabolites. The selected specific product ions (structural markers) are the *N*-ethylmethaniminium ion at *m/z* 58, (thiolan-2-ylidene)methylium ion at *m/z* 97, and thiophenylpropylium ion at *m/z* 125; the fragmentation spectra and characteristic *m/z* transitions are reported in Figure 1. Based on these structural markers and the information obtained from the literature [9], we developed the MRM method with a specific transition for each potential MPA metabolite. The predicted fragmentation patterns for MRM analysis of each metabolites were confirmed through product ion scan of the metabolites identified after full scan analysis of in vitro samples. Table 1 presents the specifically selected precursor and product ions as $[M+H]^+$ with their energies.

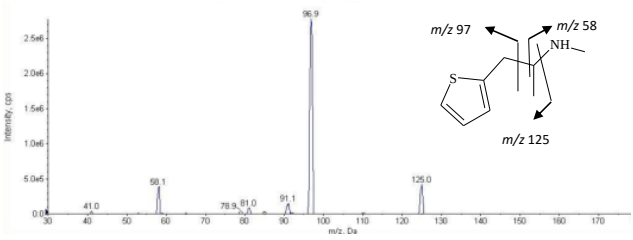

Figure III.1. Product ion spectra of MPA using a collision energy of 25 eV

Table III.1. Precursor ions, product ions and collision energies employed for MPA and its metabolites, diagnostic ions are in bold

Compound	Precursor ion(*m/z*)	Product ion (*m/z*)	Collision energy (eV)
MPA	156	58; **97; 125**	35; 30; 25
Nor-MPA	142	**97; 125**	30; 25
Hydroxy-Aryl-MPA	172	58; **113; 141**	35; 30; 25
Hydroxy-Alkyl-MPA	172	58; 97; 113; **125; 141**	35; 35; 30; 30; 25; 25
Nor-hydroxy-MPA	158	113; **141**	30; 25
Oxo-MPA	170	111; **139**	30; 25
Di-hydroxy-MPA	188	58; **74**; 113; **141**	35; 35; 30; 25
Hydroxy-oxo-MPA	186	58; 74; 111; **124;139; 155**	35; 35; 30;30; 25; 25

3.2 *In vitro* studies and optimisation of the sample preparation protocol

In vitro metabolism studies were conducted using HLM from a pool of 20 Caucasian male and female donors of different ages to minimise the effect of intra-individual variation and present the 'average' enzyme activity.

The best results were obtained using methanol as a substrate solvent (the total amount of methanol in the final assay was 1%), substrate concentration of 20 µM, protein concentration of 0.5 mg/mL, phosphate buffer of 0.1 M at pH 7.4, and incubation time of 4 h at 37 °C. Furthermore, *in vitro* metabolism studies allowed the detection of MPA metabolites by their enzymatic synthesised developing and adequate pre-treatment protocol for both MPA and its metabolites.

To optimise the pre-treatment samples, different extraction solvents (*tert*-butyl methyl-ether, diethyl ether, and ethylacetate) and pH values (7, 9 and 12) were evaluated. Three metabolites of MPA were identified: nor-MPA, nor-hydroxy-MPA, and hydroxy-alkyl-MPA.

The best recoveries (higher than 80%) for nor-MPA and MPA were obtained using *tert*-butyl methyl-ether ether at pH 12, however it was not optimal for the hydrolysed metabolites extracted with recovery higher than 80% at pH 7. At pH 7, nor-MPA and MPA were extracted in very low amounts. For this reason, samples were pre-treated using *tert*-butyl methyl-ether at pH 9. Under these conditions, MPA and its metabolites were extracted with recoveries higher than 70%. A chromatogram of HLM incubation after extraction at pH 9 is reported in Figure 2.

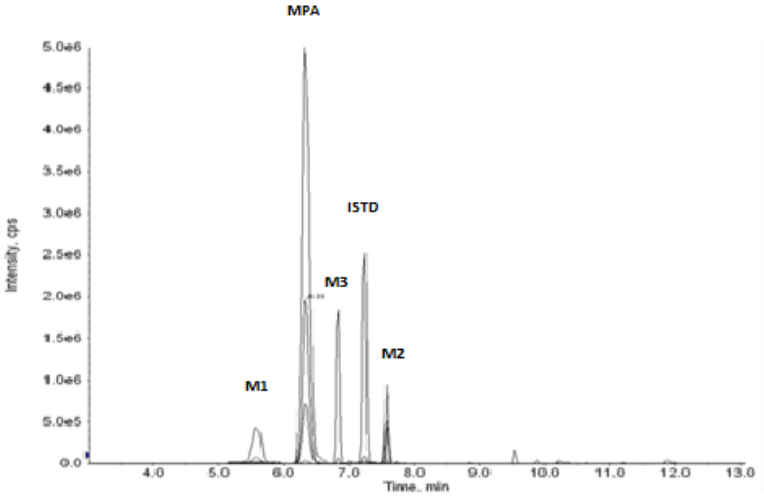

Figure III.2. Extracted chromatogram of the sample obtained after incubation of MPA with HLM for 4 h at 37 °C

3.3 *In vivo* studies after acute administration

Urine samples collected from male mice at every 3-h interval after administering 10-mg/kg MPA were analysed using the analytical protocol set up and optimised using the samples obtained after the incubation of MPA with HLM. Blank urine was also analysed to verify the possible presence of interferents.

MPA, nor-MPA (M1), hydroxy-alkyl-MPA (M3), oxo-MPA (M4), and hydroxy-aryl-MPA (M5) were identified as phase I *in vivo* metabolites, while nor-hydroxy-MPA (M2) was identified as a phase II metabolite. The excretion profile of MPA shows a metabolic pathway similar to amphetamine-like drugs with N-demethylated, hydroxylated, and hydroxyl-oxidised metabolites [31]. All metabolite structures identified *in vitro* and *in vivo* are reported in Figure 3.

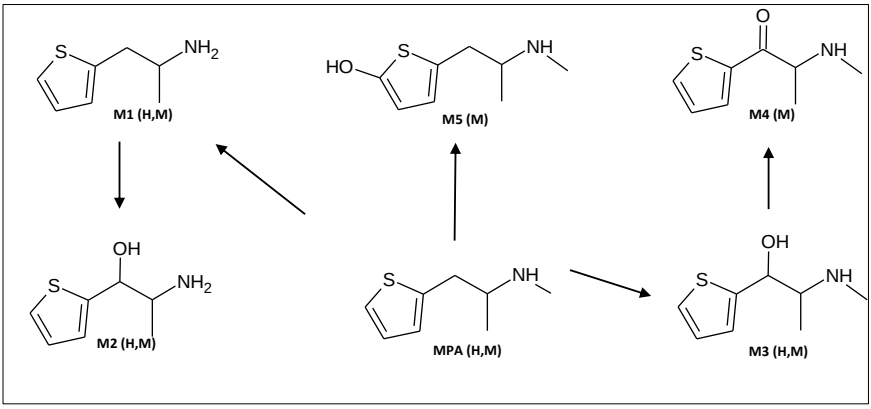

Figure III.3. metabolic profile of MPA with human liver microsomes (H) and in mouse (M) where M1 is Nor-MPA, M2 is Nor-Hydroxy-MPA, M3 is Hydroxy-Alkyl-MPA, M4 is Oxo-MPA, M5 is Hydroxy-Aryl-MPA.

Is notable that, *in vivo* and *in vitro* studies identified differences in the formation of hydroxylated and oxidised metabolites. Specifically, nor-hydroxy-MPA is a phase I metabolite of HLM incubation and a phase II metabolite in mice, and M4 is found only in mice urine samples in phase I and phase II. This result related to M4 suggests that MPA only shows conversion into oxidised metabolite in mice or rat and non in human. This hypothesis is supported by the result of Welter et al. [9]. The presence of M4 in human urine could represent an intake of 2-thiothinone, the common name of oxo-MPA, a potential new psychoactive substance [32].

-Phase I and phase II excretion profiles

In the range of 0–9 h after MPA administration, MPA itself and four principal phase I metabolites were excreted. MPA, M1, M3, M4, M5 were detectable through the time period and appeared as soon as 3 h after MPA administration. The extracted chromatograms of a representative sample for this range are reported in Figures 4 and 5 respectively, for phase I and phase II. MPA and its phase I metabolites were also excreted in the phase II metabolism as glucorono- and sulfo-conjugates.

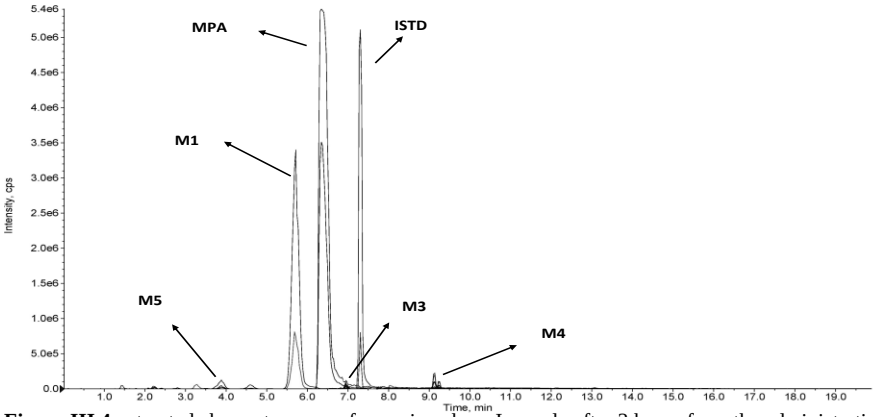

Figure III.4 extracted chromatograms of a n urine phase I sample after 3 hours from the administration of MPA.

Furthermore, M2 was only excreted in the conjugated form (Figure 5).

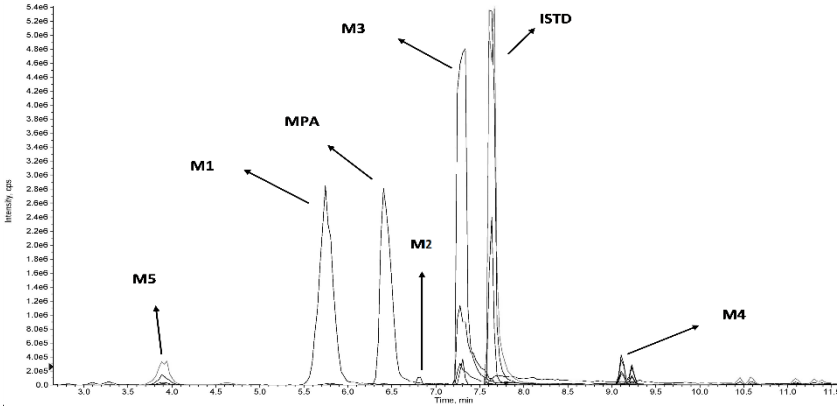

Figure III.5 extracted chromatograms of an urine phase II sample after 3 hours from the administration of MPA.

Despite the extensive metabolism, only MPA and M1 showed phase I and phase II excretion in the range of 24–36 h. The extracted chromatograms of a representative sample for this range is reported in Figures 6 for phase I.

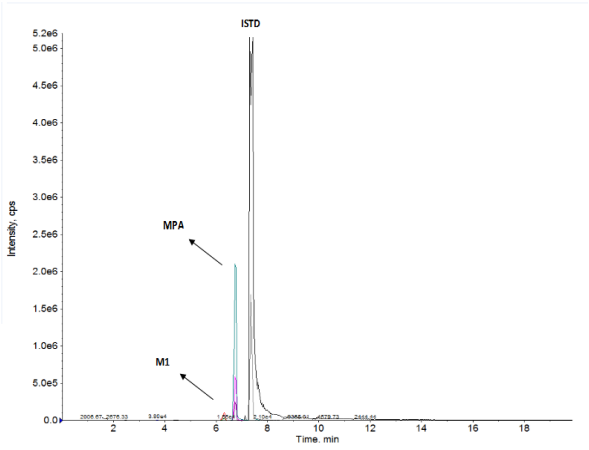

Figure III.6 extracted chromatograms of an urine phase I sample after 24 hours from the administration of MPA.

The phase II extracted chromatograms of a representative sample for the 24-36h range is shown in Figures 7.

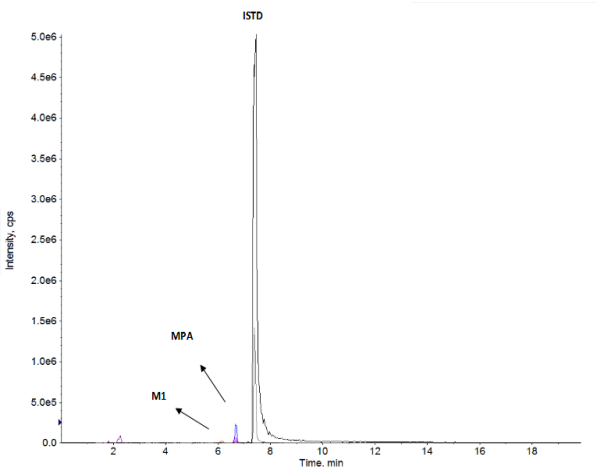

Figure III.7 extracted chromatograms of an urine phase II sample after 24 hours from the administration of MPA

The *in vivo* excretion profile of phase I metabolites in the range of 0-36 h is shown in Figure 8.

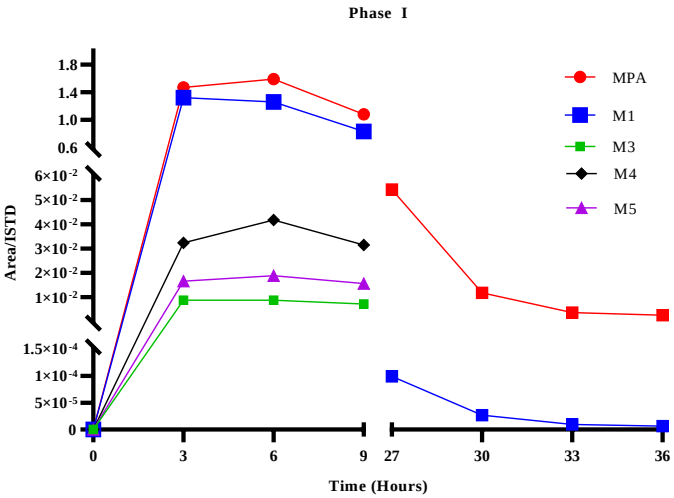

Figure III.8 Excretion profile of phase I metabolites in the range of 0-36 hours

In details, the maximum excretion for MPA and its metabolites was in the range of 3–6 h after the administration of a single dose of 10-mg/kg MPA, except for M1. It shows a maximum excretion value in the range of 0–3 h, whereas hydroxy-alkyl, hydroxy-aryl, and oxidised metabolites show their maximum excretion value in the range of 3–6 h. The difference between the excretion profiles of M1 and the other metabolites excretion in the range of 0–9 h is probably attributable to the rapid conversion of MPA into M1, followed by the hydroxylation of the structure forming the hydroxy and oxy metabolites.

However, twenty-four hours after the administration of MPA, only MPA itself and M1 are still detectable. The undetectability of the other MPA metabolites is probably attributable to the total excretion of secondary metabolites in the first 0–9 h owing to their high hydrophilicity.

Note that, the maximum excretion value of phase I metabolites in the range of 3–6 h corresponds to the minimum excretion value of phase II MPA and its metabolites.

The *in vivo* phase II metabolites are showed in Figure 9

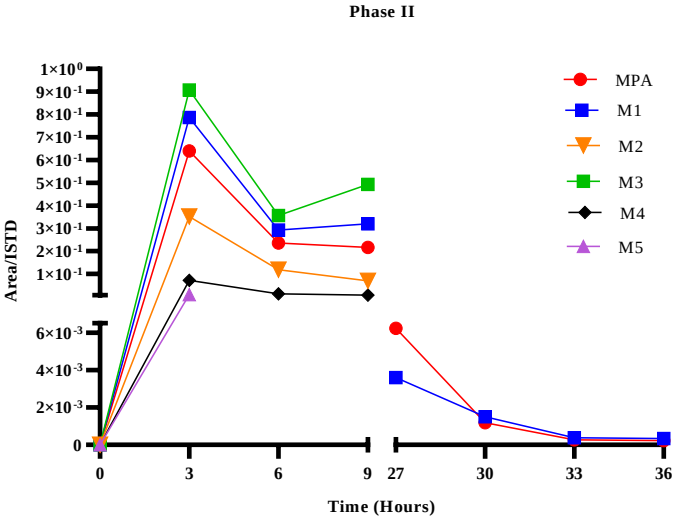

Figure III.9 Excretion profile of phase II metabolites in the range of 0-36 hours

The maximum excretion values in the range of 0–3 h for MPA, and all metabolites is due to the rapid conversion of phase I metabolites into their conjugate forms with a progressive decrease over the range. As seen as for the phase I metabolites, only MPA and M1 were detectable after 24 hours by the administration of MPA.

With the aim to better understanding the *in vivo* excretion profile, the total excretion profile, sum of phase I and phase II, are shown in Figure 10.

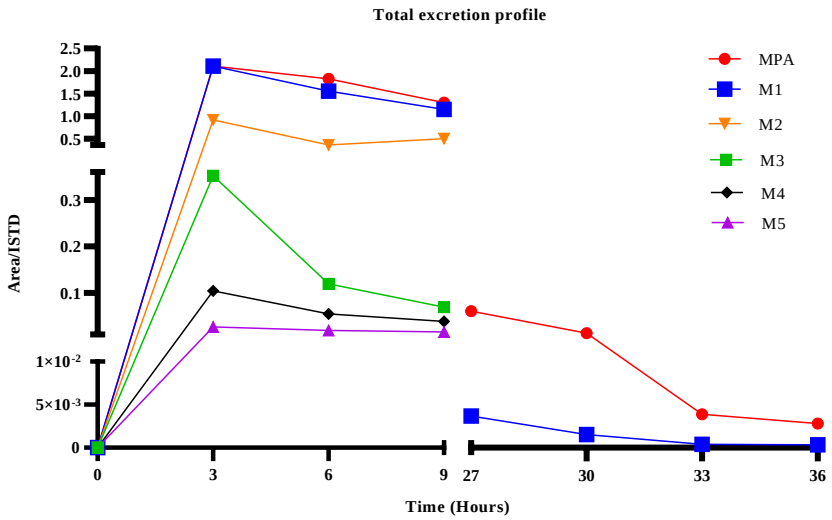

Figure III.10 Total excretion profile is reported as a sum of phase I and phase II in vivo excretion into the total range of 0-36h

Is remarkable that MPA and its metabolites show their maximum total excretion after 0-3 hours with a progressive decrease after the first time point. This decrease is markable for M3 where M2 shows a decrease at 6 h and a slight increase after 9 h. The presented method based on the hydrolysis of conjugated form into phase I metabolites proving MPA itself and M1 are the markers of intake with the high diagnostic value.

Furthermore, the excretion percentages reported in Table 2 show that MPA M1 M4 are excreted manly in phase I while M2 and largely M3 are excreted as phase II. M5 was excreted only in the first 0–3 h.

Table III.2. Percentage excretion of phase I metabolites as calculated from the total amount of excretion per hour as the sum of all phase I metabolites excreted in the range.

	Phase I % excretion					
Hours	**MPA**	**M1**	**M2**	**M3**	**M4**	**M5**
0-3	69,67	62,65	0,00	1,80	30,97	46,85
3-6	87,08	81,13	0,00	5,02	76,51	100,00
9-6	83,27	72,20	0,00	3,07	81,49	100,00
24-27	89,71	2,69				
27-30	90,91	1,77				
30-33	93,04	2,46				
33-36	92,02	1,86				

Considering the total excretion of MPA and its metabolites, shown in Table 3, on the first range 0-9h, M1 and MPA show comparable excretion. In details, MPA shows an excretion among 37-46% of the total amount of excretion, where this percentage was calculated as the sum of MPA and metabolites excretion for each time point considerate. M1 shows an excretion among 37-39 % through the entire range, the two principal markers are followed by a 9-16% ratio for M2 followed by the hydroxy and oxo-methyl-metabolites M3 and M4. The M5 metabolite is the least representative metabolite of MPA intake. On the range of 24-36 hours M1 show a ratio above 5-10 % confirming MPA itself is the best marker of intake after 24 hour by the intake.

Table III.3. Percentage of excretion of each metabolite calculated as % of total excretion, sum of each metabolite and MPA.

	Total % Excretion					
hours	**MPA**	**M1**	**M2**	**M3**	**M4**	**M5**
0-3	37,57	37,52	16,31	6,27	1,86	0,48
3-6	46,39	39,46	9,27	3,02	1,38	0,48
6-9	42,23	37,48	16,28	2,25	1,25	0,51
24-27	94,26	5,74				
27-30	89,47	10,53				
30-33	90,8	9,2				
33-36	89,15	10,85				

Table III.3. Percentage of excretion of each metabolite calculated as % of total excretion, sum of each metabolite and MPA.

3.4 Validation

-LOD

LOD was verified for both metabolites excreted during the entire time range, MPA and M1. They show similar LOD and S/N values. The LOD value was expressed as ng/ mL of urine. S/N and the lowest MRM transition are reported in Table 4.

Table III.3. Experimental data for LOD (ng/mL), signal to noise (S/N) and relative transitions.

Substance	LOD (ng/mL)	S/N	MRM
MPA	**45**	3,5	**156-58**
Nor-MPA	**40**	3,2	**142-125**

-Selectivity

Blank urine samples collected from twenty volunteers and mice control group were analysed using the protocol described in the experimental section. No interference compounds were identified at the same retention times and with the same MRM transition of the MPA and its metabolites.

-Recovery

Data obtained from the analysed recovery samples via the Shapiro-Wilk test was used and no outliers were found by the Dixon test (data not reported). MPA and M1show recovery near 80% at a concentration of three times the LOD for both compounds. The recovery results, including standard deviation, CV, variance, and confidence intervals, are reported in Table 5.

Table III.5. Recovery and related statistic data wit degree of freedom (v) and probability (α).

Substance	**Rec. %**	**SD**	**CV**	**Var.**	**Conf.**	**Degrees of freedoom**
MPA	**84**	6,3	7,5	0,40	4,5	ν=7
Nor-MPA	**80**	6,1	7,6	0,40	4,5	α=0,05

-Repeatability

Data obtained from the analysed repeatability samples were normally distributed as determined by a Shapiro-Wilk test, and no outliers were found by the Dixon test (data not reported). Based on $C_{max} \leq C_{max,\alpha,K,\nu}$ and $C_{min} \geq C_{min,\alpha,K,\nu}$, the homogeneity of variance was evaluated, and the results are reported in Table 6.

Table III.6. Statistic value for Homogeneity Variance test where: s^2_{max} and s^2_{max} are respectively maximum and minimum variance of data set, s^2_{tot} = total variance in two days, $C_{max,\alpha,K,\nu=n-1}$ and $C_{min,\alpha,K,\nu=n-1}$ are respectively the critical max and min values of *C* critical value at: α= probability, k= number of group, ν= freedom degree ;

Variance Homogeneity		**MPA**	**Nor-MPA**
s^2_{tot}		$1{,}78 \cdot 10^{-4}$	$6{,}29 \cdot 10^{-6}$
s^2_{max}		$1{,}01 \cdot 10^{-4}$	$4{,}09 \cdot 10^{-6}$
s^2_{min}		$7{,}65 \cdot 10^{-5}$	$2{,}20 \cdot 10^{-6}$
C_{max}		0,57	0,65
C_{min}		0,43	0,35
$C_{max,\alpha=0{,}05,K=2,\nu=9}$	0,80	$\boldsymbol{C_{min,\alpha=0{,}05,K=2,\nu=9}}$	0,20

The ANOVA test shows no significant difference in variance over two days analysis of repeatability (F < Ftab) for both substances, the cumulative average variances were calculated. Data obtained is reported in Table 7.

Table III.7. Statistic value for ANOVA test where: $\boldsymbol{SS_E}$ is error Sum of Squares; $\boldsymbol{SS_r}$ is model Sum of Squares; $\boldsymbol{SS_{tot}}$ is Total Sum of Squares;$\boldsymbol{MS_r}$ is model mean squares, $\boldsymbol{MS_E}$ is model error mean squares, $\boldsymbol{F_{exp}}$ is calculated F, $\boldsymbol{F_{crit,\alpha=0{,}05,N-K=18,K-1=2}}$ is the critical values at: α= probability, k= number of group, N= total freedom degree; $\boldsymbol{S_r^2}$ is the cumulative average variances.

Test Anova	MPA	Nor-MPA
SS_E	$1{,}73 \cdot 10^{-3}$	$5{,}47 \cdot 10^{-5}$
SS_r	$4{,}85 \cdot 10^{-4}$	$9{,}07 \cdot 10^{-6}$
SS_{tot}	$2{,}22 \cdot 10^{-3}$	$6{,}37 \cdot 10^{-5}$
MS_r	$2{,}43 \cdot 10^{-4}$	$9{,}07 \cdot 10^{-6}$
MS_E	$9{,}63 \cdot 10^{-5}$	$3{,}04 \cdot 10^{-6}$
F_{exp}	**2,52**	**2,98**
$F_{crit,\alpha=0{,}05,N-k=18,k-1=2}$		**4,41**
S_r^2	$\mathbf{1{,}17 \cdot 10^{-4}}$	$\mathbf{3{,}35 \cdot 10^{-6}}$

4 Conclusion

To the best of our knowledge, this is the first study of MPA urine excretion profiles that defines the markers of intake in a range of 0–36 h after the MPA administration in mice. MPA is extensively metabolized into demethylated, hydroxyl and oxo metabolites and shows conversion into conjugated forms. The maximum excretion value calculated as a sum of phase I and phase II excretion profiles, was in the time interval of 0-3 h after MPA intake. Despite these results, only MPA and nor-MPA are detectable over the entire range, qualifying as the *most accurate* markers of intake in toxicological analysis. The presented method was, therefore, validated for both MPA and nor-MPA defining: selectivity, LOD, recovery and repeatability.

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Part II

Chapter IV

Evaluation of the *in vitro* and *in vivo* metabolic profile of 4,4' DMAR

Abstract

Para-Methyl-4-methylaminorex, also known as 4,4'-DMAR, is a synthetic, substituted oxazoline derivative and an analogue of the scheduled aminorex and 4-methylaminorex, which exerts psychostimulant activity. 4,4'-DMAR exists as two different racemic (±) –*cis* and (±) –*trans* mixture or four distinct isomers; recent evidence suggests that only the *cis* isomers show a biological activity; the aim of the present work is to investigate the *in vitro* metabolism of *cis/trans*-4,4'-DMAR, after incubation with pooled human liver microsomes and recombinant isoforms of cytochrome P450, and the *in vivo* urine excretion profile in mice to identify the most suitable diagnostic marker(s) of intake. With this aim ICR CD-1 male mice were administered with a dose of 10 mg/kg of both isomers of 4,4'-DMAR and urine samples were collected at different time (2-4-6 h). Our results show that after the incubation with human liver microsomes, no metabolite was detected for the *trans* isomer, while five metabolites were found for *cis*-4,4'-DMAR as a result of mono-hydroxylation, oxidation of the hydroxylated derivatives and carboxylation; the hydrolysis product (4-methyl-norephedrine) was also detected. The main cytochrome P450 isoform involved in metabolism of this substance is cytochrome P450 2D6; the polymorphic isoforms of CYP2D6 were also evaluated considering the singular contribution of CYP2D6*1, CYP2D6*2, CYP2D6*3; the mono-hydroxylated metabolite results detectable for all the isoforms. *In vivo* excretion profile shows similar results, where parent compound and hydroxylated and carboxylate metabolites are the more suitable markers of intake, considering the higher diagnostic values.

1 Introduction

4,4'-DMAR is a synthetic psychostimulant and anorexigenic substance with the same base structure of its analogues 4-methylaminorex (4-MAR) and aminorex (2-amino-5-phenyl oxazoline)[1,2]. Aminorex and 4-MAR were first synthesized in the 1960s as appetite suppressants[3,4]. Aminorex was scheduled in 1965 as appetite suppressant and withdrawn due to fatal complications related to pulmonary hypertension [5,6].

Previous data suggested that the (±)-*cis* isomer is the most available raceme mixture for 4,4'-DMAR. In 2012, 4,4'-DMAR was detected for the first time in seized white powder (Netherlands) [7].

As reported by the World Health Organization (WHO) between 2013-2014 4,4'-DMAR was associated with 32 analytically confirmed deaths in Europe: "Hungary (8 deaths), Poland (1 death) and the United Kingdom (23 deaths)"[2,8].

It was subsequently listed in the *Schedule II* of the Convention of Psychotropic Substances of 1971 (March 2016) by Commission of Narcotic Drugs [9] [10]. 4,4'-DMAR can be purchased on the illicit market under different street names such as "4-methyl-euphoria", "4-methyl-U4Euh", "4-M-4-MAR", or "Serotoni", in powder or tablet form [11] [12]. It can be also easily synthesised from cathinone (nor-mephedrone) or from the legal compound "1-(4-methylphenyl)-propan-1-one" with a known synthetic root which allow to the separation of the two isomeric forms *cis* and *trans* based on published literature related to the chemistry of aminorex-type compounds[3,5,13,14].

The psychostimulant and entactogen properties of 4,4'-DMAR are due to its interaction with dopamine transporter (DAT), serotonin transporter (SERT) and norepinephrine transporter (NET).

Recent studies carried out on rat brain synaptosomes, have shown that the *cis*-isomer acts as a potent, non-selective releaser, leading to an increase in the synaptic concentration of monoamines.

Specifically, in comparison to aminorex and 4-methylaminorex, *cis*-4,4'-DMAR shows a higher activity on SERT. This absence of selectivity is probably linked to the *para*-substitution of the phenyl ring [10] [15] [16]. *Trans*-4,4'-DMAR exhibits a releasing effect on DAT and NET, while it acts as a re-uptake inhibitor on SERT [7]. The increased extracellular neurotransmitters levels are responsible of either desired adverse effects: the overstimulation of central serotoninergic and dopaminergic systems cause hyperthermia, agitation, nausea and convulsion, whereas the higher norepinephrine release is responsible of cardiovascular toxicity [10]. Since 4,4'-DMAR has an amphetamine-like structure and it acts as a stimulant, it is indirect included in the section S6 "Stimulants" of the list of substances and prohibited methods annually published by the WADA[17].

So far, few information is known about the pharmacokinetic of 4,4'-DMAR. A recently validated method was used to detect the main metabolites of *cis*-4,4'-DMAR in rat plasma and brain tissues: the hydroxylate, the carboxylate, the 4-methyl-norephedrine and the deamination products were detected [9]. At the data of writing no information were available on urinary excretion profile of both isomers of 4,4'-DMAR or human metabolic profile. In the present study the *in vitro* approach was used as a model of human metabolism to evaluate the metabolic pathways of both isomers [18]. Furthermore, as the elected matrix for doping analysis is urine, *in vivo* metabolism was investigated employing male mice ICR CD1- as a model of human metabolic excretion and urine samples were collected at different time. 4,4'-DMAR metabolites were detected by LC-MS based techniques to identify the most suitable marker(s) of intake to be targeted in humane urine in forensic analysis and doping controls [19].

2 Experimental

2.1 Chemicals and reagents

Cis/*trans* 4,4′-DMAR (4-methyl-5- (4-methylphenyl)-4,5-dihydrooxazol-2-amine were synthetized and provided by University of Ferrara Department of Organic Chemistry. Amphetamine-D11 employed as internal standard was purchased by Sigma–Aldrich (Milan, Italy). All chemicals (i.e., formic acid, acetic acid, ammonium formate, ammonium acetate, sodium phosphate, sodium chloride, sodium hydrogen phosphate, potassium carbonate, potassium hydrogen carbonate, acetonitrile, absolute ethanol, methanol, dimethylsulfoxide [DMSO] diethyl ether, ethylacetate, and tert-butyl methyl-ether) were of analytical or HPLC grade and provided by Carlo Erba (Milan, Italy) and Sigma–Aldrich (Milan, Italy). The ultrapure water used was of Milli-Qgrade (Millipore Italia, Vimodrone, Milan, Italy). The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian mixed male and female donors of different ages) as well as CYP recombinant isoforms (CYP1A2, CYP2C19, CYP2C9, CYP3A4, CYP3A5, CYP2D6), were purchased by Corning Incorporated (Woburn, Massachusetts, United States). The allelic isoforms of CYP2D6 as (CYP2D6*1, CYP2D6*2, CYP2D6*3) and all the reagents used for the *in vitro* metabolism studies (sodium phosphate and tris-HCl buffers and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milan, Italy). The enzyme β-glucuronidase from *E. Coli* as well as mixture β-glucuronidase/arylsulfatase (from *Helix pomatia)* were purchased from Roche (Monza, Italy).

2.2 Animals and dose selection

For the *in vivo* studies, different groups of mice were selected, and each mouse was intraperitoneally administered at 9:00 am with a dose of 10 mg/kg of *cis/trans*-4,4'-DMAR. In details, two groups of five mice were administered with respectively *cis*-4,4'-DMAR and *trans*-4,4'-DMAR at the same doses. A group of five mice was also selected for urine blank samples.

2.3 *In vitro* protocol

All incubation conditions were optimized including different solvents (methanol, DMSO and acetonitrile), buffers (phosphate and tris-HCl), pH values (5.0, 7.4, 8.0), concentration of both isomers (0.05, 0.1, 0.5 e 1 mg/mL) and of the enzymatic proteins (0.1, 0.2, 0.5 and 1.0mg/mL), and different incubation time (60, 120, 240, and 1440 min) were evaluated. The final incubation medium also contained 6.6 mM magnesium chloride, 2.6 mM NADP+, 6.6 mM glucose-6-phosphate and 4.0 U/mL glucose-6-phosphate dehydrogenase in 5 mM of sodium citrate with a total volume of 250µL. Samples were pre-warmed at 37°C for 5min and the reactions were started with the addition of the appropriate enzymatic proteins (HLM or CYP recombinant isoforms) at the final concentration of 0,5 mg/mL. After incubation at 37°C with the selected incubation time, 250µL of ice-cold acetonitrile were added to stop the reaction. The samples were then transferred into an ice bath for 3 min for further precipitation of the proteins in the assay medium. The supernatant was subsequently separated from the precipitate by centrifugation at 14.000 rpm at room temperature for 10 min. Each set of assays also included a negative control sample, containing all reaction mixture components except the enzymatic proteins to monitor the potential non-enzymatic reactions within the incubation period. Each incubation was processed in duplicate.

2.4 Sample pre-treatment

In vitro and *in vivo* samples were pre-treated with the same protocol of phase I metabolism study. For the detection of phase II metabolites in the *in vivo* samples two different enzymatic hydrolysis were employed. In details, the first extraction employed β-glucuronidase for the hydrolysis of glucorono-conjugates, and a mixture of β-glucuronidase/ arylsulphatase was added the aqueous layer for the hydrolysis of sulfo-conjugates. This allow to the conversion of phase II metabolites into phase I metabolites. After every extraction and before enzymatic hydrolysis the extraction procedure was repeated three times in order to extract all phase I metabolites to reduce interference in phase II metabolism study, and organic layer were added and evaporated.

-In vitro studies

All the samples obtained by the *in vitro* experiment (sample volume 500μL) were added with 1.5 mL of phosphate buffer (1 M, pH 7.4) and 50 μL of the internal standard solution. (Amphetamine-D11 standard solution 1 mg/mL) and a liquid/liquid extraction was carried out with 7 mL of the most appropriate solvent (*tert*-butylmethyl, ethyl acetate, chloroform were tested) at controlled pH with different pH tested (5, 7.4, 9, 11). After 10 minutes of stirring at room temperature, samples were centrifugated at 3000 rpm for 2 min and transferred into an ice bath for 5 minutes. After centrifugation the organic layer was separated and evaporated to dryness under nitrogen stream at a temperature of 30°C.

The residue was reconstituted with 50 μL of mobile phase (initial composition) and analysed with two different LC-MS techniques.

- *In vivo studies*

Sample preparation was optimized using the sample obtained after incubation of 4,4-DMAR with HLM. In details, a volume of 200 µL of urine sample was added with 50 µL of the standard solution (final concentration in urine 250 ng/mL) and the procedure developed for *in vitro* studies were employed for phase I metabolites extraction. The final residue was dissolved in 50 µL mobile phase and then analysed by the HPLC-QqQ developed method.

The aqueous layer was stored up for the phase II metabolism studies and subsequently added with 200 µL of phosphate buffer 0.8 M (pH 7.4), 50 µL of the standard solution of amphetamine-D11 and 50 µL of β-glucuronidase with an incubation time of 1 hours at 55°C. Samples were then extracted with the same procedure described for the *in vivo* phase I metabolism. The aqueous layer was stored up and employed for the detection of sulfo-conjugates metabolites.

With this aim 200 µL of acetate buffer 1 M (pH 5) were added to the mixture and 50 µL of β-glucuronidase/arylsulphatase were added for the hydrolysis of sulfo-conjugates. Samples were next extracted with the same procedure described above.

2.5 Instrumental conditions

In vitro samples were analysed with two different liquid chromatography coupled to mass spectrometric techniques (HPLC-MS) as LC coupled to triple quadrupole (LC-QqQ) and high-resolution techniques Ultra-High-Performance-LC coupled to Quadrupole-Time Of Flight technique (UHPLC-QTOF) with the aim to evaluated the phase I metabolism of both isomers of 4,4'-DMAR. The *in vitro* metabolic pathway of 4,4'-DMAR was defined by UHPLC-QTOF technique. Metabolites were confirmed through a targeted analysis of the same samples with LC-QqQ method.

The *in vivo* metabolic profiles was confirmed through UHPLC-QTOF techniques and LC-QqQ method was selected for evaluation of phase I (or hydrolysed phase II) urinary excretion.

2.5.1 UHPLC-QTOF

In vitro samples were analysed using an Agilent 1290 infinity II series UHPLC instrument equipped with a: Zorbax C18 (10 cm x2.1 mm, 1.8 µm) coupled with a QTOF 6545 (Agilent Technologies) with an ESI source. The solvents used was the same described above for the HPLC-QqQ method.

The flow rate was set to a constant flow rate of 400 µL/min starting at 2% of B up to 30% in 4 min, next to 80% in 2 min to 95% in 1 min for 2 min. The column was flushed for 2 min at 100% B and finally re-equilibrated at 2% B for 2 min. The mass spectrometric conditions as the follow: ESI+ a capillary voltage of 3,500V, a nozzle voltage of 300 V, a drying gas flow of 15 L/min at 150°C, sheath gas flow of 10 L/min at 320°C and a nebulizer pressure of 45 psi were used with nitrogen. The high-pressure ion funnel was operated at radio frequency (RF) voltage 150 V, the low-pressure funnel at RF 60 V, and the octopole at RF 750 V. Mass data were generated from *m/z* 50 to 1000 at 9300 transients per second.

The mass calibration was performed daily. Purine with an $[M+H]^+$ ion at *m/z* 121.0509 and an Agilent proprietary compound (HP0921) yielding an ion at *m/z* 922.0098 were simultaneously introduced Selected ions $[M+H]^+$ for parents compound and metabolites are reported in Tables 1.

All aspects of instrument control, tuning, method setup and parameters, sample injection and sequence operation were controlled by the Agilent Technologies Mass Hunter software version B.08.00.

2.5.2 LC-QqQ

In vitro and *in vivo* samples were analysed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO Discovery C18 column (15 cm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass spectrometer (Sciex) with an ESI source and operated in the positive-ion mode. The solvents used were: ultrapurified water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid. The

gradient program started at 5% B and increasing to 60% B in 6 min, after 3 min, to 100% B in 1 min. The column was flushed for 3 min at 100% B and finally re-equilibrated at 5% B for 3 min. The flow rate was set to a constant flow rate of 250 µL /min. The mass spectrometric condition as set as follow: ESI source operate in positive electrospray ionization using a curtain gas pressure of 25 psi, a ion source temperature of 500 °C, an ion source gas 1 pressure of 35 psi, an ion source gas 2 pressure of 40 psi, a declustering voltage of 60 V, an entrance potential of 10 V and a needle voltage of 5500 V.

Multiple reaction monitoring (MRM) was used as the acquisition mode for detection (see Tables 2 for the MRM transitions selected). For the MRM collision-induced dissociation (CID), nitrogen was used as the collision gas at 5.8 mPa, obtained from a dedicated Parker-Balston nitrogen generator system (model 75-A74) with 99.5% gas purity (CPS Analitica Milan, Italy). All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by Analyst software version 1.5.1

3 Results and discussion

3.1 Mass spectrometric conditions

The mass spectrometric parameters for both techniques as declustering and needle voltages, gas pressure, source temperature, collision cell exit potential, and collision energy were optimised by infusing the standard solution of both isomers of 4,4'-DMAR at a concentration of 10 μg/mL at a flow rate of 10 mL/ min, as well as selected protonated molecules $[M+H]^+$ and characteristic fragmentations for parent compounds in MRM mode. The predicted fragmentation patterns for MRM analysis of each metabolites were confirmed through analysis of in vitro samples.

3.1.1 UHPLC-QTOF

For the high resolution technique the $[M+H]^+$ mass was estimated through direct infusion of standard solution. The $[M+H]^+$characteristic mass for metabolites were calculated based on the predicted structures and results obtained after incubation of standard solutions of 4,4'-DMAR with human liver microsomes and full scan analysis. Here reported in Table 1 the $[M+H]^+$, elemental composition and relative mass error for metabolites of the two cannabimimetic.

Table IV.1. $[M+H]^+$ ions for 4,4'-DMAR and all metabolites, elemental composition and error with QTOF techniques

Compound	$[M+H]^+$(*m/z*)	Error (Δppm)	Elemental Composition
4,4'-DMAR	191.2465	1.23	$C_{11}H_{14}N_2O$
Mono-hydroxylated (M1, M3)	207.1154	2,89-3,86	$C_{11}H_{15}N_2O_2$
4-methyl-nor-ephedrine (M5)	166.2322	2,39	$C_{10}H_{15}NO$
Mono-Hydroxyl-Oxidated (M4)	205,0972	2,16	$C_{11}H_{13}N_2O_2$
Carboxylated (M2)	221,0972	2,59	$C_{12}H_{14}N_2O_3$

3.1.2 LC-QqQ

Different collision energies (20, 30, 40, 45, 50 and 60 eV) were evaluated in order to obtain information about the fragmentation patterns of the compounds considered in this study to predict possible fragmentation patterns for metabolites. The full-scan MS analysis was first performed, both in positive and negative ionization mode, to identify the molecular ions of compounds.

The best response was obtained in positive mode for the protonated molecular ion $[M+H]^+$ at *m/z* 191 at 25 eV collision energy for both compounds. The mass spectra fragmentation of the *cis* compound (as the same for *trans*) with the respective structures is shown in Figures 1A.

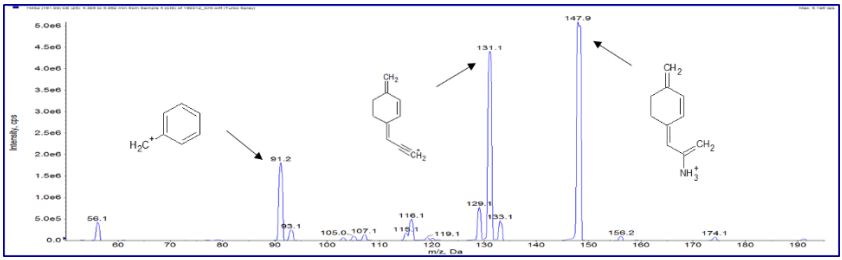


Figure IV.1A. Product ion scan spectrum obtained for *Cis*-4,4'-DMAR at a collision energy of 25eV

The above described product ions were employed as diagnostic ions for the metabolites detected in samples obtained after incubation of *cis*/*trans* 4,4'-DMAR in the presence of HLM and CYPs isoforms. The selected diagnostic MRM transition for the *cis* 4,4'-DMAR are *m/z* 91 (phenylmethylium); *m/z* 131 [3-(4-methylidenecyclohex-2-en-1-ylidene)prop-1-yn-1-ylium];

m/z 148 [3-(4-methylidenecyclohex-2-en-1-ylidene)prop-1-en-2-aminium].

Selected transitions for parent compound and metabolites are reported below in Tables 1, MRM transitions for trans-4,4'-DMAR are not reported because metabolites were not identified.

Table IV.2. Employed MRM transitions for 4,4'DMAR and its metabolites. Precursor ion, product ion and collision energy employed.. Diagnostic ions are in bold

Compound	Precursor ion(*m/z*)	Product ion (*m/z*)	Collision energy (eV)
4,4'-DMAR	191	**91**;131;**148**	40; 40; 35;
Mono-hydroxylated (M1)	207	56;**91**;**164**	30; 40;40
Mono-hydroxylated (M3)	207	56;**164**	30;40
4-methyl-nor-ephedrine (M5)	166	**148**; **91**; 131	30; 40; 35
Mono-Hydroxyl-Oxidised (M4)	205	56;**115**;**162**	50;40;30
Carboxylated (M2)	221	**178**; 56	30; 50

3.2 *In vitro* metabolism

In vitro metabolism studies were conducted using HLM from a pool of 20 Caucasian male and female donors of different ages to minimise the effect of intra-individual variation and present the 'average' enzyme activity. The protocols previously optimized were applied to all incubation samples. The best results were obtained using methanol as a substrate solvent (the total amount of methanol in the final assay was 1%), substrate concentration of 20 µM, protein concentration of 0.5 mg/mL, phosphate buffer of 0.1 M at pH 7.4, and incubation time of 4 h at 37 °C. To optimise the pre-treatment samples, different extraction solvents (*tert*-butyl methyl-ether and ethylacetate) and pH values (5, 7, 9 and 11) were evaluated. The best extraction results for parent compounds and metabolites were obtained with addiction of 50 µL of carbonate buffer (pH 11) followed by addiction of 5 mL of ethylacetate.

-HLM

In vitro samples analysis allowed to the detection of five metabolites. The extracted chromatograms of a representative sample of *in vitro* studies is shown in Figure 4 (UHPLC-QTOF technique) and in Figure 5 LC-QqQ technique).

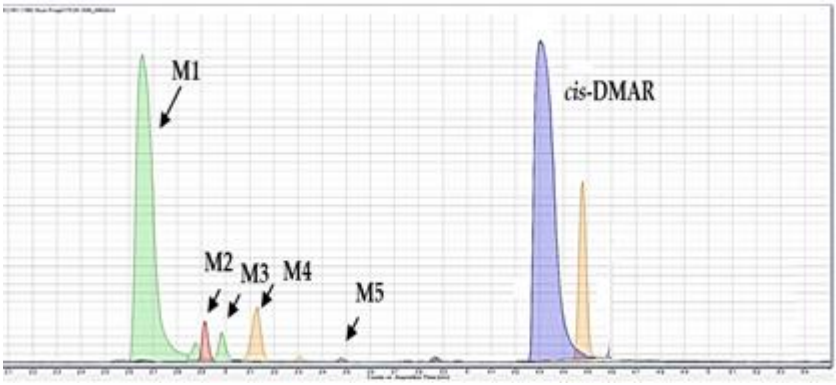


Figure IV.4. Extracted chromatogram of a representative sample after 4h of incubation in full-scan mode.

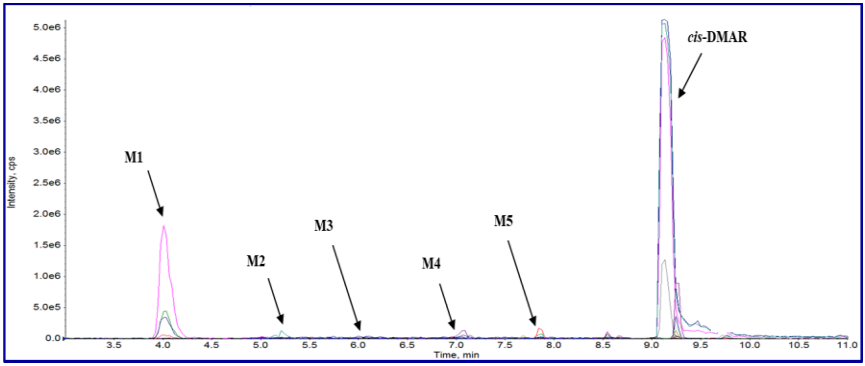


Figure IV.5. Extracted chromatogram with MRM mode (QqQ) of the same sample

Furthermore, the incubation of 4,4'-DMAR with HLM doesn't show the formation of detectable metabolites, Figure 6.

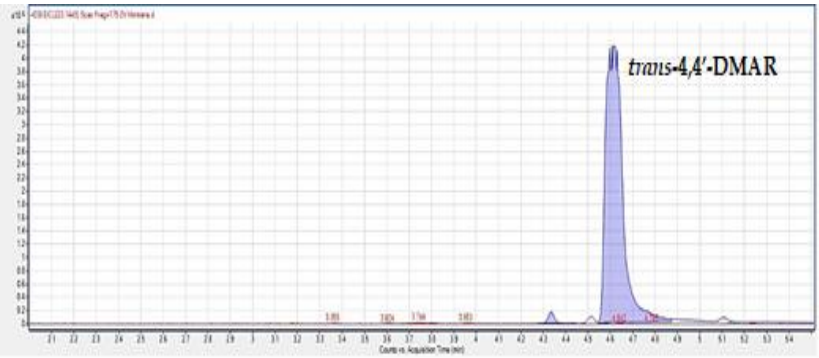


Figure IV.6. Extracted chromatogram of a representative sample of trans-4,4'-DMAR after incubation with HLM analysed through QTOF techniques.

In details, the metabolic pathways identified were: mono-hydroxylation in different positions with the formation of two different hydroxy-metabolites (M1, M3), carboxylation (M2), the formation of mono-hydroxy-oxidised metabolite (M4) and the formation of 4-nor-ephedrine (M5). Where parent compound and M1 metabolites are the most representative metabolites for *cis*-4,4'-DMAR. The metabolic pathways of *cis*-4,4'-DMAR is reported below in Figure 7.

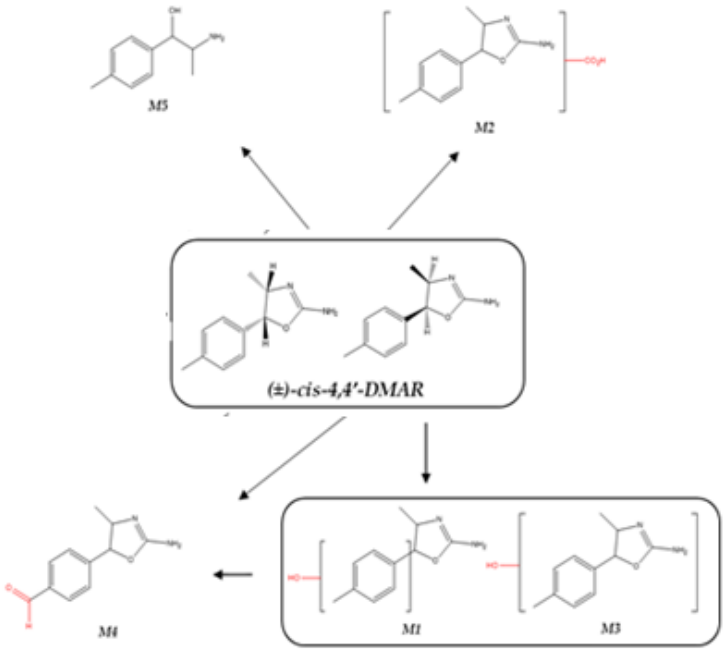


Figure IV.7. Proposed metabolic pathways for 4,4'-DMAR where M1 and M3 are the hydroxylated metabolites, M2 is the carboxylated metabolite, M4 is the hydroxy-oxidised metabolite and M5 is 4-methyl-norephedrine.

-*CYP isoforms*

The relative contribution of induvial CYPs isoforms to the metabolic reaction of *cis*-4,4'-DMAR was evaluated with the aim to estimate the CYPs involved and potential drug-drug interactions with other substances.

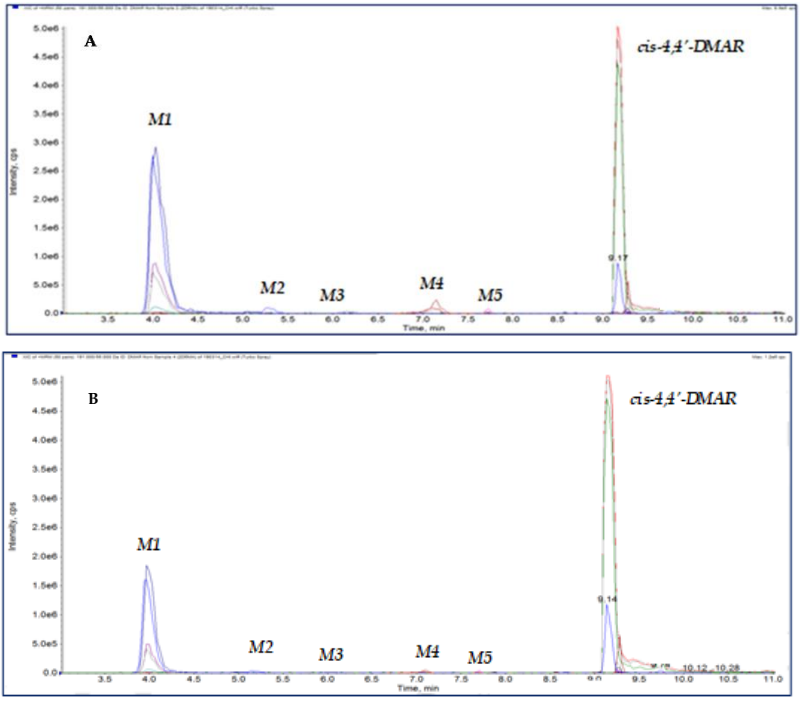
To define the relative contribution of individual CYPs isoforms to the metabolic reaction, *cis*-4,4'-DMAR standard solution (at a concentration of 1 mg/mL) was incubated with different CYP450 isoforms. The involved isoforms evaluated for the phase I metabolism are CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP3A5 and CYP2D6. Results show that the only isoform involved into *cis*-4,4'-DMAR metabolism is CYP2D6 which present different allelic variants. The three allelic variants of CYP2D6 employed are CYP2D6*1, CYP2D6*2, CYP2D6*3. They are employed *in vitro* studies with the aim to investigate possible differences in metabolic pathways among subject with different allelic variants expression, to define the best markers of intake. In details, subject with expressed CYP2D6*1 show a normal activity of this isoform (normal activity, MA), subject with genic of CYP2D6*2 show a low activity (no-activity, NA), subject which express CYP2D6*3 allelic variant show high metabolic activity (high activity, HA).

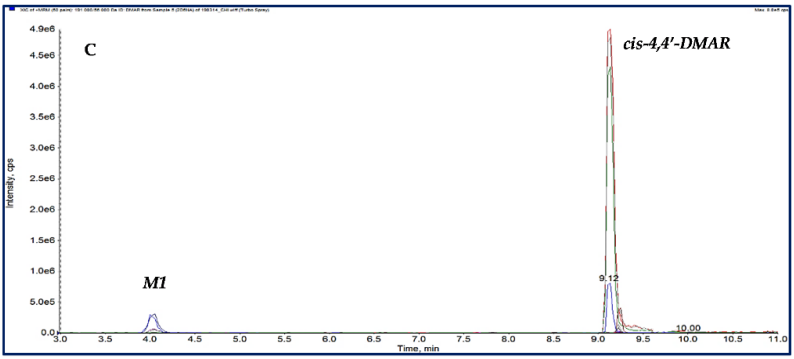
The percentage ratio of metabolites identified with the three different allelic variants of CYP2D6 are reported below in Table 3. Calculated as percentage from the total amount of excretion (sum of DMAR and metabolites peak area/area of internal standard) for each allelic variant.

Table IV.3. Percentage of excretion of DMAR and its metabolites calculated as the sum of total excretion of all metabolites and DMAR.

	DMAR	**M1**	**M2**	**M3**	**M4**	**M5**
HA	45,94	46,51	1,29	2,50	3,24	0,52
MA	69,80	27,66	0,61	1,33	0,54	0,07
NA	92,96	7,03	0,00	0,00	0,00	0,00

As described in Table 3 all metabolites were detected after incubation of *cis*-4,4'-DMAR standard solution with CYP2D6*3. The same metabolites were detectable after incubation with CYP2D6*1 at low concentration. However, only M1 metabolite is detectable in a small amount after incubation with CYP2D6*2 confirming is the most suitable marker of intake as well as the parent compound for all subject regardless of the different genic allelic expression of CYP2D6, see Figure 8 A-C for the extracted chromatograms of the incubation with the three allelic variants.





Figures IV.8 A-C. Extracted chromatograms after incubation with CYP2D6 allelic isoforms: CYP2D6*3 (A), CYP2D6*1 (B), CYP2D6*2 (C).

3.3 *In vivo* studies after acute administration

Urine samples collected from male mice at every 2-h interval for six hours after administering 10-mg/kg 4,4'-DMAR were analysed using the analytical protocol set up and optimised with the analysis of the samples obtained after the incubation of 4,4'-DMAR with HLM. Blank urine was also analysed to verify the possible presence of matrix interferents. The urine samples were analysed through the two different techniques (QTOF and QqQ) and the same metabolites were identified. Therefore, only QqQ results are shown.

-Phase I

Urine samples of mice administered with *trans*-4,4'-DMAR did not undergo phase I or phase II metabolism, as well as observed for *in vitro* studies. The analysis of *in vitro* samples, after the intake of a dose of 10 mg/kg of *cis*-4,4'-DMAR, shows the formation of the same metabolites identified for the *in vitro* studies In detail, the parent compound and metabolites M1, M2, M3, M4 and M5 were identified as phase I metabolites. They are already excreted 2 hours after the intake

The metabolic pathway identified for *in vivo* studies is comparable to the results obtained by Lucchetti et.al [9] for *cis*-4,4'DMAR in rat and by Henderson et.al for the related compound 4-Methyl-Aminorex [20].The extracted chromatogram of a representative urine samples of phase I metabolism studies is reported below in Figure 9.

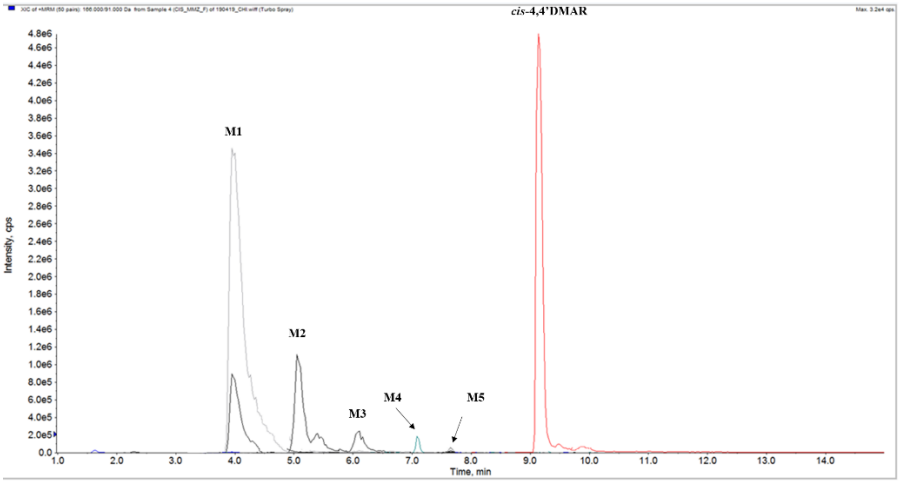


Figure IV.9. Extracted ion chromatograms of a representative urine sample of *in vivo* studies at t=2 hours after the administration of *cis*-4,4'DMAR in mice.

-Phase II

For the phase II metabolism studies samples employed for phase I studies were enzymatic hydrolysed with two different enzymatic mixture with the aim to estimate glucorono- and sulfo-conjugation separately. The procedure followed is described into experimental section. Results show that M1, M2 and M3 metabolites undergo to glucorono-conjugation where M2 and a small amount of M3 undergo to sulfo-conjugation with not significative excretion of M1 as sulfo-conjugate. The excretion profile of phase II metabolism is reported in Figure 10 respectively for glucuronidation (A) and sulfo-conjugation (B).

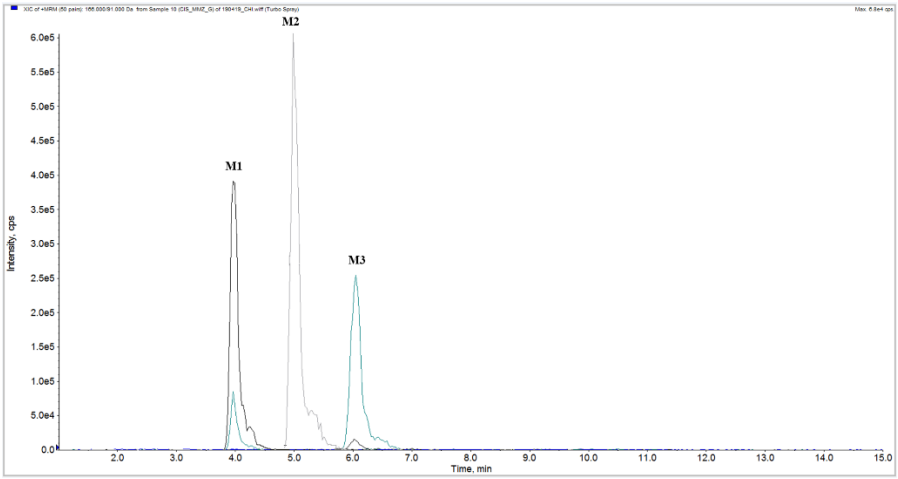


Figure IV.10 A. Extracted ion chromatograms of a representative urine sample of *in vivo* studies at t=2 hours after the administration of *cis*-4,4'DMAR in mice for phase II metabolism of glucorono-conjugates.

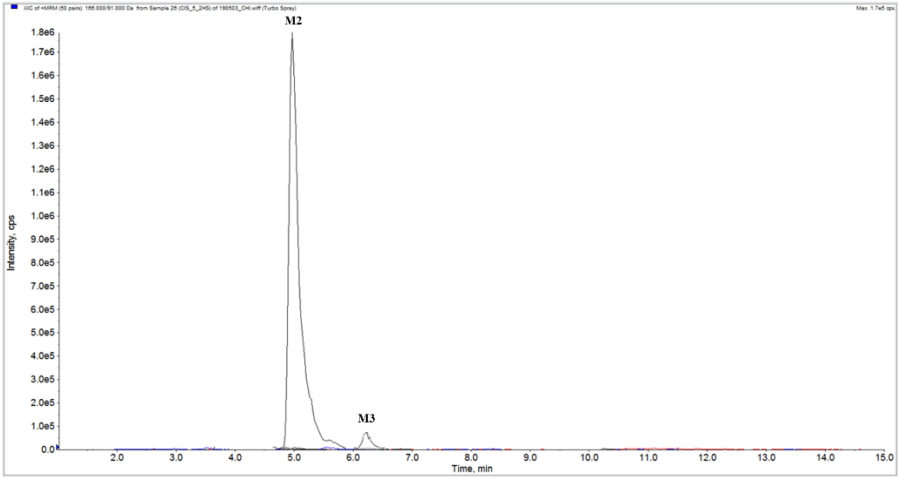


Figure IV.10 B. Extracted ion chromatograms of a representative urine sample of *in vivo* studies at t=2 hours after the administration of *cis*-4,4'DMAR in mice for phase II metabolism of sulfo-conjugates

The excretion profile of the principal metabolites M1, M2 and M3 into phase I and phase II shows that M1 is mainly excreted in phase I with small amount of phase II excretion as glucorono-conjugates (Figure 11 A). The other hydroxylated metabolite M3 shows a comparable excretion profile. However, the phase II excretion for M3 is more significant for glucorono- than sulfo- form (Figure 11 C). The excretion profile of the carboxylated metabolite M2 shows a mainly excretion as sulfo-conjugates with a small amount of glucoronate form and phase I excretion (Figure 11 C). Data were obtained by considering the variation in peak intensity ratio over time in the different phases.

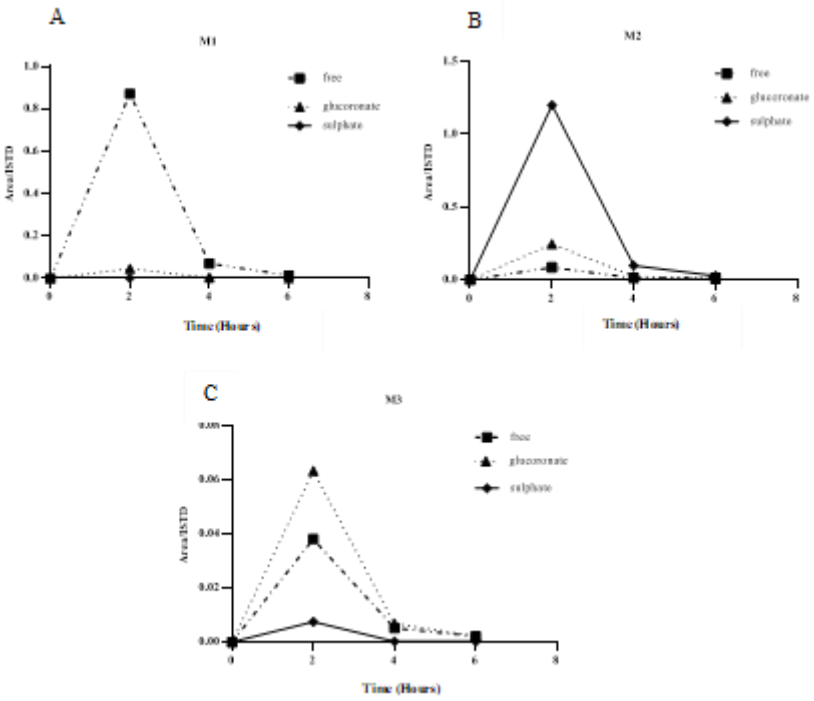


Figure IV.11. Excretion profile of the three principal metabolites of *cis*-4,4'DMAR into phase I (free) and phase II (glucoronate, sulphate) for M1 (A), M2 (B) and M3 (C).

The total excretion profile as the sum of phase I and phase I through the entire range shows that 4,4' DMAR itself, M1 and M2 are the most suitable marker of intake (Figure 12). Data were obtained by considering the variation in peak intensity ratio over time in the different phases.

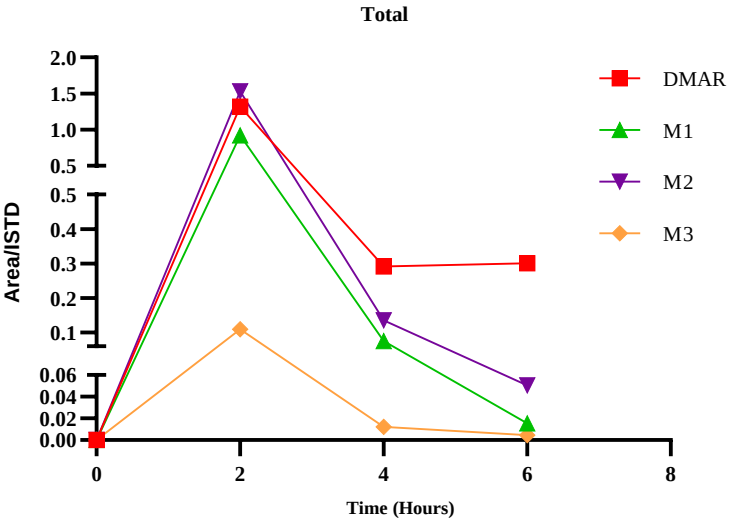


Figure IV.12. Total excretion profile is reported as a sum of phase I and phase II *in vivo* excretion

4 Conclusion

This is the first study of urine excretion profiles of *cis/trans*-4,4'-DMAR that defines the most suitable markers of intake after administration of a dose of 10 mg/kg of both isomers. Our data suggest that *trans*-4,4'-DMAR doesn't metabolize both *in vivo* and *in vitro*, where *cis*-4,4'-DMAR is metabolized into five different compounds where the two hydroxy and carboxylated metabolites are the most significant metabolic pathways identified for *in vitro* and *in vivo* studies. The most common human CYPs isoform were also evaluated, with CYP2D6 as the only isoform significantly involved into the metabolic transformation pathways. The three principal allelic variants of CYP2D6 studied as CYP2D6*(1-2-3), in order to evaluate possible different metabolism pathways. Results of *in vitro* studies show that the parent compound and one of the hydroxy metabolites are present in all the allelic variants, suggesting that they could be the proposed markers of intake for *cis*-4,4'-DMAR.

Acknowledgments

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Part II

Chapter V

Evaluation of *in vitro* metabolism of two cathinones: Pyrovalerone and Methedrone

Abstract

Pyrovalerone and methedrone are two synthetic cathinone derivatives that show stimulant effects and are prohibited “in-competition” by WADA as stimulant compounds included in prohibited list at S6 section.

The aim of the present work was to estimate the principal phase I metabolites of pyrovalerone and methedrone with the purpose to define the most suitable markers of intake as a tool in antidoping routine analysis. With this aim standard solutions of pyrovalerone and methedrone (1 mg/mL) were incubated with human liver microsomes or individual cytochrome isoforms and samples obtained after incubation were analysed through a liquid-chromatography tandem mass spectrometry technique. Our results showed that parent compounds of the two selected cathinones are detectable and are the most suitable markers of intake followed by the hydroxy metabolites. Furthermore, the cytochromes P450 isoforms (CYP2A1, CYP2B6, CYP2D6, CYP2C19, CYP2C9, CYP2E1, CYP3A4, CYP3A5) were evaluated in order to estimate possible drug-drug interaction and metabolic pathways of singular isoforms. The isoforms most involved in metabolic pathways of pyrovalerone and methedrone are CYP3A4 for both compounds followed by CYP2D6.

Introduction

Synthetic cathinones are a class of NPS derived from cathinone, a natural occurred alkaloid present in the khat shrub (*Catha edulis* plant[1]). This cathinone shows similar effects to other amphetamines, with lower effects on central stimulation potency[2–4]. First synthetic cathinones were developed starting from natural cathinone as potential therapeutic agents at the beginning of 1920s[5,6]. Actually, few cathinones were employed for therapeutic purpose due to potential abuse and dependence[4]. They were used as appetite suppressants or for chronic fatigue later withdrawn for their side effects dependency and abuse in users[4,7]. At the date, only bupropion was approved for medical use as an antidepressant, for asthenia syndrome and to support stopping smonking[8].

Since the mid-2000s, they have been employed as recreational drugs synthetized to circumvent current legislation on drug market as sold as "bath salts", "plant food" or "legal highs" labelled, as for other NPS as, "not for human consumption"[1,9–11], available recently into the "smart shops" on internet.

Chemically synthetic cathinones are a class of psychoactive substances structural related to amphetamines with a carbonyl group in α position which configures this class of substances as phenethylamine derivatives often called as "natural amphetamines", with similar stimulant effects of other amphetamine derivatives[1,3,9,12,13]. Synthetic cathinones act on monoamine reuptake transporters, increasing the level of monoamine in brain, or as transporter substrates which release monoamine through transporters[14–18].

According to Varì *et. al*[4] cathinone derivatives could be divided into three groups:

The first is *N*-alkyl compounds or compounds with an alkyl or halogen substituent at any possible position of the aromatic ring. (e.g. ephedrone, mephedrone, flephedrone, buphedrone, and pentedrone).

The second is methylenedioxy-substituted compounds with substituents at any position of aromatic ring (e.g methylone, pentylone, and butylone).

The third consists of pyrrolidine derivatives (e.g. 3,4-methylenedioxypyrovalerone (MDPV) and 3,4-methylenedioxyalphapyrrolidinopropriophenone (MDPPP)) with same skeleton of pyrovalerone.

The aim of this work was to investigate the principal phase I metabolites of pyrovalerone and methedrone, two synthetic cathinones with effects as stimulant substances prohibited in sport competition as a tool for antidoping analysis. Methedrone is a structural analogue of mephedrone first synthetized in 1933[19], entered in the market in 2009 as a recreational drug and soon related to fatal intoxication [20,21], part of the first group described above.

Pyrovalerone is an inhibitor of DAT, NET and SERT, first synthetized[22] in 1964[23]. Chemically as well as for amphetamine, pyrovalerone exists as two enantiomers where the 2S isomer is the only one that shows activity[7].

Pyrovalerone acts similarly to methylphenidate with stimulant properties proved in animal and human[7,24] employed for clinical use in chronic fatigue syndrome[25,26]. Pyrovalerone is related to pyrrolidine derivatives (also known as *"pyrovalerone*-cathinones")[27] and shows similar structure and action of the α-pyrrolidinvalerophenone (α-PVP)[28] or the most known MDPV, a potent monoamine transporter blocker and stimulant compound[29,30]. The few information about methedrone and pyrovalerone metabolism led to the establishment of an *in vitro* metabolism studies carried out with techniques employed in our laboratory as a tool to identify their principal phase I metabolic pathways with the aim to introduce these markers of intake into routine antidoping tests of our laboratory.

2 Experimental

2.1 Chemicals and reagents

Pyrovalerone *(1-(4-Methylphenyl)-2-(1-pyrrolidinyl)-1-pentanone)* and methedrone (*1-(4-Methoxy-phenyl)-2-methylamino-propan-1-one*) as well as amphetamine-D11 employed as internal standard were purchased by Sigma–Aldrich (Milan, Italy). All chemicals (i.e., formic acid, acetic acid, ammonium formate, ammonium acetate, sodium phosphate, sodium chloride, sodium hydrogen phosphate, potassium carbonate, potassium hydrogen carbonate, acetonitrile, absolute ethanol, methanol, dimethylsulfoxide [DMSO] diethyl ether, ethylacetate, and tert-butyl methyl-ether) were of analytical or HPLC grade and provided by Carlo Erba (Milan, Italy) and Sigma–Aldrich (Milan, Italy). The ultrapurified water used was of Milli-Qgrade (Millipore Italia, Vimodrone, Milan, Italy). The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian mixed male and female donors of different ages) as well as CYP recombinant isoforms (CYP2A1, CYP2B6, CYP2D6, CYP2C19, CYP2C9, CYP2E1, CYP3A4, CYP3A5), were purchased by Corning Incorporated (Woburn, Massacchussets, USA). All the reagents used for the *in vitro* metabolism studies (sodium phosphate buffer and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milan, Italy).

2.2 *In vitro* protocol

All incubation conditions were optimized including different solvents (methanol, DMSO and acetonitrile), pH values (5.0, 7.4, 8.0), concentration of both isomers (0.05, 0.1, 0.5 e 1 mg/mL) and of the enzymatic proteins (0.1, 0.2, 0.5 and 1.0mg/mL), and different incubation time (60, 120, 240, and 1440 min) were evaluated.

The final incubation medium also contained 6.6 mM magnesium chloride, 2.6 mM $NADP^+$, 6.6 mM glucose-6-phosphate and 4.0 U/mL glucose-6-phosphate dehydrogenase in 5 mM of sodium citrate with a total volume of 250µL. Samples were pre-warmed at 37°C for 5min and the reactions were started with the addition of the appropriate enzymatic proteins (HLM or CYP recombinant isoforms) at the final concentration of 0,5 mg/mL. After incubation at 37°C with the selected incubation time, 250µL of ice-cold acetonitrile were added to stop the reaction. The samples were then transferred into an ice bath for 3 min for further precipitation of the proteins in the assay medium. The supernatant was subsequently separated from the precipitate by centrifugation at 14.000 rpm at room temperature for 10 min. Each set of assays also included a negative control sample, containing all reaction mixture components except the enzymatic proteins to monitor the potential non-enzymatic reactions within the incubation period. Each incubation was processed in duplicate

2.3 Sample pre-treatment

All the samples obtained by the *in vitro* experiment (sample volume 500µL) were added with 1.5 mL of phosphate buffer (1 M, pH 7.4) and 50 µL of the internal standard solution (Amphetamine-D11 standard solution 1 mg/mL) and a liquid/liquid extraction was carried out with 7 mL of the most appropriate solvent (*tert*-butylmethyl, ethyl acetate, chloroform were tested) at controlled pH with different pH tested (5, 7,4, 9, 11). After 10 minutes of stirring at room temperature, samples were centrifugated at 3000 rpm for 2 min and transferred into an ice bath for 5 minutes. After centrifugation the organic layer was separated and evaporated to dryness under nitrogen stream at a temperature of 30°C.

The residue was reconstituted with 50µL of mobile phase (initial composition) and analysed with LC-MS techniques.

2.4 Instrumental conditions

Samples were analysed using an Agilent 1200 series HPLC instrument equipped with a DISCOVERY C18 column (15 cm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass spectrometer (Sciex) with an ESI source, and operated in the positive-ion mode. Detailed analyses were carried out at constant flow rate of 250 µL/min using mobile-phase ultra purified water, 0.1% formic acid (A), and acetonitrile 0.1% formic acid (B). The selected gradient program started at 10% of B and increased to 60% of B in 7 min, after 6 min up to 100% B, in 1 min and stored to 100% for 6 min. The column was then re-equilibrate in 2 min to 10 % of B for 6 min.

Multiple reaction monitoring (MRM) was used as the acquisition mode for detection (see Tables 1-2 for the ion transitions selected)- For the MRM collision-induced dissociation (CID), nitrogen was used as the collision gas at 5.8 mPa, obtained from a dedicated Parker-Balston nitrogen generator system (model 75-A74) with 99.5% gas purity (CPS Analitica Milan, Italy). The mass spectrometric parameters (declustering and needle voltages, gas pressure, source temperature, collision cell exit potential, and collision energy) were optimised by infusing the standard solution of methedrone and pyrovalerone at a concentration of 10 µg/mL.

All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by Analyst software version 1.6.1

3 Results and discussion

3.1 Instrumental conditions

The instrumental parameters were optimised by infusing the standard solutions of pyrovalerone and methedrone dissolved in the mobile phase at a concentration of 10 µg/mL. The best ionisation conditions for methedrone $[M+H]^+$ ions at *m/z* 195 and pyrovalerone *m/z* 246 were obtained using a curtain gas pressure of 25 psi, a source temperature of 500 °C, an ion source gas 1 (auxiliary gas) pressure of 35 psi, an ion source gas 2 (nebuliser gas) pressure of 45 psi, a declustering voltage of 60 V, and a needle voltage of 5,500 V. Different collision energies were evaluated to select the characteristic fragmentation patterns of both cathinones and the possible product ions of their metabolites, a collision energy of 30 eV was selected.

The selected specific product ions (structural markers) for pyrovalerone are the phenylmethylium ion at *m/z* 91; 4-(pyrrolidin-1-yl)but-1-en-1-ylium,at *m/z* 119; and [(4-methylidenecyclohexylidene)methylidene]oxidanium ion at *m/z* 126.

For Methedrone, the diagnostic ions are *N*-methylethenaminium *m/z* 58; methylidene(phenyl)oxidanium; *m/z* 107; (4-formylphenyl-methylidene) oxidanium *m/z* 135. The fragmentation patterns of parent compounds and relative *m/z* are reported in Figure 1A and 1B. Based on these structural markers, was developed the MRM method with a specific transition for each potential metabolites. The predicted fragmentation patterns for MRM analysis of each metabolites are reported in Tables 1-2 with the specifically selected precursor and product ions as $[M+H]^+$ and collision energies employed.

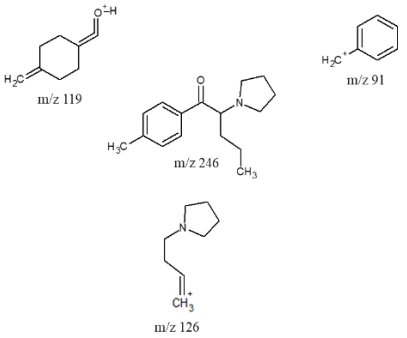


Figure V.1A. Fragmentation pattern of Pyrovalerone using a collision energy of 30 eV

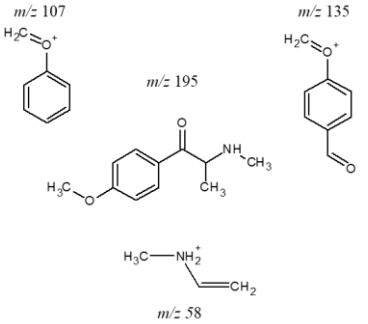


Figure V.1B. Fragmentation pattern of Methedrone using a collision energy of 30 eV

Table V.1. Precursor ions, product ions and collision energies of Pyrovalerone and its metabolites, in bold for diagnostic ions transition.

Compound	Precursor ion(*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Pyrovalerone	246	**119;** 91**;126**	30; 30; 25
M1 (alkyl-hyrodxy)	262	**135**; **107**; 126	35; 30; 25
M2 (alkyl-hydroxy)	262	91; **119**	30; 40
M3 (hydroxy-oxidation)	260	91; **119**	35; 40; 30
M4 (*N*-demethyl)	192	91; **119**	30; 35
M5 (aryl-hydroxy)	262	**107**; 135; **123**	30; 25;30

Compound	Precursor ion(*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Methedrone	195	**58; 107;135**	30; 30; 25
M1 (*N*-demethyl)	180	135; **107**; **72**	35; 30; 25
M2 (*O*-demethyl)	180	**58**; **121**	30; 40
M3 (alkyl-hydroxy)	210	58; **107**; 135;**102**	35; 40; 30

Table V.2. Precursor ions, product ions and collision energies of Methedrone and its metabolites, in bold for diagnostic ions transition.

3.2 *In vitro* metabolism

In vitro metabolism studies were conducted using HLM from a pool of 20 Caucasian male and female donors of different ages to minimise the effect of intra-individual variation and present the 'average' enzyme activity. The relative contribution of induvial CYPs isoforms to the metabolic reactions was evaluated with the aim to estimate the CYPs involved and potential drug-drug interactions with other substances. The best results were obtained using methanol as a substrate solvent (the total amount of methanol in the final assay was 1%), substrate concentration of 20 µM, protein concentration of 0.5 mg/mL, phosphate buffer of 0.1 M at pH 7.4, and incubation time of 4 h at 37 °C. To optimise the pre-treatment samples, different extraction solvents (*tert*-butyl methyl-ether and ethylacetate) and pH values (5, 7.4 , 9 and 11) were evaluated. The best extraction results for parent compounds and metabolites were obtained with addiction of 50 µL of phosphate buffer (pH 7,4) followed by addiction of 5 mL of *tert*-butyl-methylether.

-*Pyrovalerone*

The analysis of *in vitro* samples after 4 h of incubation with HLM shows the formation of five principal phase I metabolites. The metabolic reactions identified are: alkyl-hydroxylation on para-methyl group of benzene ring (M1), alkyl-hydroxylation on side chain (M2), aryl-hydroxylation (M5), *N*-demethylation (M4) and hydroxylation on alky-chain followed by oxidation (M3)

The postulated structures for metabolites identified are reported

below in Figure 2

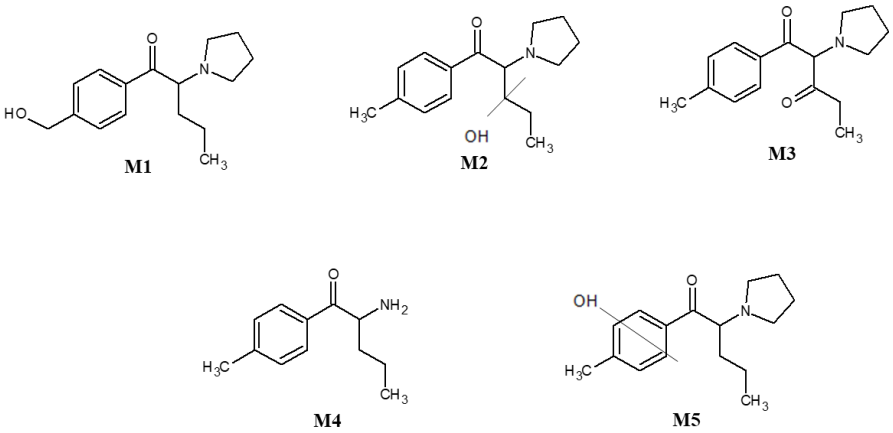


Figure V.2. Postulated structures of the phase I metabolites identified for Pyrovalerone.

Here reported in Figure 3 the extracted chromatogram after incubation of pyrovalerone with HLM for 4 h of incubation. Were the parent compound and M5 metabolite are the most suitable markers of intake.

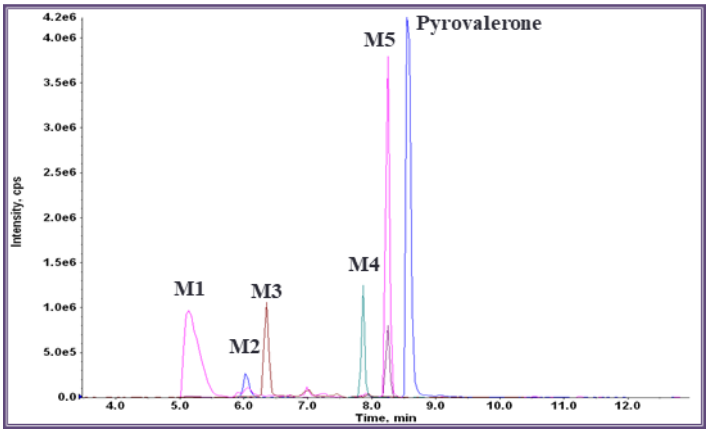


Figure V.3. Extracted chromatogram with MRM mode of a representative sample after 4h of incubation of Pyrovalerone with HLM

To define the relative contribution of individual CYPs isoforms to the metabolic reactions, The standard solution of pyrovalerone standard (concentration of 1 mg/mL) was incubated with different CYP450 isoforms. The involved isoforms evaluated for the phase I metabolism are: CYP3A4, CYP2D9. For all isoforms the relative contribution to single metabolic reaction were evaluated and calculated as percentage by the total amount of metabolites identified (peak intensity ratio), see Figure 4. The percentage was calculated from total amount (peak intensity ratio) of each metabolite formed by the allelic variant considered.

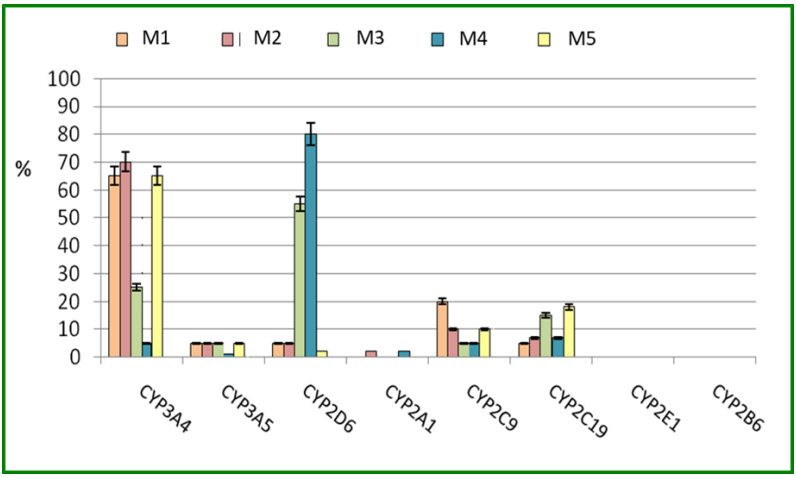
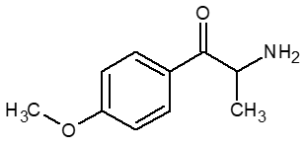


Figure V.4. Percentage contribution of each CYPs isoform to metabolism of Pyrovalerone

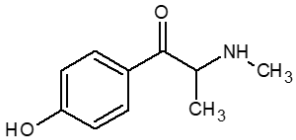
As described in Figure 4 CYP3A4 is the most involved isoform into phase I metabolic reactions for the three hydroxy-metabolites found (M1, M2, M5) with contribution higher than 60%. The *N*-demethylation and hydroxy-oxidation are mainly formed by CYP2C6 with contribution higher than 50%. The secondary isoforms involved in the formation of all metabolites are CYP2C19 and CYP2C9 with contribution ranged from 5% to 20% of total amount.

-*Methedrone*

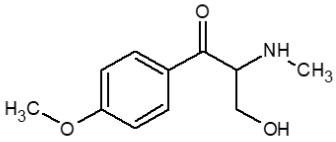
The analysis of the *in vitro* samples allowed to the detection of three different principal metabolites. In detail, for methedrone were identified the *N*-demethylated metabolite (M1) the *O*-demethylated metabolite (M2) and the hydroxy-alkyl metabolite (M3). The best incubation results were obtained after 4 h of incubation with HLM. Postulated structures of metabolites identified are reported in Figure 5.



M1



M2



M3

Figure V. 5. Postulated structures of the phase I metabolites identified

The extracted chromatogram of a representative sample analysed after 4h of incubation with HLM is reported below in Figure 6. methedrone itself and M3 are the markers of intake

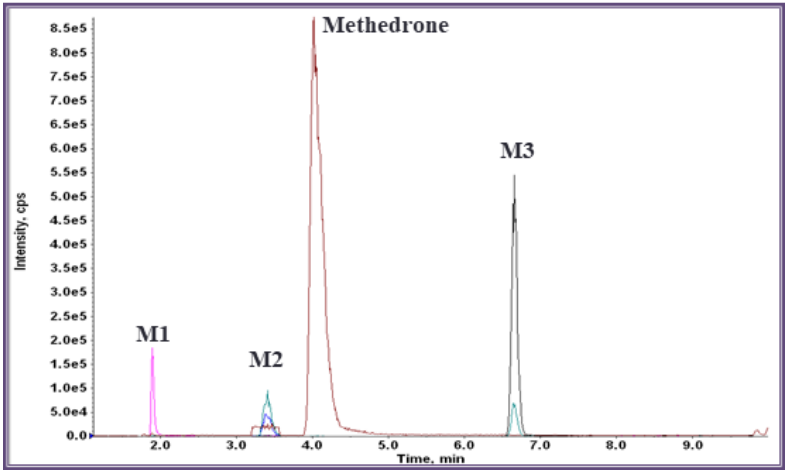


Figure V.6. Extracted chromatogram with MRM mode of a representative sample after 4h of incubation

The relative contribution of individual CYPs to the metabolic reactions of methedrone shows that the most involved isoforms are: CYP3A4, CYP2C9, CYP2D6, see Figure 7. The percentage was calculated from total amount (peak intensity ratio) of each metabolite formed by the allelic variant considered.

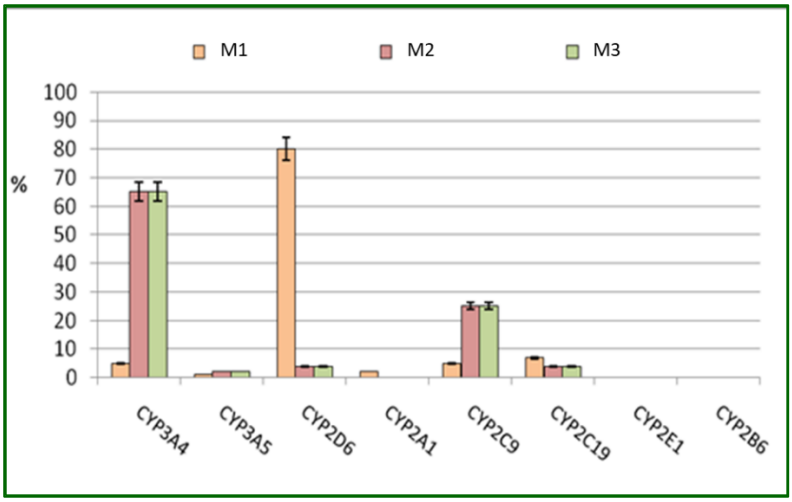


Figure V.7. Percentage contribution of each CYPs isoform to metabolism of Methedrone

As described in Figure 7 CYP3A4 is the most involved isoform into phase I metabolic reactions in particular for *O*-demethylation(M2) and hydroxylation (M3) with contribution higher than 60% followed by CYP2C6 with contribution higher than 20%. The *N*-demethylation reaction seem to be catalysed almost by CYP2D6 with a contribution near 80% of total amount of M1 metabolite, followed by CYP2C19, CYP2C9 and CYP3A4. The secondary isoforms involved in the formation of all metabolites are CYP2C19 and CYP3A5 with contribution ranged by 1% to 5%. of total amount

4. Conclusion

The presented work estimates the principal phase I metabolic reactions of two synthetic cathinones with the aim to establish the most suitable markers of intake for methedrone and pyrovalerone as a tool in doping analysis. Methedrone forms three principal metabolites, methedrone itself and its alky-hydroxy metabolite are the most suitable markers of intake. For pyrovalerone five different metabolites were identified with parent compound and hydroxy-aryl metabolites as the most suitable markers. Furthermore, results of singular CYPs isoform incubation show for both compound that CYP3A4 and CYP2D6 are the most involved isoforms in metabolism with significant differences among metabolites percentage formation.

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Part II

Chapter VI

Excretion profile of Gamma-valero-lactone and affection of testosterone and IGF-1 level in mice.

Adapted from:

C. Camuto, X. Raffaella Arfè, Micaela Tirri, de la Torre, F. Donati, M.Mazzarino, M. Marti, F. De-Giorgio, F. Botrè. “Gamma-valero-lactone (GVL) a potential underestimated drug facilitated sexual assault: study on urinary excretion window and affection of visual placing response in mice. Submitted paper

Abstract

Gamma-valero-lactone is a potential drug facilitated sexual assault that shows structural and functional similarities with the known γ-hydroxy-butyric-acid. The aim of the present work is to evaluate the excretion profile of γ-valerolactone and the affection on urine testosterone level and blood insulin growth factor 1 level after γ-valerolactone intake. A dose of 400 mg/kg of γ-valerolactone was selected and administered to a mice group in order to evaluate the urine excretion profile of γ-valerolactone in the range of 0-36 h. Urine samples were analysed by a newly developed and validated procedure based on rapid and effectiveness liquid/liquid extraction without derivatization, followed by instrumental analysis by gas chromatography coupled to mass spectrometry. Testosterone urinary levels were detected through a developed and validated method applied in our laboratory for determination of steroid excretion profile. insulin growth factor 1 analysis was set through a specific ELISA kit for mouse isoform of insulin growth factor 1 in serum, a dose of 100 mg/kg of γ-valerolactone was selected for both studies.

Our results of excretion study show that γ-valerolactone is excreted only in the first 0-8 h. Furthermore, γ-valerolactone is extensively converted into phase II form in the first 3 hours by

the administration, despite this phase II γ-valerolactone is non-detectable after 5h by the administration. Testosterone is decreased after γ-valerolactone intake as well as insulin growth factor 1.

1 Introduction

The use of γ-hydroxy-butyric-acid (GHB) in cases of drug facilitated sexual assault (DFSA) has been known and reported in several case [1–3]. As well as reported use of GHB as a recreational drug that enhances mood and pro-social behaviour [4,5], often involved in intoxication and fatal case [6–8], is not easily detectable with an excretion window in urine of 0-6 h after intake [9–11]. Therefore, GHB is a controlled substance over different countries including Italy where is contained in Table IV of Italian law DPR 309/90 [12]. This brings consumer to find possible legal analogue such as γ-butyrolactone (GBL) or 1,4 butanediol (BD)[13–15]. On this trend, γ-valero-lactone (GVL) is a potential substitute with a cyclic ester containing a methyl group at the fifth position of a dihydrofuran-2(3H)-one ring. The methyl group differentiates GVL from GBL. The lactone form of GVL shows a pH dependent equilibrium with is open ring form γ-hydroxy-valeric-acid (GHV) [16,17] as known as 4-methyl-GHB [18], as well as for GHB and its lactone GBL. Their similarities suggest that GVL could be a legal substitute of GHB, available on internet as "Tranquilli G" [19], or as a green solvent as well as used by the food industry frequently as a food additive[20]. Effect of GVL as a potential DFSA or recreational drug was investigated in different works [21–23]. However, until now only three cases of uptake of γ-valero-lactone in human were reported, in one of these drug-facilitated sexual assault (DFSA) was assumed [24], this poor number of cases reported was probably imputable to routine drug test in hospitals non set for this substance.

Studies on GVL activity show the conversion of GVL and GBL into their open ring and active forms GHV and GHB, that occurs by human lactonase with a higher maximum velocity for GVL than for GBL [21]. Nevertheless, GHV is not metabolized into GHB or GABA [21,25], where GHB is considered both a metabolite and precursor of GABA[26], suggesting different interaction of GVL with GABA receptor from GHB. With affinity of GVL for GABAa and GHB receptors is a half than GHB [21,25].

Furthermore, as known GHB is an endogenous substance with various neurobiological effects on neuroendocrine system that could affect steroidal profile (i.e. oxytocin, or sexual steroid) or Growth Hormone and the hypothalamic–pituitary–adrenal axis and subsequently its intake is possible related to variation of glucorticod profile (i.e. cortisol) [27]. Bosch et al. showed how intake of low dosage of GHB in human volunteers affects level of progesterone while nor plasma levels of oxytocin, testosterone, cortisol, ACTH and aldosterone [5]. While Bacoflen, a GABAa inhibitor, shows a decrease in testosterone level in mice [28]. Furthermore, GH and subsequently IGF-1 were increased after GHB [29] or Bacoflen intake [30,31].

The aim of the present work is to estimate the urine excretion profile of GVL to understand how is excreted through a range of 0-36 h after the administration, with the purpose to set a rapid and cost effectiveness method helpful in forensic toxicological analysis and compare this data with GHB excretion. IGF-1, testosterone and epitestosterone level were also evaluated in order to understand if GVL, as a GABAa inhibitors, could affect their level likewise GHB. For this purpose, we have selected three different mice groups for the study of testosterone urinary level and IGF-1 blood level and excretion studies. For the excretion studies urine samples were collected every hour in the interval of 0 to 8 hours and subsequently in the interval of 24 to 36 hours after the administration. For testosterone and epitestosterone blank samples were collected every day for 3 days as well as treated samples collected every day for 4 days. Blood samples for IGF-1 studies were collected with the same range. A dose of 100 mg/kg was selected for both studies and daily administered for 4 days for both studies.

2 Experimental

2.1 Chemical and reagents

γ-valero-lactone (GVL) and γ-caprolactone (used as internal standard for excretion study) as well as Testosterone (T), Epitestosterone (E), 17a- methyltestosterone (used as internal standard for steroid profile) were from Sigma-Aldrich (Milan,Italy); deuterated standards (T-d3, E-d3, (used to monitor the yield of the enzymatic hydrolysis reaction) were from the National Measurement Institute (NIM, Pymble, Australia). The derivatizing agent was a mixture of N-methyl-N-trimethylsyliltrifluoroacetamide (MSTFA) / mercaptoethanol / ammonium iodide (NH4I) (1000:6:4 V/V/w) stored in screw-cap vials at 4 °C for a maximum of two weeks. MSTFA was supplied by Chemische Fabrik Karl Bucher GmbH (Waldstetten, Germany); NH4I and mercaptoethanol were from Sigma-Aldrich (Milan, Italy). All the reagents (acetic acid, ammonium acetate, sodium phosphate, sodium chloride, sodium hydrogen phosphate, acetonitrile, methanol, *tert*-butyl methyl-ether) were of analytical grade and provided by Sigma-Aldrich (Milan, Italy). The enzyme mixture β-glucuronidase/arylsulfatase (from *Helix Pomatia*) used for the enzymatic hydrolysis of both the glucurono-conjugates and the sulfo-conjugates as well as β-glucuronidase from *E. Coli* for hydrolysis of steroid were purchased from Roche (Monza, Italy).

The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian mixed male and female donors of different ages) as well as all the reagents used for the *in vitro* metabolism studies (sodium phosphate and tris-HCl buffers and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milan, Italy). Invitrogen IGF-1 Mouse ELISA Kit was purchased form Thermo Fisher Scientific (Milan, Italy).

Vacumed (13x75mm with gel separator + clot activator x3,5 mL of blood) for the separation of whole blood and serum was purchased from FL MEDICAL s.r.l. (Padua, Italy).

2.2. Animals, dose selection and samples collection

For all protocols set into this studies GVL was administered through gastric gavage 5-10 minutes before collecting of urine or blood samples

-In vivo studies

For the *in vivo* studies a group of 5 mice was selected, a single dose of 400 mg/kg of GVL was administered and collecting of samples started at 9:00 a.m. for each mouse. A group of 5 mice was also selected as control group for urine blank samples. Urine samples were collected every hour in the range 0-8 hours after the injection of GVL and in the range 24-36 hours with the aim to define the best marker(s) of intake and their urinary excretion profile. Urine blank samples were also collected in the same range from the control mice group.

-Steroid profile

For the steroid profile study, a group of 4 mice was selected. Urine blank samples of each mouse were collected every day for 3 days at 9:00 a.m. for 3 hours. After 3 days the same mice where subsequently daily administered for 4 days with a dose of 100 mg/Kg of GVL, in order to evaluate the variation of testosterone level at low dosage of GVL corresponding to a stimulant action of GVL. Urine positive samples were daily collected for each mouse, next to the administration, in a range of 3 hours after the intake reflecting the same protocol for the blank samples.

-IGF-1 immunoassay

For the IGF-1 level a group of 5 mice was selected. Blood blank samples of each mouse were collected every day for 3 days at 9:00 a.m. After 3 days of blank samples mice were daily administered with a dose of 100 mg/Kg of GVL. Blood samples were collected after 10 minutes by the intake. For the immunoassay method 0.5 mL of whole blood was collected into Vacumed tube to obtain approximately 150 µL of blood serum next stored into a 1,5 mL tube and stored at -80°C.

2.3 Excretion studies

2.3.1 Protocol for the *in vitro* studies

All incubation conditions for GVL, i.e. proteins and substrate concentrations, buffer and solvent types, incubation time (evaluated in the range 0-48h), were optimized, starting from protocols already published and used by our group [32,33]. The final incubation medium also contained 2 µL of GVL standard solution (0.1 mg/mL in MeOH), 3.3 mM magnesium chloride, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase, in a total volume of 250 µL. Samples were pre-warmed at 37 °C for 5 min and the phase I reactions were started with the addition of HLM. After incubation at 37°C, 250 µL of ice-cold acetonitrile were added to terminate the phase I reactions. The samples were then transferred into an ice bath for further precipitation of the proteins in the assay medium. The precipitate was subsequently separated from the supernatant by centrifugation at 21.000 g (15.000 rpm) at room temperature for 10 min. Each set of assays also included a negative control sample, containing all reaction mixture components except the enzymatic proteins, to monitor the potential non-enzymatic reactions, leading to the possible formation of degradation products. Each incubation experiment was performed in triplicate.

2.3.2 Protocol for the *in vivo* studies

-Samples pre-treatment

GVL establishes an equilibrium between its lactone form and its γ-hydroxy-acid open ring form called 4-hydroxy-valeric-acid (4-HVA) (Figure 1). This is a pH dependent equilibrium where the lactone form is predominant and stable at pH 7 in aqueous solution [16,17].

For the sample treatment lactone equilibrium of GVL was considered and all samples were extracted at a controlled pH with 0.8 M phosphate buffer (pH 7).

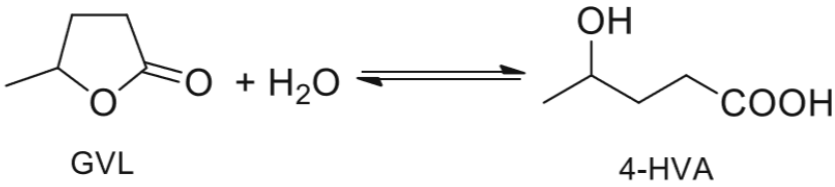

Figure VI.1 Aqueous equilibrium of GVL

-Phase I-II metabolism

Sample preparation was optimized using the sample obtained after incubation of GVL with HLM testing different conditions (pH, buffer, solvent of extraction). In details, a volume of 10 up to 200 µL of urine sample was added with 50 µL of the standard solution of γ-caprolactone (final concentration 100 ng/mL). Sample was then buffered with 100 µL of phosphate buffer 0.8 M (pH 7) and added with 5 mL of *tert*-butyl-methyl-ether. After 20 minutes of mild stirring at room temperature, samples were centrifugated at 3000 rpm for 2 min and transferred into an ice bath for 5 minutes, this procedure was repeated three times in order to extract all phase I GVL to reduce interference in phase II metabolism study after the enzymatic hydrolysis.

The three organic layers were collected and evaporated to dryness under a nitrogen flow at room temperature. The final residue was dissolved in 50 µL of *tert*-butyl-methyl-ether and then analysed by GC-MS.

The aqueous layer was stored up for the phase II metabolism studies and subsequently added with 200 µL of acetate buffer 1 M (pH 5), 50 µL of the standard solution of γ-caprolactone (final concentration 100 ng/mL) and 50 µL of β-glucuronidase/Arylsulfatase for the hydrolysis of glucorono-conjugates and sulphate-conjugates metabolites, converted into phase I form after 1.5 hours of incubation at 55°C. Samples were then added with 500 µL of phosphate buffer 0.8 M following the same procedure described for the phase I metabolism.

2.3.3 Instrumental conditions

Samples were analysed using an Agilent 6890/5973A gas chromatography-mass spectrometry (GC-MS) system (Agilent Technologies, Milan, Italy), in electron ionization (70 eV) mode, using an HP-5 fused silica capillary column (cross-linked (5%-Phenyl)-methylpolysiloxane, length 17 m, i.d. 0.20 mm, film thickness 0.33 µm; Agilent Technologies). The GC condition were as follows: the carrier gas helium at a constant pressure rate of 15 psi; injection volume was 2.0 µL with inlet operating in pulsed splitless mode, purge flow split 10 mL/min at 0.5 min injection pulse pressure 25 psi until 0,30 min ; temperature program, 50 °C (0.5 min-hold), 10 °C/min to 100 °C, 40 °C/min to 310 °C hold for 3 min; the transfer line and injection temperature operated at 280 °C. The acquisition was performed in selected ion monitoring (SIM) mode, specific retention time and diagnostic ions transition for GVL and internal standard y-Capro-lactone were reported in Table 1.

Table VI.1. Mass of compounds, Diagnostic ions and retention time of GVL and internal standard. Diagnostic ions selected to check LOD were in Bold

Compound	Mass	Diagnostic ions (*m/z*)	Retention time (min)
GVL	100	**56**, **85**, 86, 99, **100**	4.04
y-capro-lactone	114	**56**, **70**, 85, 86, **114**	5.52

Here reported below the extracted mass fragmentation patterns for GVL in GC-MS

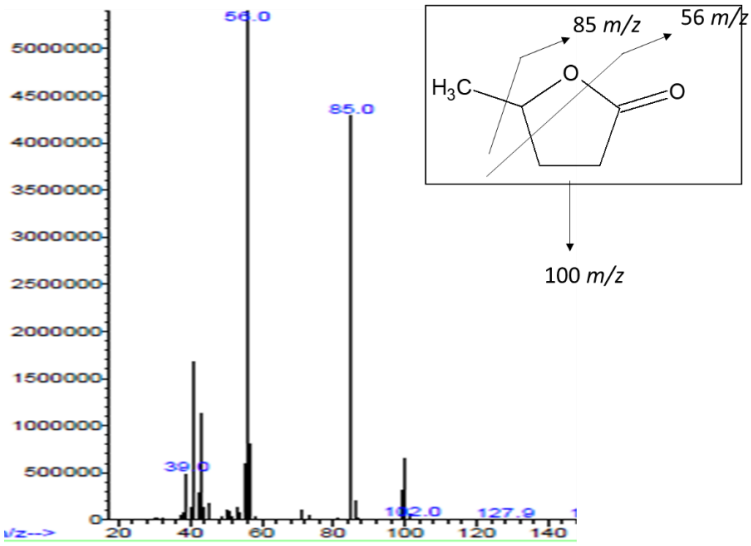

Figure VI.2 Fragmentation pattern of GVL

2.3.4 Method Validation

The method was qualitatively validated for GVL, as defined by sensitivity, limit of detection (LOD), recovery, and repeatability. Results are in accordance with WADA guidelines for minimum required performance level for non-threshold substances identified in urine. For non-threshold substances quantification of analytes and relative parameters (e.g LOQ, linearity) are not required by WADA.

-Selectivity

Selectivity was evaluated with the analysis of blank human urine collected from twenty volunteers and mice blank samples. Samples were analysed with the same protocol of positive samples verifying the presence of possible interfering compounds at the same retention time with the specific diagnostic ions of SIM mode.

-LOD

Limit of Detection (LOD) was evaluated with blank urine samples spiked with GVL starting at a concentration of 500 ng/mL with progressive dilution. LOD was defined as the concentration giving a S/N value greater than 3 for all the three diagnostic ion fragments.

-Recovery

Recovery was evaluated with 8 urine samples spiked with GVL at a concentration of 3 times the LOD. The normality of data distribution was evaluated with Shapiro Wilk test, in order to apply the Dixon test for the identification of outliers. Variance, confidence interval and Coefficient of Variation (CV) were subsequently evaluated.

-Repeatability

Repeatability was evaluated on 20 blank urine samples spiked with GVL at a concentration of 3 times the LOD, calculating the ratio between substance and internal standard.

Ten samples were analysed in the first day and the other ten after two days to evaluate the repeatability in two days of analysis with the same operator. Statistics parameters were defined following the UNICHIM directives [34], in details:

Check of normality of data distribution was evaluated with Shapiro-Wilk test (formulas [35]) to apply statistical tests that request normality of distributions like Dixon test for outliers [36], Homogeneity Variance Tests [37] and ANOVA test [38]. Variance, confidence interval and CV were also calculated.

Variance Homogeneity of the two distribution of data in the two days of analysis was evaluated with two different variance homogeneity tests as Cochran's C Variance test and Minimum Variance test [37]. Variance Homogeneity allows the application of ANOVA One-Ways Test for repeatability.

2.4 Steroid profile

Urinary Testosterone and Epitestosterone level were detected with the aim to establish possible variation of their level. T/E ratio was also evaluated to check the possible variation of ratio values. Specific gravity of urine samples was measured to normalize the urinary concentrations according to the WADA Technical Document [39].

-*Sample pre-treatment*

Samples collected under sterile conditions were stored at -80°C until analysis. Sample preparation was performed following the validated protocol adopted in our laboratory for the detection of steroids in human urine [40]. A volume of 1 mL for each sample was added with 50 µL of β-glucuronidase from *E. Coli* to perform enzymatic hydrolysis of the glucuronidated steroids, 1 mL of phosphate buffer (1 M, pH 7), 50 µL of the internal standard (see Table 2 for final concentrations in urine).

Samples were incubated for 1 h at 55 °C. After this time, 1 mL of carbonate/bicarbonate buffer (0.8 M, pH 9) was added and liquid/liquid extraction was carried out with 7 mL of *tert*-butyl-methyl ether for 5 min on a mechanical shaker. Samples were centrifuged and the organic layer was transferred to a 10 mL tube and evaporated to dryness under nitrogen stream at 70 °C.

The residue was reconstituted in 50 µL of the derivatizing mixture, and the sample was maintained at 70 °C for 20 min. Then, a 1 µL aliquot of it was injected into the GC–MS system.

-Instrumental conditions

The analysis of target testosterone and epitestosterone was carried out according to previously published method [40] already applied to assess the influence on the steroid profile of other xenobiotics [41,42] and according to the guidelines reported in the WADA Technical Document TD2018EAAS "Endogenous Anabolic Androgenic Steroid. Measurement and Reporting"[39].

Briefly, quantitative analysis was performed on an Agilent 6890/5973A gas chromatography–mass spectrometry (GC–MS) system (Agilent Technologies, Milan, Italy), in electron ionisation (70 eV) mode, using an HP-1 17-m fused silica capillary column (cross-linked methyl silicone, i.d. 0.20 mm, film thickness 0.11 µm; Agilent Technologies).

Identification was based on the specific retention times and on the diagnostic ions listed in Table 1 (fragments for quantification shown in boldface). Quantitative determination of the urinary concentration was based on the peak area ratio of the analyte to the corresponding internal standard (i.e., the corresponding deuterated compounds, where available, or 17a-methyltestosterone for all the other target analytes). Calibration samples were prepared in synthetic urine. The final concentrations of target compounds in calibration samples and of internal standards in all samples are shown in Table 1. Calibration samples were re-analysed every 15 injections along the sequence and used also as quality controls.

The linearity range was investigated by injection of each steroid at different concentrations (5, 50, 100, 200, 2000 ng/mL), to cover the entire range of the expected concentrations. Different urine samples spiked with increasing steroid concentrations were also evaluated. For all analytes the limit of quantification (LOQ) was B5 ng/mL, (calculated as ten times the standard deviation of the background noise).

Table VI. 2. Diagnostic ions used for quantitation are shown in bold. The final concentrations of the target compounds in the calibration samples and of internal standards in all samples are shown in column 3.

Target Analyte	Diagnostic Ions (m/z)	Final concentration in urine (ng/mL)
Testosterone	**432**, 417, 301	40
Epitestosterone	**432**, 417, 301	10
Internal standards		
17a-Methyltestosterone	**446**, 431, 301	250
Testosterone-d3	**435**, 420, 301	100
Epitestosterone-d3	**435**, 420, 301	25

2.5 IGF-1 immunoassay

The Elisa Kit procedure was followed for the detection of IGF-1 level in serum. Briefly, 100µL of serum was added with 100 µL of standard followed by incubation for 2.5h, then 100 µL of antibody and incubation for 1h, then 100 µL of Streptavidin HRP Reagent and incubation for 45 min, followed by 100 µL TMB substrate and dark incubation for 30 min, then 50µL of stop solution followed by the measure of absorbance at 450nm through a Perkin Elmer VICTOR X3 MultiLabel Plate Reader.

3 Results

3.1 GVL metabolism study

In vitro metabolism studies were performed with the aim to study phase I metabolism of GVL. Only GVL itself was identify after 48 h of incubation with HLM. Subsequently GVL was selected as the only marker of intake for *in vivo* studies both of phase I and phase II.

-*In vitro metabolism study*

In vitro metabolism studies were carried out through HLM from a pool of 20 Caucasian mixed male and female donors of different ages in order to minimize the effect of intra-individual variation and to present an "average enzyme activity". A substrate concentration of 20 µM, a proteins concentration of 0.5 mg/mL, phosphate buffer 0.1 M at pH 7.4 were employed. After 48 h by the incubation with HLM no phase I metabolites were detected.

Subsequently the decrease of GVL over different incubation time was evaluated in order to understand if GVL did not metabolize. With this aim different incubation time was selected starting after 0 min to 48 hours, a one sample t-test was performed to estimate variation by incubated samples and 0 min control sample.

P value (P=0.411) show no significant difference between this two groups. These results indicate the non-metabolization of GVL. Data are reported in Table 3.

Table VI. 3. Relative Area, time interval, average, standard variation (Sd) and CV% for GVL incubation with HLM. Result of one-sample t-test are reported as P value (P), t calculated (t) and degrees of free (df).

Time	R. Area
0'	0,0514
15'	0,0580
30'	0,0690
60'	0,0494
90'	0,0621
180'	0,0590
240'	0,0411
360'	0,0407
12h	0,0617
24h	0,0515
48h	0,0473

Average	**0,0537**
Sd	**0,0008**
CV%	**1,5147**

one-sample t-test

P=0,411
t=0,8619
df=9
0' vs incubation not significantly different

-In vivo metabolism study

Urine samples collected from mice every 1 h after a dose of 400 mg/kg of GVL were analysed with the analytical protocol set up and optimized using the samples obtained after incubation of GVL with HLM. Blank urines were also analysed in order to verify the possible presence of interferents.

For the i*n vivo* studies an appropriate volume of samples for the different hours range was selected due to the high concentration of GVL in the first 0-2 hours and its progressively decreased over the entire range. Results show only GVL as the principal marker of intake both in phase I and in phase II samples. The extracted chromatogram of a representative sample of *in vivo* studies is shown in Figure 3 with GC-MS operating in full scan mode.

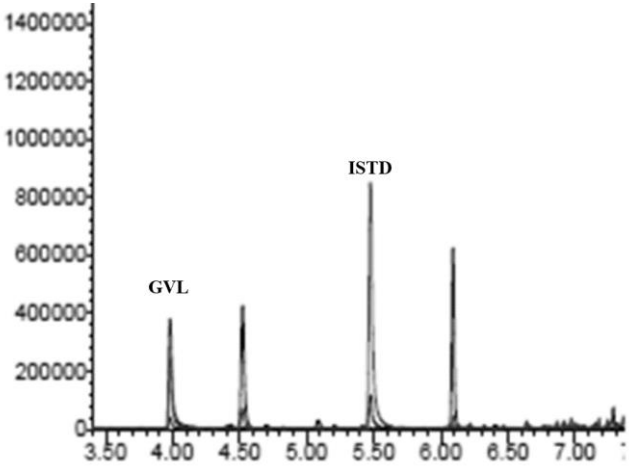

Figure VI.3. Extracted chromatogram of an urine sample with GC-MS operating in full scan mode.

Data of excretion show the progressive decrease of GVL over the first range and the undetectability of GVL in the range of 24-36 h after the administration.

Data were reported in Figure 4 for both phase I and phase II GVL excretion, as expressed as ratio between GVL and internal standard over the range studied, corrected for the volume of samples used for analysis.

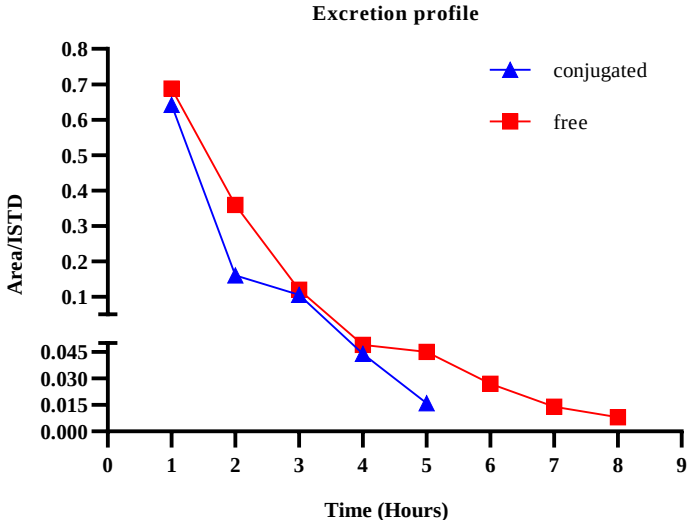

Figure VI.4. Excretion profile of GVL both of phase I (free) and phase II (conjugated).

In vivo studies showed that GVL does not undergo phase I metabolism, these data are also fully supported by the *in vitro* data, where the incubation of GVL for 48 h did not show any statistically significant decrease of study samples compared to blank control samples, see Table 3. This was probably due to the high hydrophilicity of the γ-valerolactone and its equivalent 4-hydroxy-valeric-acid (4-HVA) that was converted into GVL with the sample pre-treatment protocol. In the first 2 hours after the administration of GVL in mice an amount of 10 µL of samples was found to be the optimal volume giving an adequate GC-MS signal in terms of peak intensity and shape.

Increasing volumes have been used in the range of 2-8 hours (20 µL up to 200 µL) with the same purpose. All volume of samples was employed after 24 hours by the administration. However, no GVL were detected in the range of 24-36 h after intake, despite the LOD of method was evaluated as 6.25 ng/mL.

It is an expected result because, it is known that GHB, closely similar to GVL, shows a detectability around 3-6 hours by the intake and then going down to concentrations lower than endogenous level [9–11]

Concerning the phase II excretion, a high conversion of GVL was observed in the first hour after the administration, in details after 1h GVL phase II excretion was above the same of phase I. After 2 hours by the administration the GVL phase I excretion return to be the most representative. In details, phase II GVL was about half of GVL phase I excretion. From the 3th to the 5th by the administration of GVL phase I and phase II excretion show similar excretion intensity, see Figure 4. Phase II GVL was non detectable after the 5th hour (Figure 4-5).

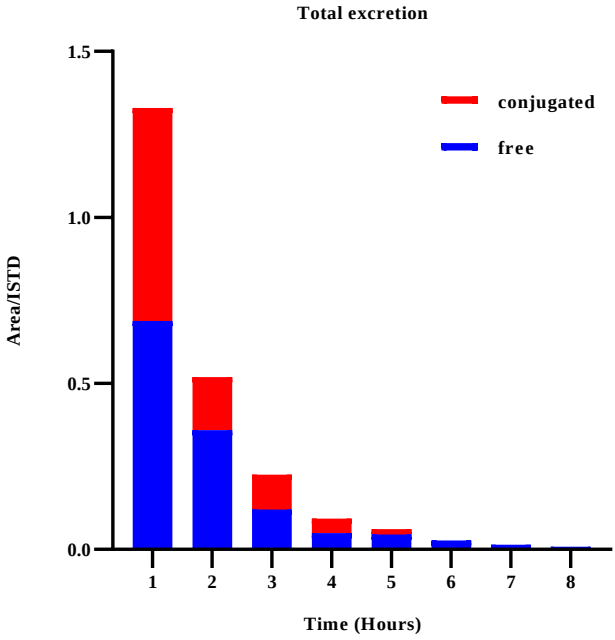

Figure VI.5. Total **e**xcretion profile of GVL of phase I (free) and phase II (conjugated).

These data show how an administration of GVL bring to a quick conversion of this substance into is phase II form, as probably a response of mice organism to the high dosage of administration. The undetected of phase II GVL after the 5th hour is possibly imputable to the remarkable decrease in circulating levels of GVL, that does not bring to is conversion into phase II form. Indeed, GVL is a high-polarity substance that does not request a mandatory conversion into phase II form to be excreted. Comparing these data with GHB it is known that endogenous GHB as well as exogenous GHB is converted to is glucorono- and sulfo- conjugates forms [43–45]. GHB-Gluc urine excretion profile clearly demonstrated the conversion of GHB into GHB-Gluc with an excretion lower than GHB free [46].

Despite this, the urine excretion profile of GHB-Gluc is incomparable with GVL cause of the variable endogenous excretion of GHB-Gluc and inter-variability among subject after GHB intake [46].

3.1.1 Validation results

- *LOD*

LOD was verified for GVL. LOD value as expressed as ng/mL of urine, S/N and selected lowest diagnostic ion are reported on Table 4.

Table VI.4. Experimental data for LOD, selected lowest ion and relative signal to noise value (S/N)

Substance	LOD (ng/mL)	S/N	Ion (*m/z*)
GVL	6.5	3.5	**100**

- *Selectivity*

The urine blank samples from twenty volunteers were analysed with the protocol described in the experimental part. No interference compounds were identified at the same retention time e with the same diagnostic ions of the GVL.

- *Recovery*

Data obtained from the ten recovery samples analysed have showed a normal distribution of data checked with Shapiro-Wilk test, no outliers were found with Dixon test (data not reported). GVL shows a recovery near 60% at a concentration of 3-times LOD. Results of: recovery, standard deviation, CV, variance, and confidence interval; are reported below in Table 5.

Table VI.5. Recovery and related statistic data at a degree of freedom of 9 and 95% of probability

Substance	Rec. %	SD	CV	Var.	Conf.	Degrees of freedom
GVL	**64**	0,04	9,8	0,001	0,03	ν=9 α=0,05

- *Repeatability*

Data obtained from the repeatability samples analysed show a normal distribution of data checked with Shapiro-Wilk test, no outliers were found with Dixon test (data not reported). Due to $C_{max} \leq C_{max,\alpha,K,\nu}$, and $C_{min} \geq C_{min,\alpha,K,\nu}$ homogeneity of variance was checked.

Here reported in Table 6 the data for Variance Homogeneity test.

Table VI.6. Statistic value for Homogeneity Variance test where: s^2_{max} and s^2_{max} are respectively maximum and minimum variance of data set, s^2_{tot} = total variance in two days, $C_{max,\alpha,K,\nu=n-1}$ and $C_{min,\alpha,K,\nu=n-1}$ are respectively the critical max and min values of *C* critical value at: α= probability, k= number of group, ν= freedom degree ;

Variance Homogeneity		GVL
s^2_{tot}		$2{,}59 \cdot 10^{-3}$
s^2_{max}		$1{,}49 \cdot 10^{-3}$
s^2_{min}		$1{,}11 \cdot 10^{-3}$
C_{max}		0,57
C_{min}		0,43
$C_{max,\alpha=0,05,K=2,\nu=9}$	0,80	$C_{min,\alpha=0,05,K=2,\nu=9}$

ANOVA test shows no significant difference in variance over two days analysis of repeatability ($F<F_{tab}$) for GVL S^2_r, the cumulative average variance was calculated. Data obtained are reported below in Table 7.

Table VI.7. Statistic value for ANOVA test where: SS_E is error Sum of Squares; SS_r is model Sum of Squares; SS_{tot} is Total Sum of Squares; MS_r is model mean squares, MS_E is model error mean squares, F_{exp} is calculated F, $F_{crit,\alpha=0,05,N-K=18,K-1=2}$ is the critical values at: α= probability, k= number of group, N= total freedom degree; S^2_r is the cumulative average variances.

SS_E	SS_r	SS_{tot}	MS_r	MS_E	F_{exp}	$F_{crit,\alpha=0,05,N-k=18,k-1=2}$	S^2_r
$2{,}34 \cdot 10^{-2}$	$5{,}34 \cdot 10^{-4}$	$2{,}39 \cdot 10^{-2}$	$1{,}3 \cdot 10^{-3}$	$2{,}68 \cdot 10^{-4}$	**0,21**	**4,41**	$3{,}55 \cdot 10^{-2}$

3.2 Steroid profile

Testosterone and epitestosterone excretion profiles were evaluated in 3 days before GVL intake and after 4 days of daily administration of a dose of 100 mg/Kg of GVL. Samples were collected at the same range of hours both for blank and positive samples every day, with the aim to reduce the variation of data due to the natural fluctuation of steroids excretion.

The results of testosterone excretion are reported in Figure 4 as concentration of testosterone over days. T/E values were also calculated with the aim to check possible variation.

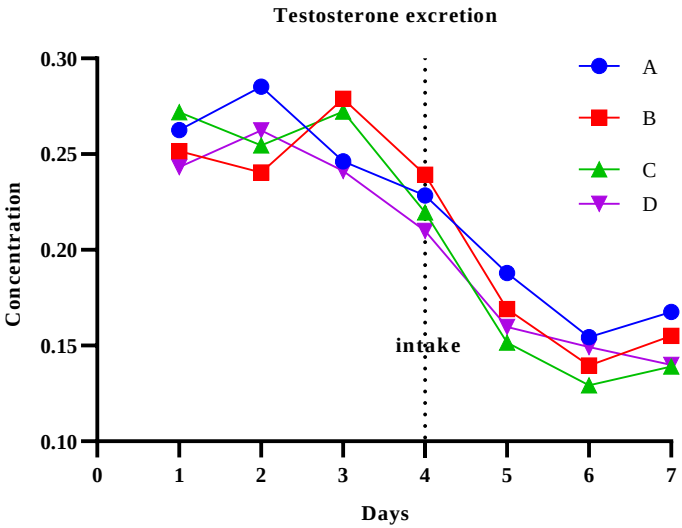

Figure VI.4. Testosterone excretion profile of four treated mouse

After the intake of GVL, testosterone and epitestosterone seem to be equally and negatively affected with a decrease of both T and E concentrations. Testosterone variation was considered grouping the mice into blank and treated groups where the variation in the 3 days of blank are imputable to normal fluctuation of testosterone levels. The unpaired t-test for the two groups, with Welch's correction for statistic different variance, shows significant difference between blank and treated samples (P=0.0172), data are reported in Table 8.

Table VI.8. Value of Testosterone over the days, average and Standard deviation (Sd) for blank and positive sample. Result of T-Test are reported as P value(T), calculated t and degrees of freedom (df) with Welch's correction for different variance of the two groups estimated with F test (PF).

		Mice			
	Days	**A**	**B**	**C**	**D**
blank	**1**	0,263	0,252	0,272	0,243
	2	0,285	0,240	0,255	0,262
	3	0,246	0,279	0,272	0,241
	Average	**0,265**	**0,257**	**0,266**	**0,249**
	Sd	**0,020**	**0,020**	**0,010**	**0,012**
treated	**4**	0,228	0,239	0,220	0,210
	5	0,188	0,169	0,152	0,160
	6	0,154	0,140	0,129	0,149
	7	0,168	0,155	0,139	0,140
	Average	**0,185**	**0,176**	**0,160**	**0,165**
	Sd	**0,032**	**0,044**	**0,041**	**0,031**
Unpaired t test Welch's correction		**P=0,0172 PF=0,0042**	**t=4,779 df=3,017**	**blank vs treated significantly different**	

The equal variation of T and E is reflected in a non-variation of T/E values, as expected by a xenobiotic substance that affect steroid profile [41], see Figure 5.

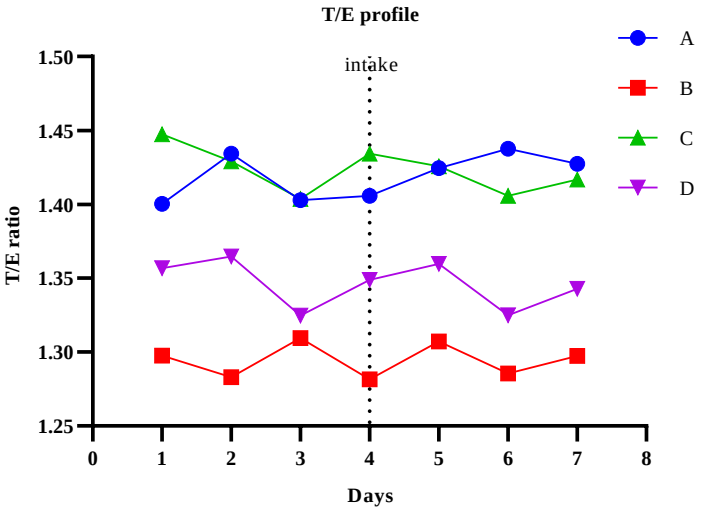

Figure VI.5. T/E excretion profile of four treated mouse

The significance of the data was checked as well as reported for testosterone values. T/E values were not significant different, see Table 9.

Table VI.9. Value of T/E over the days, average and Standard deviation (Sd) for blank and positive sample. Result of T-Test are reported as P value(T), calculated t and degrees of freedom (df).

		Mice			
	Days	**A**	**B**	**C**	**D**
blank	**1**	1,401	1,298	1,447	1,357
	2	1,434	1,283	1,429	1,365
	3	1,403	1,309	1,404	1,325
	Average	**1,413**	**1,297**	**1,427**	**1,349**
	Sd	**0,019**	**0,013**	**0,022**	**0,021**
treated	**4**	1,406	1,282	1,434	1,349
	5	1,424	1,307	1,426	1,36
	6	1,438	1,286	1,406	1,325
	7	1,427	1,297	1,417	1,343
	Average	**1,424**	**1,293**	**1,421**	**1,344**
	Sd	**0,013**	**0,011**	**0,012**	**0,015**
Unpaired t test		**P=0,9058**	**t=0,1245 df=5**	**blank vs treated not significantly different**	

Data of testosterone excretion show significant difference between GVL and GHB action on steroid profile, where GHB does not affect testosterone level after its intake [5]. However, other GABA inhibitor such as Baclofen as known to affect negatively testosterone levels in mice after intake [28], suggesting GVL interaction with GABA receptor is more similar to Baclofen rather than GHB.

3.3 IGF-1 immunoassay

IGF-1 was selected as a target of GH variation more stable than GH itself. Each serum sample was processed in duplicate and final concentration of the samples was expressed as the average of both measures. Data of excretion are reported in Figure 6.

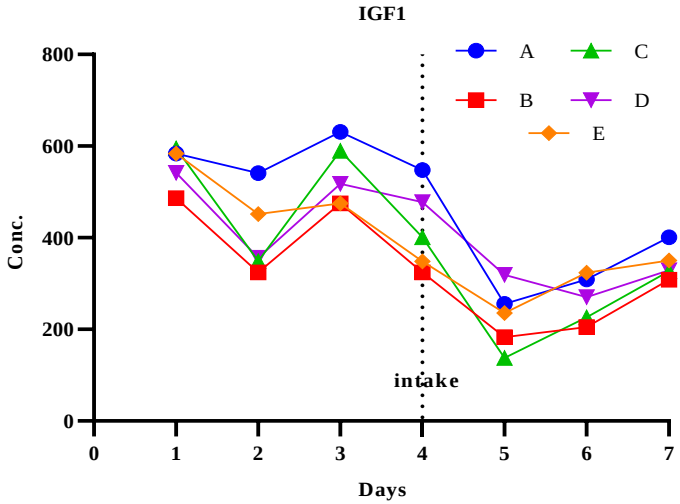

Figure VI.6. Excretion of IGF-1 in five different mice (A-E) before and after the intake of a dose of 100mg/Kg of GVL at the 4th day.

IGF-1 shows and inter-individual and inter-days variation of IGF-1 excretion among days before and after the administration. However, after GVL intake a decrease in IGF-1 value is verifiable and markable after 1 days by administration (5th day). With a progressive increase in IGF-1 values after the 2th and 3th day by the intake.

Unpaired t-test shows a significant variance between blank and treated groups, see Table 10.

Compare to GHB or Baclofen that positively affect GH and IGF1 level [29–31], GVL seem to decrease blood level of IGF-1.

Table VI.9. Value of IGF1 over the days, average and Standard deviation (Sd) for blank and positive sample. Result of T-Test are reported as P value(T), calculated t and degrees of freedom (df).

		Mice				
	Days	**A**	**B**	**C**	**D**	**E**
blank	**1**	583,40	486,44	595,05	541,89	583,40
	2	540,93	324,33	349,20	356,24	451,82
	3	630,70	475,04	589,71	517,44	475,04
	Average	**585,01**	**428,60**	**511,32**	**471,86**	**503,42**
	Sd	**44,91**	**90,49**	**140,43**	**100,87**	**70,23**
treated	**4**	547,49	324,33	400,87	477,41	348,26
	5	255,60	183,03	137,40	318,69	357,65
	6	309,30	204,69	226,19	270,24	323,86
	7	401,33	308,36	327,04	329,02	350,14
	Average	**378,43**	**255,10**	**272,88**	**348,84**	**344,98**
	Sd	**127,76**	**71,56**	**115,25**	**89,46**	**14,65**
Unpaired t test		**P=0,0321**	**t=2,944 df=5**	**blank vs treated significantly different**		

4 Conclusion

The presented method for urine excretion profile is able to identify a GVL intake in mouse after 8 h by the administration of a dose of 400 mg/kg of GVL. *In vivo* phase I and phase II excretion was studied and GVL was selected as the marker of intake, subsequently method was validated for GVL estimate selectivity, LOD, recovery and repeatability. The presented sample pre-treatment is rapid and effective without solid phase excretion and derivatization. The pre-treatment protocol occurs with a total conversion of phase II (glucorono or sulfo)-GVL into GVL, simplifying the analysis and increasing the capability of method to detect GVL intake adding GVL phase I and phase II excretion. As the author best knowledge, this is the first work which evaluates urine excretion profile after GVL intake and possible affection of GVL on testosterone and IGF-1 levels with a decrease of both parameters. However, further studies are requested to estimate the affection of GVL on testosterone and IGF-1 at different doses of intake and with a larger group.

Acknowledgments

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Part II

Chapter VII

Evaluation of *in vitro* metabolism of two new synthetic cannabinoids ADB-CHMICA and CUMYL-THPINACA

Adapted from

Monica Mazzarino, Cristian Camuto, Fabio De-Giorgio, Xavier de la Torre, Matteo Marti, Sara Samperi, Francesco Botrè. "Poster presentation at VI NPS conference, Maastricht :" In vitro characterization of the metabolic pathways of four new synthetic cannabinoids"

Abstract

Synthetic cannabinoids are a class of NPS originally developed to explore the function and structure of cannabinoid receptors. At present, they have found great popularity into the world of "recreational" drug use, being among the most widely diffused new psychoactive substances. Indeed, more than a hundred different varieties of synthetic cannabinoids have been synthetized so far, making their detection in herbal products and in biological fluids a demanding challenge for forensic laboratories.

Here the phase I biochemical reactions involved in the biotransformation pathways of two new cannabinoids (CUMYL-THPINACA, ADB-CHMICA), part of aminoalkyl-indole-indazole derivatives. The metabolic pathways were characterized by *in vitro* models by liquid chromatography-mass spectrometric based techniques employing two different techniques as Triple Quadrupole and Time of Flight, not only to select the most appropriate markers of intake, but also to increase knowledge on metabolism of this variety of synthetic cannabinoids and make more reliable prediction of the metabolic profiles of similar compounds.

Our data show that the selected compounds are extensively metabolized mainly by CYP3A4 isoform in more than 10 oxidised metabolites, considering the mono-hydroxylation the most abundant biotransformation pathway detected.

1. Introduction

Synthetic cannabinoids (SCs) are a class of NPS originally developed to explore the function and structure of cannabinoid receptors[1] as potential pharmaceutical drugs for different diseases [2–5]. The interest of medicine into the field of SCs is due to their action which involves endocannabinoid receptors, both central (CB1) and peripheral (CB2) where they exert action as receptor agonists[6] with higher binding affinity for CB1 and CB2 than classic cannabinoids, such as Δ9-tetrahydrocannabinol (THC)[6–9].

SCs are an inhomogeneous class of substances with a wide variety of structures and effects. Some of the most known are ascribable to stimulant, narcotic and hallucinogenic effects with still unknow adverse effects [6,10] that could led to death [11–14]. When a new substance appears for the first time, there are not enough pharmacological or toxicological data: it could be an issue for both individual and public health[15]. SCs have been started to be available since the beginning of 2000s and have become soon popular [16] as legal highs around 2008[17]. First SCs were sold with the name of Spice, a smokable herbal bland contained a mixture of SCs cannabinoids such as JWH-018, CP 47497, JWH-073 and HU-210[18]. Soon, they have started to be employed in sport as doping agent due to their stimulant activity. Among them, the most abused (especially for doping purpose) are JWH-018 and JWH-073[19,20]. This has led to the introduction of these substances into specific schedule of prohibited list of each country and also into the list of prohibited substances by WADA in sport[21]. Nowadays, SCs have found great popularity into the world of "recreational" drug use, considering their diffusion worldwide. A large variety of new compounds that mimes the effects of scheduled NPS are produced in clandestine laboratories.

They are sold with different labelling without a clear description of the name or structure into the packaging of the substance. SCs are easily found on internet until they are declared illicit and subsequently introduced into specific schedules of prohibited list of each country, becoming an emerging health risk problem around Europe like the other NPS [22]. In Europe, seizures of new psychoactive substances are typically dominated by synthetic cannabinoids and cathinones with 51% of the total seizure for cannabinoids in 2017. At the date, more than a hundred of different SCs have been first reported by the EMCDDA among 2005-2018 with a significant prevalence after 2010 [23] . These data are referred only to the European market which is our referring market. Therefore, these first introduced compounds are only an amount of the total number of SCs available in the world market, synthetized and sold as recreational drugs. Data of 2019 drug market will be available in the EMCDDA report 2020. This continuous growth and appearance of new SCs is leading to an incessant variation of SCs classification and structure, therefore a classification of SCs quickly becomes obsolete and beyond of this thesis scope. Therefore, this fasting growth make SCs detection in herbal products and in biological fluids a demanding challenge for forensic laboratories [24–29].

The aim of the present work is to investigate the *in vitro* metabolism of two different SCs based on a 3-carboxamide structure with indazole or indole ring, a still valid classification for SCs based on the presence of the indole/indazole ring linked with a carboxamide function. The aim was to define the best markers of intake and the typical fragmentation patterns for these class of substances as a tool in forensics toxicological analysis. Carboxamide SCs could be synthetized starting from cumyl amine (α,α-Dimethylbenzylamine), and their distribution were reported by EMCDDA in 2014[30]. Among these, CUMYL-THPINACA and ADB-CHMICA were selected to evaluate their phase I *in vitro* metabolism.

Both CUMYL-THPINACA and ADB-CHMICA were first notified to early warning system in 2014[30]. However, at the date, no metabolism studies were carried out for these two SCs. CUMYL-THPINACA is an indazole based SC part of CUMYL-indazoles derivatives (Figure 1). Different CUMYL indazole or indole compounds were under law restriction in several countries[8], and a case of poisoning related to the similar CUMYLPINACA was reported in Slovenia in 2017[31]. Furthermore, CUMYL-THPINACA activity as a CB1 and CB2 receptor agonist was evaluated by Asada et al (2017)[32]. ADB-CHMICA is an indole SC, its structure is related to MDMB-CHMICA (Figure 1), a potent agonist of cannabinoids receptors first appeared in Europe in 2014 [30]and soon involved in 42 acute intoxications which occurred in 9 European countries between 2014 and 2016 and 29 deaths (Germany (5), Hungary (3), Poland (1), Sweden (9), United Kingdom (10), Norway (1)) [33–35]..

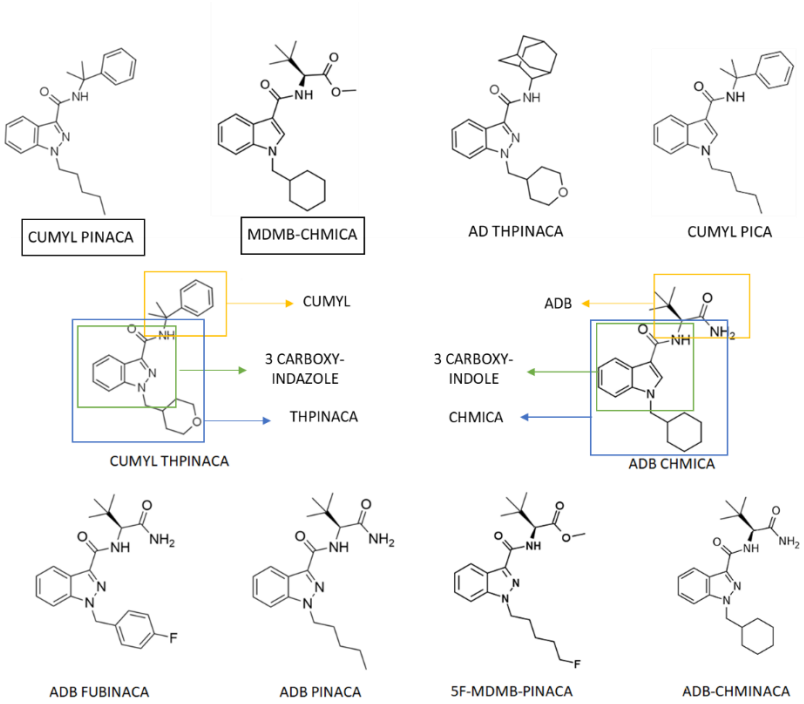

Figure VII.1. Structures of CUMYL-THPINACA and ADB-CHMICA and their related compounds MDMB-CHMICA and CUMYL-PINACA and others representative compound of indoles/indazoles class

2 Experimental

2.1 Chemicals and reagents

Amino alkyl-indole/indazole analogues ADB-CHMICA(N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H-indole-3-carboxamide) and CUMYL-THPINACA (N-(1-methyl-1-phenylethyl)-1-[(tetrahydro-2H-pyran-4-yl)methyl]-1H-indazole-3-carboxamide) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). All chemicals (ammonium formate, formic acid, acetonitrile, methanol, dimethylsufoxide, sodium phosphate, sodium hydrogen phosphate, tert-butylmethyl ether) were of analytical or HPLC grade and provided by Carlo Erba (Milan, Italy) and Sigma–Aldrich (Milan, Italy). The ultrapure water used was of Milli-Qgrade (Millipore Italia, Vimodrone, Milan, Italy). The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian mixed male and female donors of different ages) as well as (CYP recombinant isoforms CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6, and all the reagents used for the *in vitro* metabolism studies (sodium phosphate and tris-HCl buffers and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milan, Italy).

2.2 *In vitro* protocol

All incubation conditions were optimized (protein and substrate concentrations, buffer and solvent types, incubation time) considering the protocols already used in previous studies carried out in our laboratory [36–38]. Different solvents (methanol, DMSO and acetonitrile), buffers (phosphate and tris-HCl), pH values (5.0, 7.4, 8.0), concentration of both Synthetic Cannabinoids (0.1, 0.5 and 1mg/mL) and of the enzymatic proteins (0.1, 0.2, 0.5 and 1.0 mg/mL), and different incubation time (30, 60, 120, 240, 480 and 720min) were evaluated.

The final incubation medium also contained 3.3mM magnesium chloride, 1.3mM NADP+, 3.3 mM glucose-6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase in a total volume of 250µL. Samples were pre-warmed at 37°C for 5min and the reactions were started with the addition of the appropriate enzymatic proteins (HLM or CYP recombinant isoforms). After incubation at 37°C, 250 µL of ice-cold acetonitrile were added to stop the reaction. The samples were then transferred into an ice bath for 3 min for further precipitation of the proteins in the assay medium. The supernatant was subsequently separated from the precipitate by centrifugation at 14.000 rpm at room temperature for 10min. Each set of assays also included a negative control sample, containing all reaction mixture components except the enzymatic proteins in order to monitor the potential non-enzymatic reactions within the incubation period. Each incubation was processed in duplicate.

2.3 Sample pre-treatment

All the samples obtained by the *in vitro* experiment (sample volume 500µL) were added with 1,5 mL of phosphate buffer (1 M, pH 7.4) and 50 mL of the internal standard (ISTD: deuterated JWH015 final concentration 5 ng/mL) and a liquid/liquid extraction was carried out with 5 mL of the most appropriate solvent (*tert*-butylmethyl, ethyl acetate, chloroform were tested) at controlled pH with different pH tested (5, 7.4, 9, 12). After 10 minutes of stirring at room temperature, samples were centrifugated at 3000 rpm for 2 min and transferred into an ice bath for 5 minutes. After centrifugation the organic layer was separated and evaporated to dryness under nitrogen stream at a temperature of 30°C. The residue was reconstituted with 50µL of mobile phase (initial composition) and an aliquot of 10µL was injected in the liquid chromatography-mass spectrometry systems for the detection of phase I metabolites.

2.4 Instrumental conditions

Samples were analysed with two different liquid chromatography coupled to mass spectrometric techniques (HPLC-MS) as LC coupled to triple quadrupole (LC-QqQ) and high-resolution techniques Ultra-High-Performance-LC coupled to Quadrupole-Time Of Flight technique (UHPLC-QTOF). The *in vitro* metabolic pathway of cannabimimetics were defined by UHPLC-QTOF technique. Metabolites were confirmed through a targeted analysis of the same samples with LC-QqQ method.

2.4.1 UHPLC-QTOF

Samples were analysed using an Agilent 1290 infinity II series UHPLC instrument equipped with a: Zorbax C18 (10 cm x2.1 mm, 1.8 µm) coupled with a QTOF 6545 (Agilent Technologies) with an ESI source. The solvents used and the gradient programme was the same described above for the HPLC-QqQ method. The flow rate was set to a constant flow rate of 400 µL/min.

The mass spectrometric conditions as the follow: ESI operated in positive mode using a Jet Stream ESI source and Ion Funnel. In ESI+ a capillary voltage of 3.500V, a nozzle voltage of 300 V, a drying gas flow of 15 L/min at 150°C, sheath gas flow of 10 L/min at 350°C and a nebulizer pressure of 45 psi were used with nitrogen. The high-pressure ion funnel was operated at radio frequency (RF) voltage 150 V, the low-pressure funnel at RF 60 V, and the octopole at RF 750 V. Mass data were generated from *m/z* 100 to 1100 at 9300 transients per second.

The mass calibration was performed daily, at the beginning of every analytical session, using a calibration solution provided by the manufacturer. Purine with an [M+H]+ ion at *m/z* 121.0509 and an Agilent proprietary compound (HP0921) yielding an ion at *m/z* 922.0098 were simultaneously introduced via a second orthogonal sprayer, and these ions were used as internal calibrants along all the analysis. Selected ions [M+H]+ for parents compound and metabolites are reported in Tables 3-4.

All aspects of instrument control, tuning, method setup and parameters, sample injection and sequence operation were controlled by the Agilent Technologies Mass Hunter software version B.08.00.

2.4.2 LC-QqQ

Samples were analysed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO C18 column (15 cm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass spectrometer (Sciex) with an ESI source and operated in the positive-ion mode.

Detailed, the solvents used were: ultrapurified water (eluent A) and acetonitrile (eluent B), both containing 0.1% formic acid. The gradient program started at 15% B and increasing to 60% B in 7min, after 6 min, to 100% B in 4 min. The column was flushed for 4min at 100% B and finally re-equilibrated at 15% B for 4min. The flow rate was set to a constant flow rate of 250 µL /min.

The mass spectrometric condition were set as follow: ESI source operate in positive electrospray ionization using a curtain gas pressure of 25 psi, a ion source temperature of 500 °C, an ion source gas 1 pressure of 35 psi, an ion source gas 2 pressure of 40 psi, a declustering voltage of 80 V, an entrance potential of 10 V and a needle voltage of 5000 V. Multiple reaction monitoring (MRM) was used as the acquisition mode for detection (see Tables 1-2 for the ion transitions selected). For the MRM collision-induced dissociation (CID), nitrogen was used as the collision gas at 5.8 mPa, obtained from a dedicated Parker-Balston nitrogen generator system (model 75-A74) with 99.5% gas purity (CPS Analitica Milan, Italy). All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by Analyst software version 1.6.1

3 Results and discussion

3.1 Mass spectrometric conditions

The mass spectrometric parameters for both technique as declustering and needle voltages, gas pressure, source temperature, collision cell exit potential, and collision energy were optimised by infusing the standard solution of both cannabinoids at a concentration of 10 μg/mL, as well as selected protonated molecules [M+H]+ and characteristic fragmentations for parent compounds in MRM mode. For this purpose, 1 mL syringe operated by a syringe pump at a flow rate of 10 mL/min was employed.

3.1.1 QTOF

For the high resolution technique the $[M+H]^+$ mass was estimated through direct infusion of standard solutions. The $[M+H]^+$ characteristic mass for metabolites were calculated based on the predicted structures and results obtained after incubation of standard solutions of ADB-CHMICA and CUMYL-THPINACA with human liver microsomes and full scan analysis.

Here reported in Table 1-2 the $[M+H]^+$, elemental composition and relative mass error for metabolites of the two cannabimimetic.

Table VII.1. $[M+H]^+$ ions for all metabolites of ADB-CHMICA, elemental composition and error.

Metabolic reaction	**$[M+H]^+$ (*m/z*)**	**Elemental Composition**	**Error (Δppm)**
Di-hydroxylation (M1-M6, M8)	402.2387	$C_{22}H_{31}N_3O_4$	1.23-2.01
Mono-hydroxylation (M7, M9-M10, M12-M17)	386.2438	$C_{22}H_{31}N_3O_3$	1.55-2.51
Hydrogenation (M18)	372.2646	$C_{22}H_{33}N_3O_2$	3.24
Mono-hydroxylation dehydrogenation (M11)	384.2282	$C_{22}H_{29}N_3O_3$	1.51
Deamination (M19)	371.2329	---	---

Table VII.2. $[M+H]^+$ ions for all metabolites of CUMYL-THPINACA, elemental composition and error.

Metabolic reaction	$[M+H]^+$ (*m/z*)	Elemental Composition	Error (Δppm)
Di-hydroxylation (M1-M7)	410.2074	$C_{23}H_{27}N_3O_4$	1.08-2.97
Mono-hydroxylation (M8-10, M12)	394.2125	$C_{23}H_{27}N_3O_3$	1.78-2.33
Hydrogenation (M14)	380.2333	$C_{23}H_{29}N_3O_2$	2.14
Mono-hydroxylation dehydrogenation (M11, M13)	392.1969	$C_{23}H_{25}N_3O_3$	2.11-3.13

3.1.2 LC-QqQ

Different collision energies (20, 30, 40, 45, 50 and 60 eV) were evaluated in order to obtain information about the fragmentation patterns of the compounds considered in this study and to select and optimize the mass spectral fragments formed from the characteristic portions of the molecular structure that is common to the aminoalkyl-indole/indazole analogues. The full-scan MS analysis was first performed, both in positive and negative ionization mode, to identify the molecular ions of compounds. The best response was obtained in positive mode for the protonated molecular ion $[M+H]^+$ at *m/z* 396.5 for ADB-CHMICA and at *m/z* 378.5 for CUMYL-THPINACA. The most reproducible fragmentation patterns of parent compounds were obtained at a collision energy of 30eV for both compounds. The mass spectra fragmentations of the two compounds with the respective structures are reported in Figures 2-3.

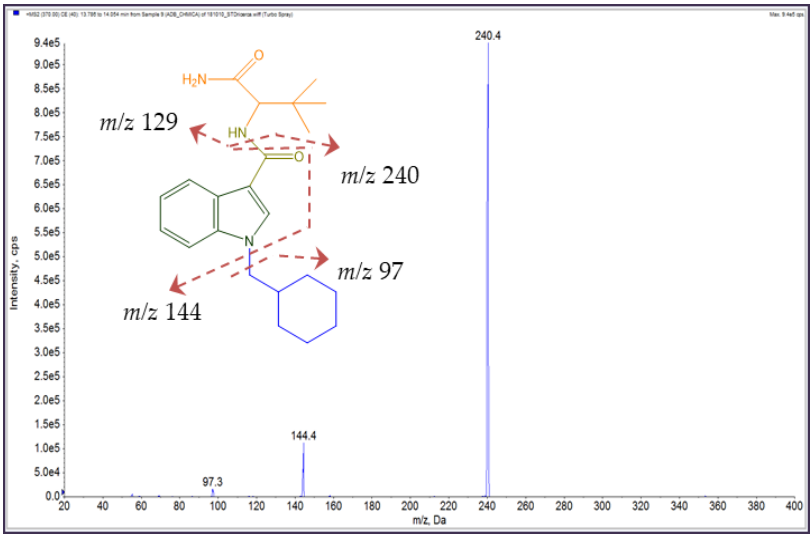

Figure VII.2. Product ion scan spectrum obtained for ADB-CHMICA at a collision energy of 30eV

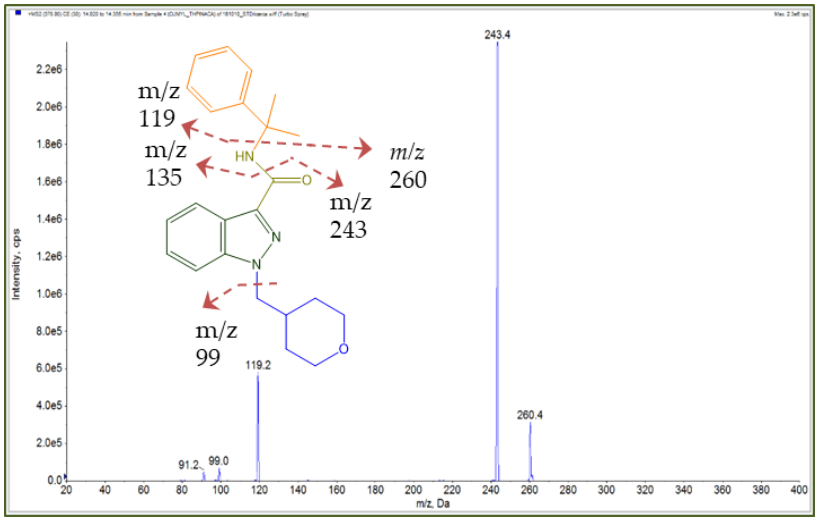

Figure VII.3. Product ion scan spectrum obtained for CUMYL-THIPINACA at a collision energy of 30eV

The above described product ions were utilized as diagnostic ions (structural markers: characteristic portions of the molecular structure that is common to the two cannabinoids and their metabolites) to obtain structural information of the analytes detected in either the samples obtained after incubation of ADB-CHMICA and CUMYL-THPINACA in the presence of HLM and CYPs isoforms. The MRM transitions for the compounds are reported below in Tables 3-4.

Table VII.3. Employed ion transitions for ADB-CHMICA metabolites

Metabolic reaction	Precursor ion (*m/z*)	Product ion (*m/z*)	Identified metabolites
Di-hydroxylation	402	97; 113; 129; 144; 160; 176; 240; 256; 272	M1-M6, M8
Mono-hydroxylation	386	97; 113; 144; 160; 240; 256	M/7, M9-M10, M12-M17
Hydrogenation	372	97; 144; 142; 238; 240	M18
Mono-hydroxylation-dehydrogenation	384	97; 111; 144; 158; 240; 238	M11
Deamination	371	97; 144; 240	M19

Table VII.3. Employed ion transitions for CUMYL-THPINACA metabolites

Metabolic reaction	Precursor ion(*m/z*)	Product ion (*m/z*)	Identified metabolites
Di-hydroxylation	410	99; 115; 131; 119; 135; 151; 243; 260; 259	M1-M7
Mono-hydroxylation	394	99; 115; 135; 151; 243; 259	M8-M10, M12
Hydrogenation	380	99; 241;	M14
Mono-hydroxylation-Dehydrogenation	392	99; 113; 119; 243; 257;	M11,M13

3.2 *In vitro* metabolism

In vitro metabolism studies were conducted using HLM from a pool of 20 Caucasian male and female donors of different ages to minimise the effect of intra-individual variation and present the 'average' enzyme activity. The protocols previously optimized were applied to all incubation samples. The best results were obtained using methanol as a substrate solvent (the total amount of methanol in the final assay was 1%), substrate concentration of 20 µM, protein concentration of 0.5 mg/mL, phosphate buffer of 0.1 M at pH 7.4, and incubation time of 4 h at 37 °C. To optimize the pre-treatment samples, different extraction solvents (*tert*-butyl methyl-ether, diethyl ether, and ethylacetate) and pH values (5, 7, 9 and 12) were evaluated. The best extraction results for parent compounds and metabolites were obtained with addiction of 200 µL of phosphate buffer (pH 7.4) followed by addiction of 5 mL of ethylacetate. The relative contribution of individual CYPs isoforms to the metabolic reaction of both cannabinoids was evaluated with the aim to estimate the CYPs involved and potential drug-drug interactions with other substances. The protocol followed for CYPs incubation was the same as described for HLM incubation.

-ADB-CHMICA

The analysis of the *in vitro* samples using both mass spectrometric techniques allow the detection of different metabolic reactions. In detail, for ADB-CHMICA 18 different metabolites were identified with QTOF technique (Figure 4).

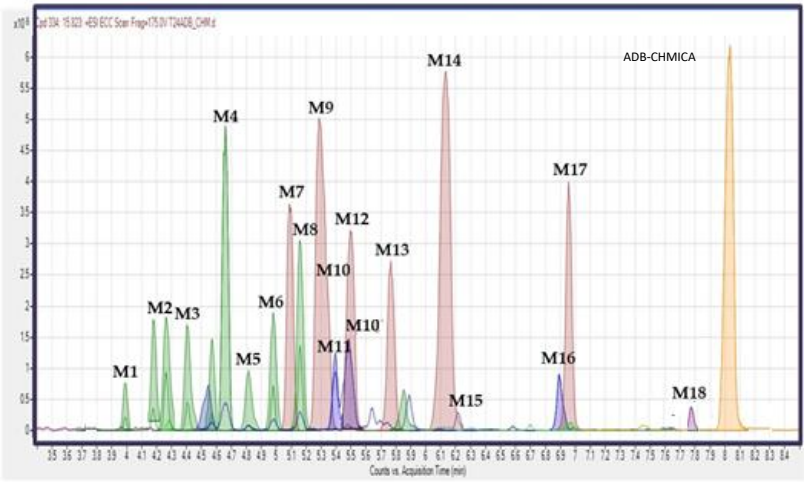

Figure VII.4. Extracted chromatogram with QTOF technique of a representative sample after 4h of incubation

The same metabolites were confirmed through QqQ techniques, except for deaminated metabolite (M19) (Figure 5).

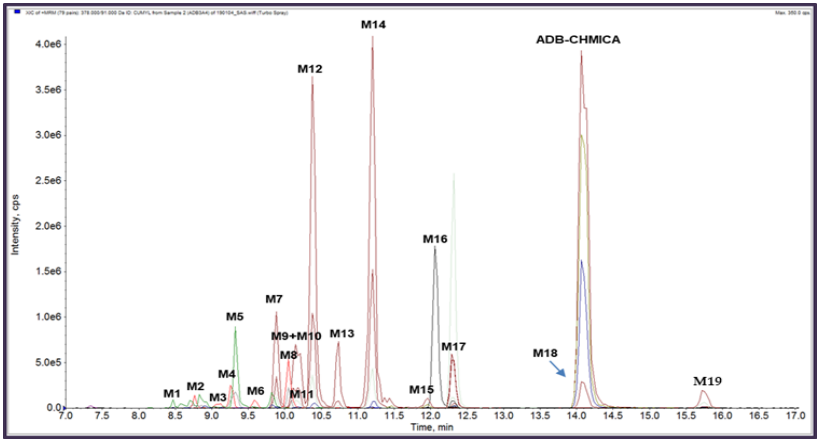

Figure VII.4. Extracted chromatogram with MRM mode (QqQ) of the same sample analysed with QTOF technique

In details, the metabolic pathways identified were: hydroxylation (both mono and di) in different positions as the most representative metabolic pathways followed by dehydrogenation hydrogenation and deamination. The chemical pathways of ADB-CHMICA are reported below in Figure 6.

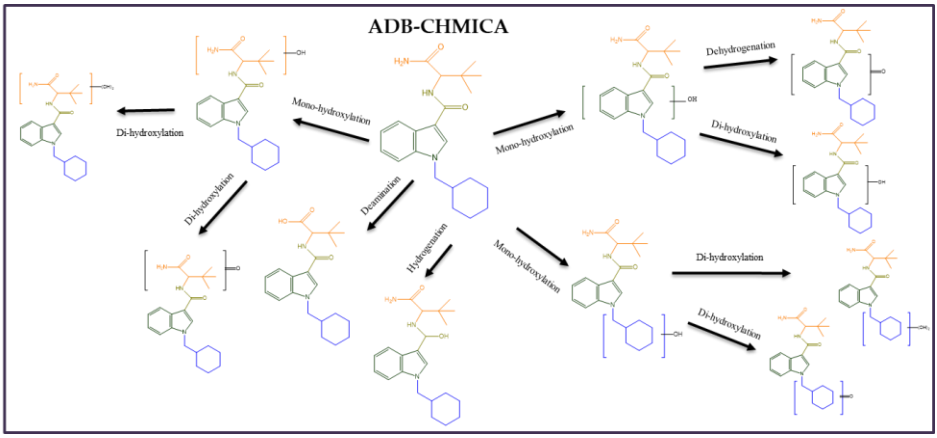

Figure VII.6. Postulated structures of the phase I metabolites identified

To define the relative contribution of individual CYPs isoforms to the metabolic reaction, ADB-CHMICA standard solution (at a concentration of 1 mg/mL) was incubated with different CYP450 isoforms. The involved isoforms evaluated for the phase I metabolism of ADB-CHMICA are CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6. For all the isoforms the relative contribution to single metabolic reaction was evaluated and calculated as percentage, see Figure 7. The percentage was calculated from total amount (peak intensity ratio) of each metabolite formed by the allelic variant considered.

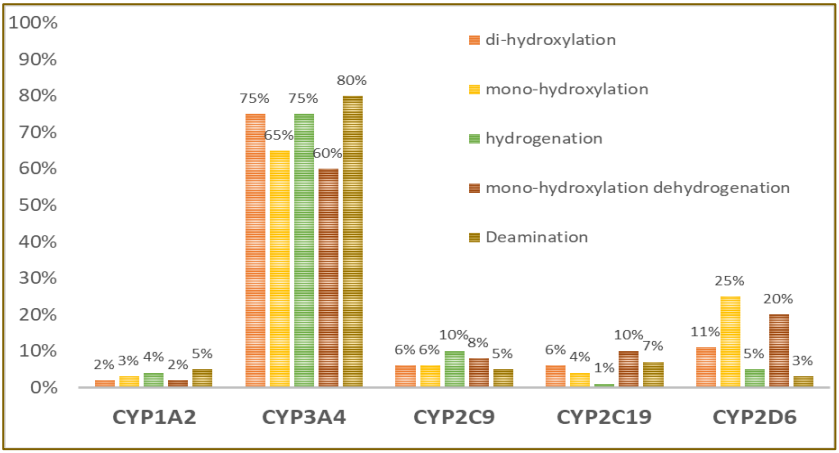

Figure VII.7. Percentage contribution of each CYPs isoform to metabolism of ADB-CHMICA

As described in Figure 7, CYP3A4 is the most involved isoform into phase I metabolic reactions in particular for deamination and with contribution higher than 60% for all reactions, followed by CYP2D6. The secondary isoforms involved are CYP2C19 and CYP2C9 with contribution ranged by 1% to 10%. The contribution of CYP2D6 to hydroxylation and dehydrogenation reaction ranged from 11% up to 25%. In particular, the mono-hydroxy reaction (followed by dehydrogenation) give a contribution to the metabolic pathways major than 20%.

-*CUMYL-THPINACA*

The analysis of the *in vitro* samples allows the detection of 14 different metabolites after the incubation of a standard solution of CUMYL-THPINACA with HLM. Here reported below an extracted chromatogram of in vitro sample with QTOF after 4h of incubation with HLM (Figure 8).

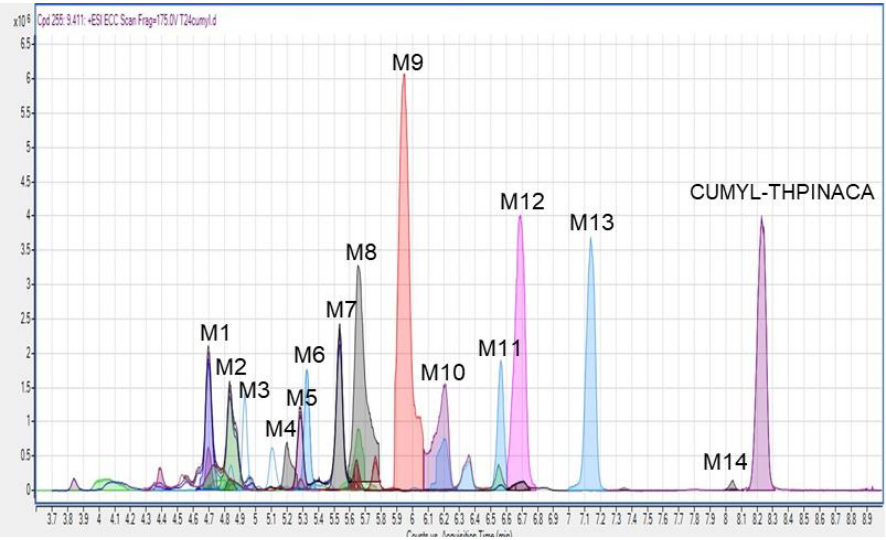

Figure VII.8. Extracted chromatogram of a representative sample with QTOF technique

The same in vitro samples were analysed through QqQ technique operating in MRM mode. All the metabolites identified with QTOF technique were confirmed (Figure 9)

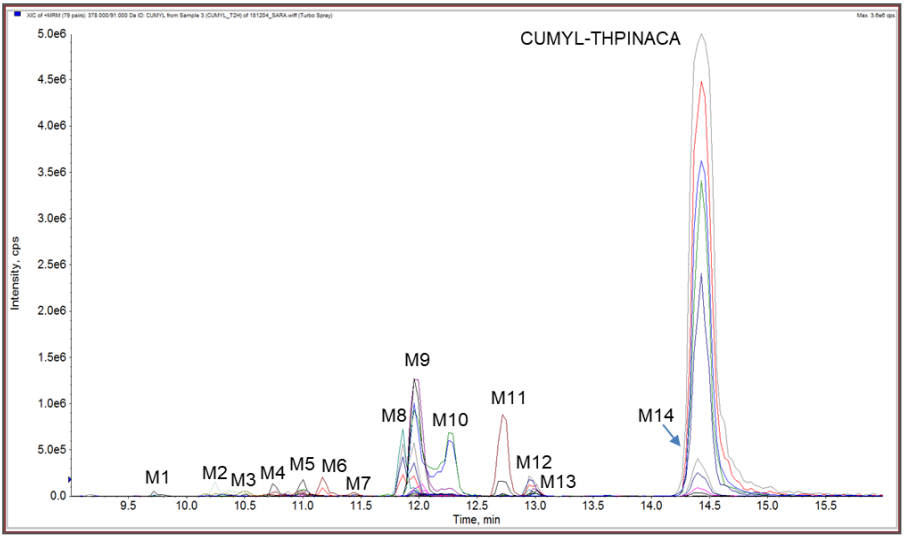

Figure VII.9. Extracted chromatogram with MRM mode (QqQ) of a representative sample of CUMYL-THPINACA

In details, the metabolic pathways identified for CUMYL-THPINACA were mono- and di- hydroxylation in different positions as well as described for ADB-CHMICA. Furthermore, like ADB-CHMICA, for CUMYL-THPINACA the most representative metabolic pathway is hydroxylation. This data is full comparable with other aminoalkyl-indole/indazole cannabinoids where the hydroxy metabolites are the most representatives [39,40]. The postulated chemical structures of metabolic pathways are reported below in Figure 10.

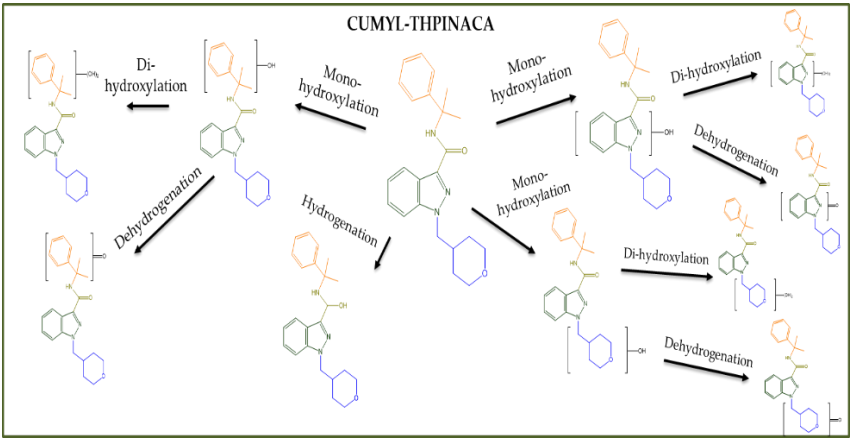

Figure VII.10. Postulated structures of the phase I metabolites identified for CUMYL-THPINACA

To define the relative contribution of individual CYPs isoforms to the metabolic reaction, CUMYL-THPINACA (at a concentration of 1 mg/mL) was incubated with the same CYP450 isoforms employed for ADB-CHMICA, see Figure 11. The percentage was calculated from total amount (peak intensity ratio) of each metabolite formed by the allelic variant considered.

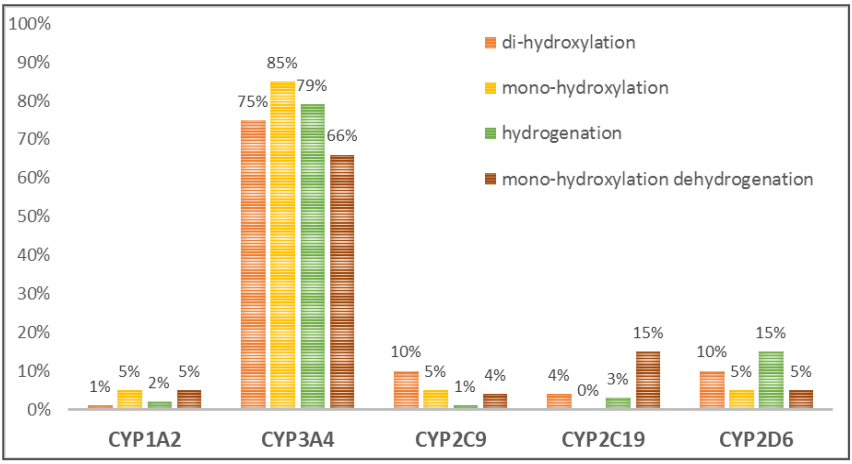

Figure VII.11. Percentage contribution of each CYPs isoform to metabolism of CUMYL-THPINACA

As described in Figure 11 CYP3A4 is the most involved isoform into phase I metabolic reactions in particular for mono-hydroxylation (85%) followed by hydrogenation (79%), di-hydroxylation (75%), and mono-hydroxylation dehydrogenation (66%). CYP2D6 is the secondary involved isoform with major contribution for di-hydroxylation and hydrogenation. The secondary isoforms involved are CYP2C19 (for mono-hydroxylation dehydrogenation about 15%) and CYP2C9 with contribution ranged by 1% to 10%. CYP1A2, as well as observed for ADB-CHMICA, represents only a few amounts of total percentage of metabolism.

4 Conclusion

As the author best knowledge this is the first study on metabolism of two novel cannabimimetic ADB-CHMICA and CUMYL-THPINACA. The presented work estimates the principal metabolic pathways through two different mass spectrometric techniques after incubation with human liver microsomes. ADB-CHIMICA e CUMYL-THPINACA show an extensive phase I metabolism with the formation of respectively 19 and 14 phase I metabolites. The hydroxylation metabolic pathways are the principal ones, therefore for all the compounds considered the hydroxylated metabolites could be considered as markers of intake. Furthermore, the contribution of CYPs isoforms were evaluated CYP3A4 results the most involved isoforms into the phase I metabolism of both cannabinoids.

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General Conclusion and Future Perspective

The principal metabolic reactions of seven NPS were successful estimated through *in vitro* techniques allow to estimate the common phase I reactions of the selected compounds. Furthermore the *in vivo* metabolism of three of these substances (Methiopropamine, 4,4'-DMAR and GVL) was estimated employing the murine model as a model for human metabolism. The results obtained and collected with the other groups involved (Università di Ferrara, Università Cattolica) were or will be added to EWS database as a fundamental overview of these compounds.

Methiopropamine, Methedrone, Pyrovalerone and 4,4'-DMAR are phenethylamine derivatives which show the typical metabolic pathways of this class of drugs. In details, *N*-demethylation and aromatic/alkyl hydroxylation were detected as the principal metabolic reactions involved, as well as for other phenethylamine derivatives.

For Methiopropamine and 4,4'-DMAR the metabolic pathways were confirmed through *in vivo* studies and the excretion profile was followed after the intake of a dose of 10 mg/kg (dose common selected by University of Ferrara for behavioural studies where phenethylamine derivatives are involved).

For both substances the *in vivo* studies were able to identify the proper markers of intake.

- For Methiopropamine a significant difference among *in vitro* and *in vivo* studies is observable. With the undetectability of M2-M5 metabolites after 24 h by the intake, where after *in vitro* studies M2 and M3 appear to be the suitable makers of intake instead of M1 (known as thiopropamine). Furthermore, the formation of the M4 metabolite (known as thiothinone) only in mice represent an important information. In details, thiothinone seem to be formed and excreted only in mice.

Therefore, the detection of thiothinone in human biological matrices might to be linked to an intake of thiothinone (a potential new underestimated cathinone derivatives part of both cathinone, thiophen, and phenethylamine derivatives) with still unknow metabolism, excretion and toxicological/behavioural data. Our future perspective for this project is to extend the *in vitro* and *in vivo* metabolism studies to this drug and support and link this data with behavioural test and evaluation of tissue damage conducted by the University of Ferrara and University Cattolica.

- For 4,4'-DMAR the *in vitro* studies show that CYP2D6 is the only isoform involved in the metabolism of 4,4'-DMAR. For this isoform three allelic variants were studied and results demonstrate that only parent compound and M1 metabolite are formed with both variants. Therefore, parent compound and M1 metabolite are the markers of intake. However, the *in vivo* excretion profile show that M2 metabolite could be an important marker of intake. Our future purpose is to estimate the excretion profile of 4,4'-DMAR in a range of 36 hours after the intake. Furthermore, an important future development is to estimate the combined action of *trans* and *cis* isomers. In details, our recent results indicate that the contemporary administration of *trans* and *cis* isomers could increase 4,4'-DMAR effects through the inhibition of *cis* metabolism despite *trans* isomers do not show pharmacology activity (data obtained with behavioural studies conducted by University of Ferrara), further data are requested for a properly estimation of their combined effects.

ADB-CHMICA and CUMYL-THPINACA are synthetic cannabinoids of indole/indazole class which show an extensive phase I metabolism with hydroxylation pathways are the most common.

- Author suggest that hydroxylated metabolites are the suitable markers of intake for these compounds. Furthermore, results obtained can increase knowledge on metabolism of this variety of synthetic cannabinoids and make more reliable prediction of the metabolic profiles of similar and still unknown compounds. Future perspective of this project is to estimate the *in vivo* excretion profile of both cannabinoids with the aim to verify the to confirm the most appropriate markers of intake for these compounds.

GVL is a potential DFSA selected for its dose-dependent action with both stimulant and narcotic effects. The aim was not only to estimate its *in vitro/in vivo* metabolism but to estimate possible affection on testosterone and IGF-1 level.

- The presented data could be helpful to the understand of the action of this compound on hypothalamic–pituitary–adrenal axis not only as relevant information for potential doping implication but as an important tool for potential medical implication in view of the recent and widespread employ of GABA analogues compound for treatment of different neurological disease. Our metabolism data confirm that GVL as well GHB represent a problem for toxicology analysis because of their rapid excretion and subsequently undetectability after few hours by the intake. Future perspectives are related to a deep investigation of hypothalamic–pituitary–adrenal axis affection with the aim to develop a properly method for detection of different target steroids and their level evaluation after intake of different dosages of GVL. At the same doses the affection of IGF-1 level will be also estimated, with a larger group employed.