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Clinical and toxicological significance of the involvement of the cytochrome P450 system in the metabolism of 3,4-Methylenedioxymethamphetamine

(MDMA, Ecstasy)

DEPARTAMENT DE CIÈNCIES EXPERIMENTALS I DE LA SALUT
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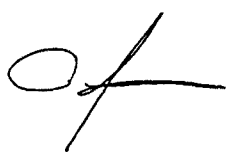
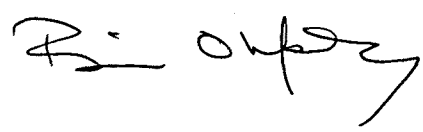
Clinical and toxicological significance of the involvement of the cytochrome P450 system in the metabolism of 3,4-Methylenedioxymethamphetamine (MDMA, ECSTASY)

Memòria presentada per Brian O'Mahony per optar al títol de doctor per la Universitat Pompeu Fabra. Treball realitzat sota la direcció del Dr. Rafael de la Torre Fornell, en el Grup de Recerca clínica en farmacologia humana i neurociències, IMIM-Hospital del Mar, Parc de Recerca Biomèdica de Barcelona. Programa de Doctorat de la Universitat Pompeu Fabra.



Signatura del Director de tesi
(Dr. de la Torre Fornell)



Signatura de la doctorand
(Brian O'Mahony)

Se'm caigué un floc de neu a la mà
Delicat i intricat com un cor humà
Assentat en la seva gàbia d'animal
Vaig intentar que no se'm fongués
Però se'm va desfer tot d'obsessió
I res s'anava solucionant
Fins que el vaig deixar volar.

For my family and for my friends from Cabinteely

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137X,	1,3,7-trimethylxathine
17U,	1,7-dimethyluric acid
17X,	1,7-dimethylxanthine
1U,	1-methyluric acid
1X,	1-methylxanthine
2CB,	4-bromo-2,5-dimethoxymethamphetamine.
5-HIAA,	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HT,	5-hydroxytryptamine
AAMU,	5-acetylamino-6-amino-3-methyluracil
aCSF,	Artificial cerebrospinal fluid
ACTH,	Vasopressin
ARCI,	Addiction Research Centre Inventory
AUC,	Area Ander the Curve
BDZ,	Benzodiazepine
BP,	Blood pressure
$CaCl_2$,	Calcium Chloride
CL,	Clearance
C_{max},	Maximum Concentration
CNS,	Central Nervous System
COMT,	Catechol-o-methyltransferase
CPK,	Creatinine Phosphokinase
CYP,	Cytochrome P450 isozyme
DAT,	Dopamine Transporter
DEX,	Dextromethorphan
DOB,	4-bromo-2,5-dimethoxyamphetamine
DOM,	4-methyl-2,5-dimethoxymethamphetamine

Abbreviations

DOR,	Dextrorphan
DSM-IV,	Diagnostic and Statistical Manual of Mental Disorders
DSST,	Digit for Symbols Substitution Test
DXEs/XREs,	dioxin/xenobiotic responsive elements
ECG,	Electrocardiogram
EM,	Extensive metabolizer
EMCDDA,	European Monitoring Centre for Drugs and Drug Addiction
GHB,	γ-hydroxybutyric acid
H_2SO_4,	Sulphuric acid
HCl,	Hydrochloric acid
HHA,	3,4-dihydroxyamphetamine
HHMA,	3,4-dihydroxymethamphetamine
HIV,	Human immunodeficiency virus
HM,	Hydroxymorphinan
HMA,	4-methoxy-3-hydroxyamphetamine
HMMA,	4-methoxy-3-hydroxymethamphetamine
HR,	Heart Rate
IM,	Intermediate metabolizer
KCl,	Potassium Choride
K_{deg},	Enzyme degradation constant
K_e,	Elimination Rate constant
k_{inact},	Maximum inhibition rate
k_{obs},	Observed inhibition constant
LDH,	Lactose Dihydrogenase
MAO,	Monoamine oxidase
MBI,	Mechanism-based inhibition
MBTFA,	N-methyl-bis-trifluoracetamide

MDA,	3,4-mehtylenedioxyamphetamine
MDEA,	3,4-methylenedioxyethylamphetamine
MDMA,	3,4-methylenedioxymethamphetamine
MDP,	Methylenedioxyphenyl
$MgCl_2$,	*Magnesium Chloride*
MM,	Methoxymorphinan
MR,	Molar Metabolic Ratio
NaCl,	Sodium Chloride
NADPH,	Nicotinamide adenine dinucleotide phosphate
NaOH,	Sodium hydroxide
NET,	Norepinephrine Transporter
NH_4Cl/NH_3,	*Ammonium chloride/ammonium hydroxide*
ODS,	Octododecyl silicone
PM,	Poor metabolizer
PMA,	Paramethoxyamphetamine
PRISM,	Psychiatric Research Interview for Substance and Mental Disorders
PRL,	Prolactin
PTT,	Partial Thromboblastin time
ROS,	*Reactive Oxigen Species*
RT,	Retention Time
SERT,	Serotonin Transporter
SOD,	Superoxide Dismutase
SSRI,	Selective Serotonin Inhibitor
SULT,	Sulphotransferase
$t½_e$,	Half-life of elimination
$t½_{inh}$,	*Half-life of inhibition*
TMA,	2,4,5-trimehtoxyamphetamine

TP,	Prothrombin time
UDGPT,	Glucuronosyl transferase
UM,	Utra-rapid metabolizer
VAS,	Visual Analog Scale
Vd,	Volume of Distribution
VMAT-2,	Vesicular Monoamine Transporter
VSG,	Gobular sendimentation velocity

JUSTIFICATION

3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is a substituted amphetamine, used for its euphoric effects. Its use reached its zenith in the mid to late 1990's, but it is still widely abused today, especially among the youth. MDMA is one of the most intensely studied drugs of abuse and although there is a growing consensus that MDMA is toxic to humans, the mechanisms underlying its toxic effects remain to be elucidated. Among the theories for the acute and neurotoxic effects of MDMA, clinical and experimental data from animal models support the view that systemic metabolism contributes significantly to toxicity[1]. As with any drug of abuse, MDMA research is highly politicised. Its eventual prohibition in the 1980's was contested by certain sectors in psychotherapy, while on the other hand, it is still subjected to regular demonization in the popular press. Hence, MDMA as a substance which causes a complex pharmacological response still deserves objective investigation.

In vitro evidence has suggested that cytochrome P450 isoform 2D6 (CYP2D6), the enzyme that contributes to MDMA phase I metabolism, is itself inhibited by MDMA[2]. This mechanism-based inhibition of the enzyme, leads to a quasi irreversible inactivation whose activity recovers with newly synthesised enzyme and is independent of MDMA pharmacokinetics. This was further studied *in vivo* in a clinical trial designed to study the pharmacology of two consecutive doses of MDMA[3]. It was seen in this study that CYP2D6 contributed 30% towards MDMA metabolism. Considering MDMA acute toxic effects, it would therefore be prudent to study the duration of inhibition of CYP2D6 by MDMA to allow an estimate of the incurred risk by MDMA users of experiencing pharmacological interactions with substrates of the same enzyme that may endanger their lives.

MDMA is both O-demethylenated and N-demethylated by CYP2D6, CYP1A2, CYP3A4 and another cytochrome P450 isoform, CYP2B6. These P450 systems demonstrate differing affinities for the substrate and differ in their contribution to O-demethylenation and N-demethylation. The contributions of these and possible

changes in the activities of these enzymes following MDMA administration have never been studied *in vivo*.

Furthermore, the *CYP2D6* gene is highly polymorphic with some alleles coding for a completely inactive protein and others for proteins of lower activity and different substrate affinities. Following the fortunate inclusion of a CYP2D6 poor metabolizer in a clinical trial studying two repeated doses of MDMA, data were reanalysed according to *CYP2D6* genotype and it was observed that possession of *CYP2D6*4* allele affects MDMA pharmacology[4]. Thus, any study of CYP2D6 substrate metabolism must take into account the interindividual differences in its activity caused by genetic polymorphisms.

Although there is much evidence in animals and humans suggesting MDMA to be a serotonergic neurotoxin, there is still much debate as to what is the cause of these long-term changes. Research points towards the excessive production of reactive oxygen species (ROS) in the brain following MDMA administration[5]. MDMA induces hyperthermia, excessive cerebral dopamine release and leads dysregulation of energy metabolism[6-10]. Furthermore, the metabolism of MDMA leads to the production of a reactive catechol, products of which have been found to cause serotonergic neurotoxicity in rats[11]. The source of this excessive ROS production and consequent long-term serotonergic changes could be caused by any of these different pharmacological factors. Animal models, especially rats have been used for many years to study these *sequelae*. Consequently, it would be interesting to potentiate neurotoxicity by interfering in MDMA and dopamine metabolism and by changing body temperature to investigate which of these previously mentioned factors hold more importance in the development of long-term serotonergic changes in the brain.

1. INTRODUCTION

1.1. Designer Drugs

Designer drugs are synthesised by chemically modifying the structure of already known natural products or medical drugs resulting in similar if not heightened pharmacological effects of the precursor. The synthesis of designer drugs is usually relatively simple, since their production is carried out in a clandestine setting with limited resources. They are frequently sold in the form of pills of various colours and imprinted with a variety of designs to target their sale towards a current illegal drugs market. In function of their chemical structure, designer drugs can be divided into five categories: phenylethylamines, synthetic opiates, arylhexylamines, derivatives of methaqualone and others.

1.1.1. A brief history of MDMA

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is a phenylethylamine structurally similar to amphetamine and mescaline. Other structurally similar methylenedioxy compounds are also found as aromatic constituents of oils and spices. Some more commonly mentioned examples are safrole, piperine and myristicin which are found in sassafras, nutmeg and pepper, respectively (Figure 1.1). MDMA was first synthesised in 1912 by Merck and first patented in 1914 (patent nº 274.350) as an anorexic. The first reported study was in animals in 1953 by the U.S. Army. In 1965 Alexander Shulgin synthesised the compound once again, during his investigations into new psychoactive compounds deriving from the essential oils of nutmeg[12]. In many ways, he and his contemporaries can be credited with the introduction of its use in psychotropic therapy in the 1960's and 1970's. Its properties were considered useful by psychotherapists due to its induction of feelings of euphoria, friendliness, closeness to others, and empathy after its administration. Although MDMA shares properties with both hallucinogens and stimulants it has been classed as an "entactogen" by some authors[13]. The recreational use of MDMA began in the late 1970's leading to it

being classed as a controlled substance in the UK in 1977. However, use continued during the 1980's and in 1984 the World Health Organisation (WHO) recommended that MDMA be included in a list of substances with a high abuse potential leading the way for a decision of the North American Drug Enforcement Administration to include it on the Schedule I of illegal substances (those with a high potential of abuse with a lack of medical application). Finally, in 1986 and after some debate between authorities and psychotherapists, MDMA was considered internationally illegal by the WHO Special Committee on Drug Dependencies[14]. In Spain, approximately one month following the announcement by the WHO, the use, fabrication, importation, transportation and sale of MDMA was prohibited by ministerial order in the Boletín Oficial del Estado. Its prohibition did not stop its increase in use in Europe and North America in the 1990's. It is primarily consumed by young people in large dance and music environments ("raves") and to a lesser extent in smaller social settings[15]. Until the middle of the 1990's it was not possible to conduct clinical studies with MDMA. Currently, the countries where such studies can be carried out are the USA (by authorisation of the FDA), Switzerland, Great Britain and Spain. In Germany it is also authorised to conduct studies with the pharmacologically similar, MDEA.

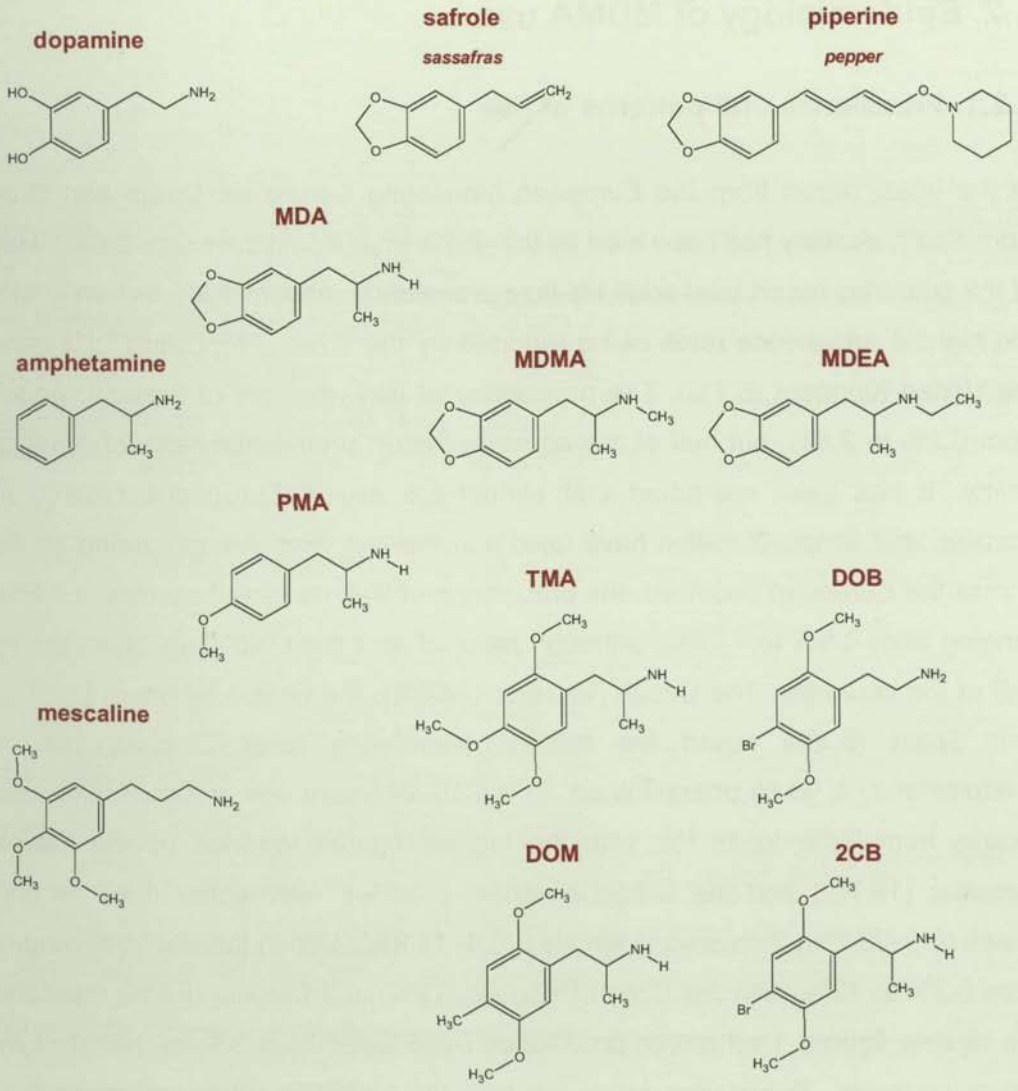


Figure 1.1. Amphetamine, common substituted amphetamines and naturally occurring compounds structurally related to MDMA. MDA = 3,4-methylenedioxyamphetamine; MDMA = 3,4-methylenedioxymethamphetamine; MDEA = 3,4-methylenedioxyethylamphetamine; PMA = paramethoxyamphetamine; TMA = 2,4,5-trimehtoxyamphetamine; DOB = 4-bromo-2,5-dimethoxyamphetamine; DOM = 4-methyl-2,5-dimethoxymethamphetamine; 2CB = 4-bromo-2,5-dimethoxymethamphetamine.

1.2. Epidemiology of MDMA use

1.2.1. Prevalence and patterns of use

In the latest report from the European Monitoring Centre for Drugs and Drug Addiction[16], ecstasy has been tried by 0.2–7.1% of all adults (average 2.6%). Half of the countries report total adult life-time prevalence rates of 1.8% or lower, with the highest prevalence rates being reported by the Czech Republic (7.1%) and the United Kingdom (6.7%). The prevalence of last year use of ecstasy ranges from 0.2% to 3.5%, but half of the countries report prevalence rates of 0.5% or below. It has been estimated that almost 8.5 million Europeans have tried ecstasy, and almost 3 million have used it in the last year. Among young adults across the European countries, the prevalence of lifetime use of ecstasy is 5.2%, ranging from 0.5% to 14.6%, although rates of less than 3.6% are reported by half of the countries. The Czech Republic (14.6%), the United Kingdom (12.7%) and Spain (8.3%) report the highest prevalence rates. Ecstasy use is predominantly a youth phenomenon. In the 15–24 years age group, lifetime use ranges from 0.4% to 18.7%, with the highest figures reported by the Czech Republic (18.7%) and the United Kingdom (10.7%), with higher rates among males (0.3–23.2%) than among females (0.4–13.9%). Use in the last year ranges from 0.3% to 12%, with the Czech Republic (12%) and Estonia (6.1%) reporting the highest figures. Last month prevalence rates lower than 3% are reported by seven countries. Prevalence rates are typically higher in urban areas, and in particular among people frequenting discos, clubs or dancing events. Among 15 to 16-year-old school students, surveys show that overall lifetime prevalence of ecstasy use increased over the period 1995–2003, with the greatest increases occurring in the Czech Republic and most of the new EU Member States. Even though its use may be still increasing among young people, its escalation among the general population seems to be limited, at least for the time being.

For comparison, the 2006 United States National Survey on Drug Use and Health reports that lifetime experience of ecstasy use among adults (defined as 12 years and older) rose from 4.3% in 2002 to 5.0% in 2006. However, recent use of ecstasy (last 12 months) decreased from 1.3% to 0.9% in the same period. Males and females had similar rates of past month use of ecstasy (0.2 percent for both). Among persons aged 12 to 49, the average age at first use was 20.6 years[17].

MDMA users are known to on average take more than one pill per session, with doses averaging from 1.8 to 2.9 per session reaching a maximum of 6.56 depending on the type of user[18]. It has also been documented that users may "binge" on ecstasy[19]. Solowij et al., observed in a group of 100 individuals that 15% had at some time used ecstasy for a few days in a row[20]. It was later reported by same research group that heaviest users can take up to 30 pills in one session, with 35% admitting that they had binged on ecstasy in the preceding 6 months[21]. In the UK, heavy users may binge with some 15-25 pills per session[22]. A report from users in Scotland referred to two distinct activities. First "stacking", which is taking several doses at once, and second "boosting", which is taking several tablets but at intervals over a period of time. During a binge, users may both stack and boost. In this study 39% of the users admitted that they binged for 1-2 days, with 38% admitting that they would binge again[23].

Demands for treatment related to ecstasy use are reported to account for less than 1% of all treatment demands in most countries, with the exception of Cyprus, Hungary, Ireland and Turkey, where ecstasy clients constitute between 4% and 6% of all clients seeking treatment for drug dependence. The number of deaths associated with MDMA consumption is relatively low when compared to the large population exposed to this substance. However, the frequency of recorded incidents associated with its acute toxicity is high. In the United States, MDMA-related hospital emergency room incidents increased from 253 in 1994 to 4,511 in 2000[24].

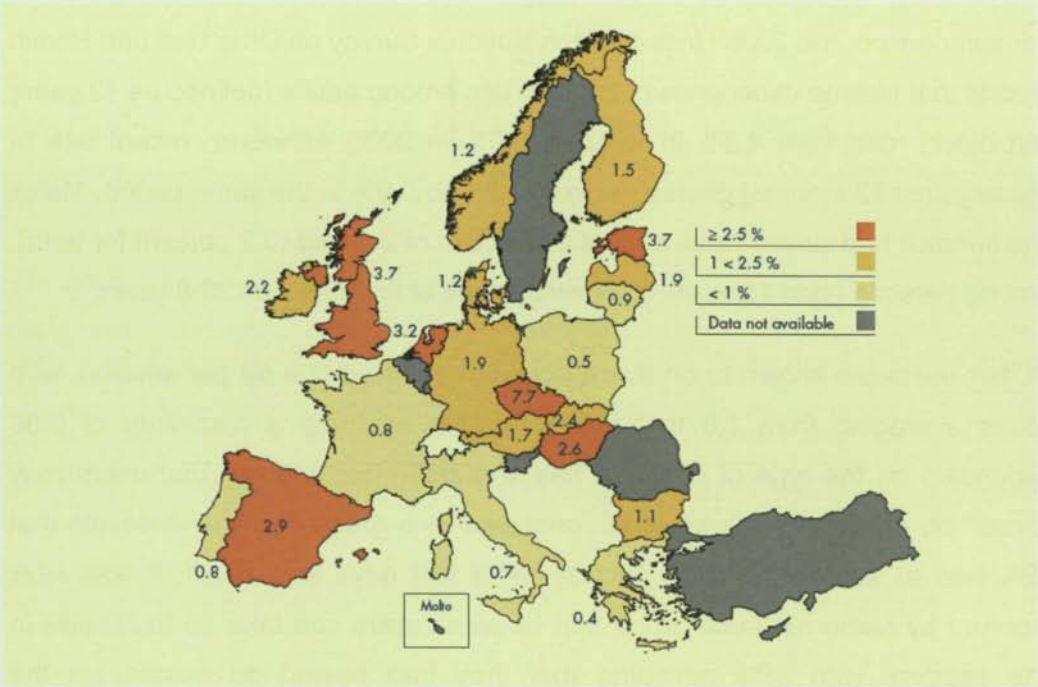


Figure 1.2. Last year prevalence of ecstasy use in young adults (15-34). Source: The State of the Drugs Problem in Europe, 2006 report, EMCDDA, p 53

1.2.2. MDMA use in Spain

In a recently presented school survey about drugs, which was conducted in 2006, 26.454 youths between 14 and 18 years participated, 3.3% of the interviewees admitted that they had taken ecstasy at least once, 2.4% in the last year and 1.4% in the last month (Observatorio Español sobre las Drogas, PNSD, Madrid, Informe de la Encuesta Estatal Sobre Uso de Drogas en Estudiantes de Enseñanzas Secundarias (ESTUDES) 2006-2007, http://www.pnsd.msc.es/Categoria2/observa/pdf/Estudes2006-2007.pdf). These data compared to those of previous years show a decrease in consumption. The prevalence of consumption was found to be greater in males than it is in females.

In the home survey concerning drugs conducted in 2006, 27.934 people participated between the ages of 15 and 64, 4.4% of the interviewees recalled that they had taken ecstasy at least once, 1.2% in the last year and 0.6% in the last month (Plan Nacional sobre Drogas). Those that consumed cocaine and cannabis in the last year also reported the similtaneous of ecstasy: cannabis (9.1%), cocaine (27.5%). The average age of initial consumption in this population was 20.1 years.

In a report conducted by the Plan Nacional Sobre Drogas in 1997[25], various data were presented concerning the development and extent of use of MDMA in universities and secondary schools in Spain in the previous years. A revision was done of the existing epidemiological data and results were presented of a survey carried out by the investigating authors of the report of 418 consumers of pills in five Spanish Autonomous Communities (Madrid, Valencia, Andalucía, Murcia, and Baleares). Among the most noticeable sociodemographic factors reported was that two thirds of the interviewed consumers were male. The average age of the sample was 21.9 years, with a range of ages between 18 and 53 years, but the majority of consumers comprised of those between the ages of 18 and 24. The educational and social levels of those surveyed varied; the majority (35%) being students, followed by waiters and people who worked in restaurants and clubs. It was also pointed out that a large majority of people took the substance in the company of other people or with a partner, while only 4% of consumers took it alone. The drug was found to be used at parties or discos by nearly 80% of those surveyed. Some 32% were experimental or occasional consumers (between 10 and 49 times in their lives), up to 24% said they had used these types of substances less than 9 times, while only 14% admitted using the drug more than 100 times. On the other hand, 76% consumed ecstasy and alcohol simultaneously. The simultaneous use of cannabis (76%), other amphetamines (34%) or LSD (33%) was also reported. Finally it was pointed that those who

used the drug had a decreased ability to drive a vehicle while under its influence. The results of this survey seemed to demonstrate that these substances are frequently used along with alcohol, although the percentages could vary according to the sample. This seems to contradict the opinion that users only drink water while taking the drug. Another noticeable result was the dose taken and the intervals between distinct doses. It seems that users in this study also admitted taking one or more dose of ecstasy per session. A considerable percentage of the subjects took an initial dose (half or one whole pill) and then another dose between thirty minutes and two hours later (a half pill). Although some subjects only take one dose, more than two doses are frequently taken, including as many as six in one session. The average is 1.5 to 2.5 pills of ecstasy per session or party, with 25% of the users taking more than 4 pills.

Ecstasy is sold as the racemate or mixture of the enantiomers (*R*)-MDMA and (*S*)-MDMA. The content of ecstasy pills varies, but the proportion of falsifications is relatively low, more than half contain MDMA, 30% contain other analogous phenylethylamines (MDE, MDA), the rest contain other psychostimulants (amphetamine or caffeine) or substances without psychoactive effects. The average MDMA content of a pill is 80 mg, ranging between 40 and 200 mg. On occasions pills can contain hallucinogenic and other more dangerous substances (e.g. paramethoxyamphetamine or PMA)[26].

1.3. The pharmacology of MDMA

As occurs with the majority of drugs of abuse, some of the pharmacological effects of MDMA are directly sought by users (euphoria, well-being) and others are clearly unwanted effects and moreover are manifestations of intoxication. MDMA and other designer drugs produce euphoria and psychostimulation, increase empathy and alter levels of perception. The sought effect is euphoria, the sensation of well-being and pleasure, along with the psychostimulant effects

of an increase in energy, talkativeness, decrease in tiredness and appetite. Users refer to an induction of a positive emotional state and pleasure, characterised by an increase in empathy. Tendencies to be intimate with those around them, a greater facility for communication and for interpersonal relation, are those referred to as entactogenic effects. MDMA is not an aphrodisiac although it appears to increase sensuality. In comparison with amphetamine it does not improve psychomotor performance or concentration, and can even worsen it at high doses. Regarding some of the perceptive effects, the most frequent are a certain amount of sensory hypersensitivity (frequently of a tactile nature), an alteration in the visual chromatics with changes in the intensity of colours, alterations in the subjective perception of time and a greater auditory sharpness. MDMA and its derivatives are not hallucinogenic substances, although descriptions do exist in cases of intoxication and when it is administered at high doses. Some of the derived hallucinogens like DOB, DOM, TMA, or PMA produce hallucinations at lower doses. When the euphoric effects diminish, sensations of intense relapse including fatigue and disinterest are felt. Many subjects pass the entire week in this dysphoric state until they take the drug again the following weekend[27].

MDMA and other designer drugs produce a stimulation of the sympathetic nervous system with an increase in the pupil diameter (mydriasis), which may cause blurred vision and greater sensitivity to light. They also produce dry mouth, sweating, trembling, tension in the jaw (trismus), chewing movements and grinding of the teeth (bruxism), and a slight increase in body temperature. They increase arterial pressure and heart rate (palpitations and tachycardia). The majority of these effects are considered by users to be unwanted [27].

It has been reported that MDMA can increase the plasma concentrations of prolactin, cortisol and ACTH, and vasopressin (antidiuretic hormone)[28]. It seems that following a single dose of MDMA a reversible immunosuppression is

produced (a reduction in $CD4^+$ T cell concentration) but may last some days following repetitive dosing[29]. In chronic users a higher risk of contracting infectious disease has been reported[30]. It is not clear the effects MDMA has on pregnancy. There are citings of increases in the incidences of malformations but contradictory data also exist[31].

1.3.1. MDMA pharmacodynamics

The mechanism of action of MDMA and its derivatives are similar to amphetamine, causing the release of the monoamines norepinephrine, dopamine and serotonin into the synaptic cleft. The difference is that while amphetamine has more dopaminergic and adrenergic activities than serotonergic, MDMA and other designer drugs seem to be more active in serotonergic transmission and less in dopaminergic and adrenergic. In all cases neither one nor the other are totally selective[32].

In the neurons containing monoamines, the formed neurotransmitter, for example serotonin, is stored in vesicles situated in the proximity of the synapse. When an excitatory impulse arrives the contents of these vesicles are released into the synapse and in that fashion the neurotransmitter can bind to the postsynaptic receptors to instigate a stimulus. Similarly the neurotransmitter can bind to presynaptic receptors (in the same neuron that released them and that is responsible for regulating the quantity of neurotransmitter they should release). The action of these monoamines ends with the recuperation of the majority of them by means of a transport protein responsible for collecting them at the synaptic cleft and introducing them back into the cytoplasm (uptake). Serotonin transporters (SERT) act at serotonergic neurons, dopamine transporters (DAT) at dopaminergic neurons and norepinephrine transporters (NET) act at adrenergic neurons. From the cytoplasm monoamines are introduced into storage vesicles by means of another vesicular transport system (VMAT-2). Amphetamine

analogues have the capacity to reverse the flow of the VMAT-2 system, in a way that empties the contents of the vesicles to the cytoplasm and releases them from there to the synapse. In this way they produce a release of monoamines. Just as it seems that these types of substances can enter into the neuron by means of the aforementioned membrane transporters, they also interfere in the process of uptake and because of this act as inhibitors of the same transport system. While MDMA and its derivatives bind principally to SERT rather than DAT (affinity constants of 0.35 μM and 1.14 μM respectively), other amphetamines have a higher affinity for the DAT transporters than the SERT (affinity constants for amphetamine are 0.13 μM and 4.51 μM respectively). However, taking this selectivity into account, the quantity of dopamine liberated by MDMA is more than that of serotonin because dopaminergic neurons are more plentiful than serotonergic ones in most areas of the brain[32].

Moreover, MDMA inhibits the enzyme tryptophan hydroxylase which is the limiting step in the synthesis of serotonin[33]. In this way, MDMA provokes an important release of serotonin, impedes its uptake and rapidly produces an exhaustion of the reserve serotonin vesicles combined with a decrease in its synthesis. The result is an initial increase in serotonin followed by a decrease some 2-4 h later, which persists more than 24 h. Additionally, MDMA has a certain activity as a monoamine oxidase A (MAO-A) inhibitor[34]. MDMA and its derivatives are also agonists of α_2-adrenergic receptors, M_1 muscarinic receptors and 5-HT_2 serotonergic receptors[32].

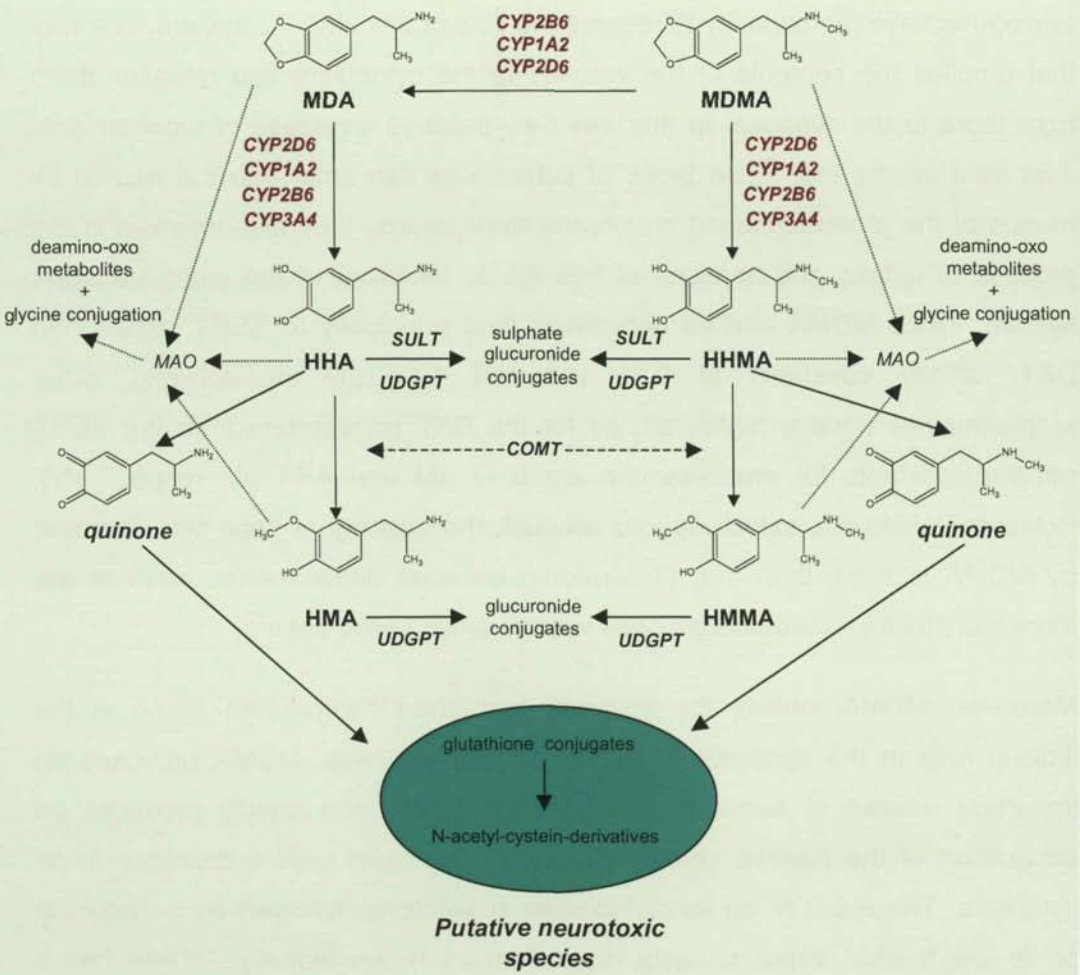


Figure 1.3. Pathways of 3,4-methylenedioxymethamphetamine (MDMA) metabolism in humans. The parent compound is N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA). HHMA is further O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA). Abbreviations: CYP = isozyme of cytochrome P450; COMT = catechol-O-methyl transferase; HMA = 3-methoxy,4-hydroxyamphetamine; MAO = monoamine oxidase; SULT = sulfotransferase; UDPGT, glucuronosyltransferase.

1.3.2. MDMA metabolism and pharmacokinetics

1.3.2.1. *In vitro* metabolism

The main pathways of MDMA metabolism are shown in Figure 1.3. MDMA is N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA) by isozymes of cytochrome P450. HHMA is highly unstable and conjugates with sulphate and glucuronic acid. HHMA can also be oxidised rapidly to its corresponding quinone and form adducts with glutathione and other thiol-containing compounds[35]. HHMA is further O-methylated to form 4-hydroxy-3-methoxymethamphetamine (HMMA) by catechol O-methyltransferase (COMT). MDMA metabolism has been studied in rat, rabbit and human liver microsomes, in cytochrome P450 expression systems and recombinant enzymes[36-39]. MDMA is predominantly N-demethylated to MDA in rat liver microsome preparations with cytochrome P450's of the CYP2D and CYP1A families involved in this step. In the rabbit, the main metabolic route is O-demethylenation to HHMA. In human systems the main cytochrome P450's identified in MDMA metabolism were CYP2D6, CYP1A2, CYP3A4 and CYP2B6, with MDMA being both N-demethylated and O-demethylenated. The O-demethylenation step is predominant and presents biphasic kinetics with the high affinity component controlled by CYP2D6 and a low affinity component controlled by CYP1A2 with contributions by CYP3A4 and CYP2B6. Human microsomal maximal velocities for N-demethylation are far lower than for O-demethylenation and are characterised by monophasic kinetics. The most important isozyme for this reaction appears to be CYP2B6. Although MDMA displays the highest affinity for CYP2D6, CYP1A2 seems to have the highest rate of O-demethylenation. Studies have also investigated MDMA's inhibition of its own metabolism. The inhibitory complex of CYP2D6 and MDMA has been observed *in vitro*[2,40]. This phenomenon is referred to as mechanism-based inhibition (MBI).

Species	Strain	Gender	Dose (mg/kg)	Admin. route	N	Collection	Endpoint (h)	C_{max} (µg/L)	t_{max} (h)	AUC (µg.h/L)	$t½_e$ (h)	V_d (L/kg)	Ref.
Rat	DA	male	5	i.p.	3	orbital vein	6	1100	0.25	2020	1.16		40
	DA	male	20	i.p.	7	cardiac	24	2835	2		6.65		41
	W	male	5	i.p.	5	brain dialysate	5	1009	0.77	7080	0.73		42
	W	male	5	i.p.	5	jugular vein	5	477	0.63	3180	0.72		42
	SD	male	5	p.o.	3	brain homogenate	10			17800	2.1		43
	SD	male	5	p.o.	3	cardiac	10			4560	2.4		43
	SD	male	10	i.v.	6	jugular vein	10			4610 (*R*) 3661 (*S*)	1.68 (*R*) 1.23 (*S*)	1.33 (R) 1.24 (*S*)	44
	SD	male	10	i.v.	3	iliac artery	12			2000 (*R*) 1500 (*S*)	1.70 (*R*) 1.70 (*S*)	6.67 (*R*) 8.00 (*S*)	45
	SD	male	10	s.c.		jugular vein	10		1.17	3329 (*R*) 3404 (*S*)		2.67 (*R*) 3.09 (*S*)	46
	SD	female	15	s.c.	3	cardiac	8	3980	1.5	15450	2.72		47
	SD	male	20	i.v.	4	iliac artery	12			3900 (*R*) 2700 (*S*)	3.50 (*R*) 3.60 (*S*)	12.67 (*R*) 19.33 (*S*)	45
	SD	male	20	s.c.	4	iliac artery	12			7600 (*R*) 5500 (*S*)	2.20 (*R*) 1.80 (*S*)	3.67 (*R*) 4.00 (*S*)	45
	SD	male	20	s.c.	5	trunk	10	5169	0.22	20250	3.99	4.61	48
	SD	male	40	s.c.	4	iliac artery	12			19000 (*R*) 12000 (*S*)	3.20 (*R*) 2.30 (*S*)	5.00 (*R*) 6.00 (*S*)	45
Monkey	Rhesus	male	10	i.m.	10	femoral vein	8	1836	0.5	7916	2.8	4.81	49
	Rhesus	male	1,5	i.v.	10	femoral vein	4	8999		1885	1.6	1.74	50
	Squirrel	male and female	7,4	p.o.	4	femoral vein	6	773		3408	3.1		51
	Squirrel	male and female	7,4	s.c.	4	femoral vein	6	1227		5006	3.5		51

Table 1.3.1 Pharmacokinetic data for MDMA in animals. DA = Dark Agouti; W = Wistar; SD = Sparague Dawley; i.p. = intraperitoneal; p.o. = oral; i.v. = intravenous; s.c. = subcutaneous; i.m. = intramuscular; C_{max} = maximal plasma concentration; t_{max} = time after administration at which concentration is maximal; AUC = area under the curve (plasma concentration vs. time after administration); $t½_e$ = half-life of elimination in plasma; Vd = volume of distribution.

Dose[1] (mg)	N	Gender	Weight (kg)	Height (cm)	Age (yr)	CYP2D6 activity	C_{max} (µg/L)	t_{max} (h)	AUC (µg/L.h)	$t^1/_{2e}$ (h)	CL_R/F (L/h)	Ref.
47.5	8	male			27	no	33.7 (*R*) 21.2 (*S*)	4.0 (*R*) 2.0 (*S*)		5.8 (*R*) 3.6 (*S*)	10.0 (*R*) 10.0 (*S*)	52
50	2	male				dex[2]	51.3	2.5	457	3.9	39.1	53
50	1	male	74		40	no	105.6	2		7.6		54
75	8	male	74.4	178	26.5	dex	130.9	1.8	1311.5	7.9	12.8	55
75	12	male and female			25.5	no	178	3				56
100	2	male				dex	199.8	2.5	1852.2	5.8	16.4	57
100	8	male				dex	222.5	2.3	2431.4	9		58
100	9	male	67.4	175	23	dex	231.4	1.5	2447.9			59
100	4	male	69.5	182	25.5	dex	181.6	1.9	1465.9	7.1		60
100	8	male	72.7	177	24.4	dex	181.4	1.5	1598.6	7.2		61
100	2	male				dex	162.6	3				62
100	6	male				dex	223	2.8	2554.8	8.5		63
100	9	male	73.3	177	23	dex	180	2	1452	7		3
100	7	male				dex	208.7	1.6	3108.5	11.8		64[3]
100	7	male				dex	116.7 (*R*) 88.8 (*S*)	3.5 (*R*) 1.9 (*S*)	2158.8 (*R*) 773.0 (*S*)	14.8 (*R*) 4.8 (*S*)		64[3]
100	7	male	64.7	173	22.6	dex	232.9	1.5	2541.8	8.3	9.4	65[4]
125	8	male	74.4	178	26.5	dex	236.4	2.4	2623.7	8.7	13	55
142	2	male and female			31.5	no	331	2				66

1 Dose given as mg of MDMA hydrochloride
2 CYP2D6 phenotype by Metabolic ratio of dextromethorphan to dextrorphan following 30 mg Romilar™ and 0-8 h urine collection, all volunteers were CYP2D6 EM
3 Values calculated from 0-48 h
4 Values calculated from 0-27 h

Table 1.3.2 Pharmacokinetic data for MDMA in humans. CYP2D6 activity = phenotyping of CYP2D6 by a probe drug; dex = dextromethorphan phenotyping; C_{max} = maximal plasma concentration; t_{max} = time after administration at which concentration is maximal; AUC = area under the curve (plasma concentration vs. time after administration); $t^1/_{2e}$ = half-life of elimination in plasma; CL_R = renal clearance.

1.3.2.2. *In vivo* metabolism and pharmacokinetics: animals

Although there are many studies reporting MDMA and metabolite concentrations in animals there are fewer presenting pharmacokinetic values due to the difficulty in serial plasma sample collection. Many differences in metabolism have been found between species, strain, gender and administration route. Some of the published pharmacokinetic parameters for MDMA are summarised in table 1.3.1. In the rat, MDMA is predominantly N-demethylated to MDA, although most of the metabolites have been identified in urine[41]. In the Sprague-Dawley rat, following a 10 mg/kg i.v. dose of MDMA hydrochloride, the elimination half-life of MDMA is 1.7 h with a volume of distribution of approximately 7 L/kg. Plasmatic concentrations of MDA are approximately 20% of MDMA in the rat with 9% dose excreted in urine. Enantioselective metabolism has also been observed, favouring the clearance of (S)-MDMA over (*R*)-MDMA[42]. In the rhesus monkey, following a 1.5 mg/kg i.v. dose of MDMA the C_{max} reaches 9000 μg/L at approximately 1.5 h, the elimination half-life is 1.6 h and a volume of distribution of 1.74 L/kg. MDMA is predominantly O-demethylenated in monkeys and non-linear pharmacokinetics has been observed in squirrel monkeys[43]. Although no pharmacokinetic data exist for mice the main metabolic route is that of N-demethylation to MDA. Concentrations of MDMA and MDA are similar to that of the rat, although the ratio of MDMA to MDA is slightly lower in the mouse[44].

1.3.2.3. *In vivo* metabolism and pharmacokinetics: humans

In humans, MDMA disposition has an important first pass hepatic metabolic component[45]. MDMA is predominantly O-demethylenated in humans with about 5% of the oral dose being N-demethylated. The majority of published pharmacokinetic parameters for MDMA are summarised in table 1.3.2. Some of the data were obtained from non-controlled or single-case studies. When interpreting analytic results in correlation with these data, broad individual

variations must be considered due to variations in metabolic or excretion rates or due to drug or food interactions. Indeed, although all data come from oral administrations, differences between whether MDMA was administered in capsule, tablet or solution will affect the absorption rate of the drug. In summary, following a 100 mg dose of MDMA hydrochloride in a soft gelatine capsule t_{max} was observed at 2 h with maximum concentrations of 200 μg/L. Mean elimination half-life is approximately 8 h. Plasma concentrations of HHMA and HMMA are similar to the parent drug with a C_{max} of approximately 180 μg/L and 160 μg/L and t_{max} of 1 h and 2 h, respectively[46]. Following a 100 mg dose peak concentration of MDA is approximately 13 μg/L and is reached at 5-7 h following administration.

MDMA possesses non-linear kinetics above a certain administered dose. Concerning this hypothesis, the pharmacokinetic results of a trial where single increasing doses of MDMA were administered pointed out a disproportionate increase in the pharmacokinetic parameters (area under the concentration curve and C_{max}) in relation to the dose interval (50-100-125-150 mg)[47]. The pharmacokinetics of MDMA is enantioselective with (*S*)-MDMA being eliminated faster than (*R*)-MDMA[48].

1.3.2.4. CYP2D6 and MDMA pharmacokinetics

Cytochrome P450 isoform 2D6 is a heme containing protein of 497 amino acids in length. It accounts for only a small percentage of hepatic P450's (~10%) but is responsible for the metabolism of approximately 20-30% of marketed pharmaceuticals, including tamoxifen, antidepressants such as imipramine, nortriptyline, maprotiline and the antianginal agent perhexiline[49,50]. In the human, CYP2D6 is postulated to be responsible for 30% of the O-demethylenation of MDMA to HHMA[3]. As has previously been mentioned, MDMA presents non-linear pharmacokinetics in humans, i.e. the plasma concentrations of the MDMA are not proportional to the administered dose and it tends to accumulate at high doses.

This is due to the inhibition of its own metabolism. This inhibition has been observed *in vivo* where MDMA was administered in two consecutive doses administered 24 h apart[3].

Interindividual differences in CYP2D6 activity were first discovered in the late 1970's and then subsequently proven to be genetic in nature. The clinical aspects of the CYP2D6 polymorphism were further studied in investigations into the adverse effects of debrisoquine and sparteine respectively[51,52]. The complete characterisation of alleles *CYP2D6*3* and *CYP2D6*4* was published in 1991[53]. Since then, over 70 different alleles of *CYP2D6* have been described. The variant *CYP2D6* alleles can be classified into categories, which cause abolished, decreased, normal, increased or qualitatively altered catalytic activity. Among the most important variants are *CYP2D6*2*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*10*, *CYP2D6*17* and *CYP2D6*41* and gene duplications. The frequency of *CYP2D6* alleles also varies ethnically. All variant alleles are presented at the home page of the human CYP allele nomenclature committee (http://www.imm.ki.se/cypalleles/cyp2d6.htm)[54]. Phenotypically, individuals can be divided into four categories: poor metabolisers (PM), intermediate metabolisers (IM), extensive metabolisers (EM) and ultrarapid metabolisers (UM) according to increasing CYP2D6 activity.

Preliminary data have shown that MDMA pharmacology differs according to *CYP2D6* genotype[4]. Studies *in vitro* have shown MDMA intrinsic clearance differs according to *CYP2D6* genotype, with *CYP2D6*17* differing in substrate specificity between dextromethorphan (DEX), a commonly known CYP2D6 substrate, and MDMA[55].

1.3.2.5. Individual differences in activity of other enzymes involved in MDMA metabolism

In vitro data show that, MDMA is also O-demethylenated by CYP1A2, CYP3A4 and CYP2B6. CYP1A2 is involved in the metabolic activation of several carcinogens and its activity differs drastically between individuals due to gender, ageing, race, genetic polymorphisms and exposure to inducers. CYP1A2 can be induced by smoking, dietary factors, several drugs and exposure to industrial chemicals[56]. CYP3A4 is the most abundant P450 isozyme found in the liver. It is responsible for the metabolism of benzodiazepines, endogenous steroids and HIV antiretrovirals among others. Indeed, the involvement in the metabolism of the latter group of substrates provides the only published observation of a harmful drug interaction with MDMA[57] on account of it being inhibited by the antiretroviral, ritonavir. Other CYP3A4 inhibitors include grapefruit juice. CYP3A4 is also inducible. CYP2B6's concentration in human liver is very low as compared to CYP1A2 and CYP3A4 and is mainly responsible for MDMA N-demethylation, a minor step in MDMA metabolism. Therefore little contribution is expected to MDMA metabolic disposition. What proportion CYP1A2 and CYP3A4 contribute to MDMA metabolism in humans has not been investigated.

Catechol-O-methyltransferase (COMT), which is involved in phase II MDMA metabolism, also displays polymorphic activity. A change at codon 158 in the COMT gene encodes for a low activity enzyme. This polymorphism exhibits autosomal inheritance in the population, with approximately 25% of individuals possessing the low activity haplotype in the Caucasian population. Another 25% possess the COMT ultrarapid haplotype. COMT inactivates HHMA giving rise to HMMA and so any differences in its activity may result in interindividual differences in the effects of this reactive catechol.

1.3.3. Controlled clinical trials with MDMA

1.3.3.1. Administration of MDMA at single doses

The first published controlled clinical trial using MDMA was by Grob et al.,[58]. In this trial MDMA was administered orally in doses of 0.25, 0.5, 0.75 and 1 mg/kg of body weight to healthy volunteers. The effects of MDMA were evaluated through physiological parameters (heart rate (HR), blood pressure (BP), body temperature (T) and neuroendocrines (PRL and ACTH)). The results showed a rise in HR, BP, PRL and ACTH following the administration of MDMA.

In 1998 a clinical trial was published in which a dose of 1.7 mg/kg of MDMA was administered orally, with a dose interval of 90-150 mg[59]. Physiological and subjective variables were studied. With respect to the physiological variables, the administration of MDMA produced an increase in systolic blood pressure of between 10 and 30 mmHg and an increase in diastolic blood pressure of between 5 and 10 mmHg. Body temperature increased somewhat although showed no evidence of significant change in respect to the placebo. In a number of the positive subjective effects a sensation of well being, an improvement in humour and an increase in emotional sensitivity were noticeable. Amongst the acute adverse effects were a slight anxiety, episodes of unawareness and depersonalisation, a moderate alteration in thought processing, alterations in spatial and temporal perception, although true hallucinations were not observed. After the first 24 hours of the clinical trial, the noticeable psychological effects were insomnia, weariness and a slight difficulty in concentration.

In 1999 and 2000, results from the first clinical trial performed in Spain with MDMA were published[60,61]. The clinical trial was conducted following a double blind, crossover, placebo-controlled design. Amphetamine sulphate (Centramina®) was also used as a positive control. The treatment was

administered randomly according to a 4 x 4 balanced Latin square system. The volunteers participated in four sessions (one for each condition of treatment) and the blank period between sessions was at least one week. The treatments received were two doses of MDMA (75 and 125 mg), one of amphetamine sulphate (40 mg) and placebo. These doses were selected following the results obtained from a pilot study in which MDMA was administered at doses of 50, 100 and 150 mg, amphetamine sulphate at doses of 20, 30, 35 and 40 mg and placebo. The variables studied consisted of physiological parameters, psychomotor output tests, questionnaires on subjective effects, neuroendocrine parameters and analysis of plasma and urine of psychoactive drugs.

For the physiological variables results showed an important increase in blood pressure and heart rate, as much for MDMA as with the administration of amphetamine sulphate. With regards to blood pressure, amphetamine sulphate produced some results comparable in magnitude and in temporality to the high doses of MDMA (maximum effect at 1.5 h post administration). The heart rate also showed an important increase during the three active conditions compared to the placebo. Once again the maximum increase was similar in magnitude for the high dose of MDMA and amphetamine sulphate, although the temporal course showed differences between the two substances. Thus, while for MDMA the maximum effect was produced around the first two hours, in the case of amphetamine sulphate it was produced from 8 hours post-administration. The temperature did not show distinct changes in respect to the placebo for any of the conditions of treatment, even if graphically a slight increase is observed, more evident with amphetamine sulphate. Evidence of a distinct increase in pupil diameter was observed in the doses of MDMA in respect to the placebo and amphetamine sulphate, reaching its maximum between 1 and 2 h post-administration. Amphetamine sulphate induced a significant increase, although being less than that observed for MDMA, in the eight hours post-administration.

The psychomotor tests showed a slight improvement in the output with amphetamine sulphate and a slight deterioration with the higher dose of MDMA. However, the results of the statistical analysis only reflected differences in comparison to the high dose of MDMA in respect to placebo for the Substitution of Digits for Symbols Test (DSST). The maximum effects were reached, for all the tests, between 1 and 2 h post-administration. A level of esophoria for the higher dose of MDMA was observed during the Maddox Wing test, its maximum effect being produced between 1 and 2 h post ingestion.

With respect to the subjective effects, MDMA induced a dose dependent increase in the representative scores for the variables of euphoria (Visual Analogue Scale): VAS "high", VAS "liking", subscale MBG (euphoria) of the Addiction Research Centre Inventory (ARCI). In VAS "stimulated", an increase was seen in the score for this scale in respect to the placebo as much for MDMA as for amphetamine sulphate. As regards the variable that expressed intellectual efficiency (ARCI-BG), amphetamine sulphate showed an increase compared with placebo and the higher dose of MDMA produced a decrease in score for this subscale. The variables that expressed sedation (VAS "drowsiness", ARCI-PCAG) did not show any statistically significant result in respect to placebo, although a slight increase (above all in the PCAG) is observed in the scores for the highest dose of MDMA and a slight decrease in sedation for amphetamine sulphate. In the variables representing dysphoria (ARCI-LSD), MDMA produced an increase in score in this subscale in comparison to placebo. Finally, with respect to the alterations in sensory perception, only the highest dose of MDMA induced noticeable increases in some of the corresponding scales (VAS "sensation of altered body state, different or unreal", VAS "changes in shapes", VAS "changes in light perception", and VAS "changes in audition"). For all of the variables of subjective effects, the maximum effect was situated between the first

and second hour post-administration. Hallucinations were not observed for any of the treatments administered.

With respect to the hormones studied, only MDMA produced noticeable increases compared to placebo in the plasma concentrations of cortisol and prolactin. As for the conclusions of the study, it can be affirmed that MDMA is a substance that presents a high potential for abuse. With respect to the subjective and psychomotor output effects, MDMA does not correspond to the profile of a typical stimulant or to one of a hallucinogen, although it possesses more characteristics of the former group than that of the latter. Finally, the alterations observed in the physiological parameters confer it with an elevated cardio toxic potential, without evidence of being a hyperthermic or hallucinogenic substance at recreational doses and in controlled conditions.

Trials conducted with MDMA have also been published in the USA. RT. Jones' group administered doses of 0.5 and 1.5 mg/kg body weight to male and female subjects[62,63]. C.R. Schuster's group administered 75, 110 and 145 mg per 70 kg body weight (1.1-2.1 mg/kg) to both male and females[64] showing similar increases physiological and subjective to previous studies, while also reporting the positive reinforcing effects of the drug at higher doses. In Great Britain, Henry and Cowan's group administered 40 mg of MDMA to male subjects[28] with the conclusion that even at low doses MDMA induces the release of vasopressin, a hormone associated with an acute hyponatraemia found in MDMA toxicity cases[65]. W.J. Riedel's group in Holland have administered single doses of 75 mg of MDMA to a group of male and female subjects[66]. Severe or non-expected effects were not presented following the administration of MDMA in any of these studies.

1.3.3.2. Administration of MDMA at multiple doses

In an attempt to mimic the "binging" practice as reported for humans in a recreational setting, three distinct clinical trials have been conducted in which two separate doses of MDMA was administered with an interval of 2 h (50 mg and 100 mg), 4 h (50 mg and 100 mg) and 24 h (100 mg and 100 mg)[3,67].

The pharmacodynamic results showed an existence of an elevated tolerance for the effects of the second dose of MDMA with respect to heart rate. As regards other cardiovascular and subjective effects, the effects of the second dose are greater than those of the first, but less than expected taking into account the plasma concentrations reached. However, in the case of body temperature, muscular tension (Maddox Wing) and psychomotor output, it seems that the effects are equal or superior to those expected considering the concentrations of MDMA in the blood. Serious undesired or unexpected effects were not observed in any of the participants.

With respect to the pharmacokinetics of MDMA and it metabolites, in all three studies it was observed that the concentrations of MDMA following the second dose were between 15% and 30% higher than those expected by pharmacokinetic superimposition. The concentrations of HMMA (in all three studies) and those of HHMA (the latter were only available for one of the studies) were much less than expected, in a way that the formation of these metabolites seemed to be inhibited. It has been previously mentioned that MDMA acts as an inhibitor of its own metabolism, by forming a complex with CYP2D6. This inhibition produced a 50% reduction in the concentrations of HMMA and lasts at least 24 hours. The result that the concentrations of MDMA only increased 15-30% while those of HMMA decreased by 50% indicates that CYP2D6 is mainly responsible for the O-demethylenation of MDMA. This suggests that the

remaining O-demethylenation of MDMA in humans is, as has been shown *in vitro*, carried out by other enzymes, presumably by CYP1A2 and CYP3A4.

1.3.3.3. Drug-Drug interaction studies

Alcohol

A clinical trial was conducted following double blind, double simulation, crossover design[68]. It was placebo controlled and randomised according to a balanced 4 x 4 Latin Square design. The volunteers participated in four sessions (one for each condition of treatment) with the wash-out period being no less than one week between sessions. The condition of treatment that the participants received were MDMA placebo + alcohol placebo (placebo condition), 100 mg MDMA + alcohol placebo (MDMA condition), MDMA placebo + 0.8 mg/kg of body weight alcohol (alcohol condition) and 100 mg MDMA + 0.8 mg/kg of body weight alcohol (interaction condition). The doses of alcohol and MDMA were selected from the results obtained from a pilot trial in which MDMA was administered at doses of 75 mg and 100 mg and alcohol at doses of 0.5 and 0.8 mg/kg of body weight. The administration schedule was the following: MDMA or placebo was administered together with 100 mL of mineral water at the basal time (0 h) and at 30 min the participant began to drink from a graduated glass containing either alcohol or placebo. The volunteers drank in a continuous rhythm (117 mL every 5 minutes) ending the ingestion of the drink at 45 min from the basal time. The drink containing alcohol contained a combination of vodka (Absolut®, Ahus, Sweden, 40% ethyl alcohol), tonic water (Schweppes®, Madrid, Spain) and lemon juice, adjusted to a fixed volume of 350 mL. The alcohol placebo was prepared in the same fashion but without the addition of 0.8 mg/kg of alcohol. With the intention of masking the fact that alcohol was missing 5 mL of the vodka was rubbed to the edge of the glass using a piece of cotton wool. Finally, to improve the deception the drinks were served very cold. The variables of the study consisted of

physiological parameters, psychomotor output tests, and questionnaires on subjective effects, neuroendocrine parameters and analysis of the substances in plasma and urine.

In a number of the results from the physiological variables, MDMA increased blood pressure as much as the combination of MDMA-alcohol, while the combination produced a greater increase in heart rate than MDMA alone. MDMA and the combination produced a similar increase in pupil diameter, while none of the active conditions produced significant changes in body temperature with respect to the placebo. Alcohol, however, produced a worse DSST result and induced exophoria. In a number of the results of subjective effects, MDMA increased the sensation of euphoria as much as the combination of MDMA-alcohol according to the ARCI-MBG questionnaire and the VAS "liking". In comparing the two conditions it was observed than the combination provoked some longer lasting effects than MDMA alone. The alcohol increased the sensation of drunkenness according to VAS "drunk" and sedation according to the ARCI-PCAG and VAS "sedation". The administration of the combination reversed the sedative effects of the alcohol but did no reverse effects of drunkenness. None of the active conditions produced hallucinogenic effects. As for conclusions of this study, it can be confirmed that the combination of MDMA and alcohol induces greater effects concerning heart rate than MDMA alone. Also, the combination produces a greater euphoric sensation than either one of the substances when taken separately. Finally, according to the results observed, the combination of MDMA and alcohol seems to have a greater potential for abuse than the two substances when taken separately. In a number of the neuroendocrine variables, the plasma concentrations of cortisol and prolactin increased following the administration of MDMA in a similar fashion to the increase observed during the single dose study. In this way, the combination did not induce changes in comparison to MDMA alone.

With respect to the pharmacokinetics, the plasma concentrations of alcohol, following the administration of the combination were less than those reached following the administration of alcohol alone. On the contrary, the concentrations of MDMA were greater and those of HMA less following the administration of the combination.

SSRI's

Vollenweider's group published a clinical trial reporting the interaction of MDMA and the SSRI citalopram. A dose of 1.5 mg/kg MDMA was administered orally along with 40 mg i.v. citalopram in a double blind placebo controlled trial in 12 male and 4 female volunteers. Citalopram pre-treatment reduced but did not completely abolish the cardiovascular and vegetative side-effects produced by MDMA[69,70].

Previous results in multiple dose studies encouraged the completion of a study to evaluate the importance of CYP2D6 in the O-demethylenation of MDMA. Consequently a clinical trial was designed in which the metabolism of MDMA was inhibited by means of administrating paroxetine[46,71] followed by MDMA administration. The trial also studied pharmacodynamic parameters with this metabolic interest in mind. It was suspected that MDMA enters the neuron by binding to SERT. Hence, previous administration of an inhibitor of this transporter, for example paroxetine may influence the effects of MDMA.

The results showed that the administration of paroxetine inhibits the metabolism of MDMA. The plasma concentrations of MDMA increase approximately 15% while the concentrations of HMMA are reduced by more than 50% and those of HHMA up to 20%. The results are very similar o those observed in the study using repetitive doses of MDMA. This reinforces the theory that MDMA inhibits CYP2D6, and this isozyme is responsible for 30% of the O-demethylenation of

MDMA. The previous administration of paroxetine produces an important decrease in the cardiovascular and pleasant subjective effects of MDMA.

Paroxetine reduced the cardiovascular effects produced by MDMA by approximately 50% and the interaction with paroxetine reduces the increase in pupil diameter mediated by MDMA by approximately 70%. During the paroxetine condition, the increase in temperature shown during the administration of MDMA alone decreased by approximately 50%. Furthermore, the concomitant administration of paroxetine produced a marked and significant reduction in the euphoric and pleasurable effects of MDMA and some feelings of dysphoria.

Recently a study has been published by Tancer and Johanson[72], where a dose of 1.5 mg/kg MDMA was administered orally following 20 mg p.o. fluoxetine in a double blind placebo controlled trial in 8 male volunteers. Fluoxetine treatment attenuated most of the positive-like subjective effects. In addition, heart rate but not blood pressure increases were reduced.

Others

Other controlled clinical trials reporting interactions are those carried by Vollenweider and Liechti with the dopamine D_2 antagonist haloperidol and the 5-$HT_{2A/C}$ antagonist ketanserin[73,74]. In the former, 14 healthy volunteers were pre-treated with a 1.4 mg i.v. dose of haloperidol before a dose of 1.5 mg/kg p.o. dose of MDMA. Haloperidol attenuated MDMA-induced positive and mania-like mood changes but had no reducing effect on other subjective and cardiovascular effects. In the latter, 50 mg p.o. ketanserin was administered previously to a dose of 1.5 mg/kg p.o. dose of MDMA in 14 healthy volunteers. The serotonin receptor antagonist attenuated the perceptual changes, emotional excitation and some acute adverse responses caused by MDMA but had little effect on positive mood, well being and extroverted behavioural changes induced by MDMA.

1.3.4. MDMA acute toxicity

Perhaps the most predominant severe acute adverse effect following ingestion of MDMA by recreational users is hyperthermia, which can lead to such problems as rhabdomyolysis, myoglobinuria and renal failure, liver damage and disseminated intravascular coagulopathy[75]. These problems are a feature of heatstroke and have been observed for other amphetamines and can lead to death in some cases. Although it is one of the major symptoms of MDMA acute toxicity with body temperatures of up to 43°C being reported, such changes generally only occur at higher doses. Mas et al.[60] did not observe changes in body temperature at a dose of 125 mg, while Vollenweider et al.,[59], giving a dose of 1.5 mg/kg, did. However, MDMA is usually consumed in dance clubs or "raves", where the hot and crowded conditions where people engage in very active dancing may alter this value.

Many studies in animals report an acute dose-dependent hyperthermic response[76]. Some investigators have reported a hypothermic response although this difference may be due to the temperature at which the animals were housed at. Higher room temperatures result in rats having a hyperthermic response whereas lower temperatures result in a hypothermic response. Both responses have also been observed in mice but the result tends to be strain and dose specific. These observations lead to the conclusion that MDMA does not cause hyperthermia *per se* but in a more general fashion interferes with thermoregulation.

The hyperthermic response has also been studied following what can be defined as a repeated binge dosing regimen. The data obtained however have been somewhat contradictory. Dafters[77] gave daily doses of 7.5 mg/kg during 13 consecutive days and reported an increase in peak temperature over time. In contrast, a more intense dose regimen of 10 mg/kg every 2 h for a total of four

injections caused an inhibition of the hyperthermic response to a subsequent MDMA challenge. A prior single dose of 12.5 mg/kg MDMA resulted in a challenge of 5 mg/kg MDMA producing a greater hyperthermic response in rats housed at 30°C than in non-pre-treated animals. Repeated MDMA administration (2, 4 or 6 mg/kg x 3) produced dose-dependent hyperthermia in rats housed at 19°C, with MDMA (2 mg/kg x 3) having little effect. However, this dose produced significant hyperthermia in rats housed at 30°C following the third dose. A prior single dose of MDMA (12.5 mg/kg) resulted in MDMA (2 mg/kg x 3) producing marked hyperthermia after the first dose and severe hyperthermia after the third dose[78].

It is generally assumed that hyperthermia is associated with increased 5-HT release, although recent evidence suggests dopamine release is the principal mechanism. Indeed, administration of 5-HT uptake inhibitor fluoxetine produces a major inhibition of MDMA induced 5-HT release but does not affect the hyperthermic response[79]. Furthermore, most 5-HT receptor antagonists fail to antagonise MDMA-induced hyperthermia. These preclinical data are generally consistent with the findings in humans[74]. However, the dopamine D1 receptor antagonist SCH23390 is an effective antagonist of the hyperthermia in rats[79]. The hyperthermic response also differs in animals which are housed separately or together[80], evidence which has consequences for the way in which MDMA is taken by humans. Conversely, the heightened locomotor response caused in animals administered with MDMA does not seem to be linked with hyperthermia.

At a cardiovascular level, MDMA can cause in humans arterial hypertension, tachycardia, arrhythmia, myocardial ischemia (angina) and acute myocardial infarction. Cephalea, trembling, muscular tension and chewing. vertigo, ataxia and dystonia can also be observed. Moreover, MDMA has been attributed to aplastic anaemia, retention of urine, mandibulary joint injury, dental erosion and

facial muscle pain. At the gastrointestinal level nausea and vomiting may be caused.

At the psychological level dysphoria, insomnia, irritability, agitation, hostility and confusion may be caused. Psychiatric trauma includes anxiety, distress and delirium. References to certain types of paranoia and hallucinations have been made. At the disappearance of the euphoric effects or at the end of use, the typical residual effects that appear are; fatigue, difficulty in concentrating, anorexia, apathy, insomnia, loss of memory, unwillingness to work/study, irritability, depression, muscular pain and a loss of sexual desire[81].

Other serious alterations are malignant neuroleptic syndrome and serotonin syndrome. due to an excess of serotonin in the CNS. It is clinically noticed by the appearance of at least three of the following symptoms: confusion, fever, trembling, diaphoresis, ataxia, hyperreflexia, myoclonus and diarrhoea[81].

Evidence suggests that MDMA use is one of the primary causes of hepatitis among young people[82]. Amongst other potentially fatal effects are subarachnoideal haemorrhage, intracranial haemorrhage, cerebral infarction and thrombosis of the cerebral ventricular cavity. Among the implicated mechanisms are, hypertension, the possible swelling of the cranial blood vessels and or dehydration.

Dehydration is produced usually due to the high temperatures of the places where MDMA is habitually consumed and the elevation of body temperature caused by dancing. In this case, the users drink water to assuage these unwanted effects. However, cases of cerebral oedema and hyponatraemia have been described, which are secondary causes of an inadequate secretion of antidiuretic hormone (vasopressin) induced by MDMA[28]. This induces thirst and the consumption of inadequately large quantities of water which leads to

hyponatraemia. Both MDMA and its metabolites, particularly HMMA are inducers of vasopressin secretion[83].

In humans, MDMA acute toxicity has been associated with elevated plasma concentrations[84]. Interindividual differences in metabolism due to differences in activity caused by the genetic polymorphisms of CYP2D6 could contribute to differences in the toxicity profile to MDMA between subjects, for example in the hyponatraemic response or hyperthermia. Indeed, an increased hyperthermic response is observed in an animal model for CYP2D6 PM[85]. The duration of CYP2D6 inhibition by MDMA must also be considered in the context of patterns of use, i.e. that of "binging". If it lasts more than one week then a subject who binges or takes MDMA in two subsequent weekends may not present a toxic response following the first dose but the following week may present such a response, especially if the hyperthermic results found in animals are to be believed.

1.3.5. MDMA neurotoxicity

The neurotoxicity associated with MDMA is concerned with long-term reductions in markers of the serotonergic system activity. These reductions have been observed in rats and non-human primates, with non-human primates being more sensitive to the neurotoxic effects. They include the decrease in cerebral 5-HT and its major metabolite 5-HIAA, decreases in 5-HT uptake transporters (SERT), decreases in tryptophan hydroxylase activity and loss of serotonergic axons in the striatum, neocortex and thalamus[86]. However the failure to demonstrate gliosis, the classic marker for neurotoxic brain injury, raises doubt on the validity of this model of brain "damage"[87]. Although it is difficult to relate the latter findings to the human there is growing consensus that MDMA is neurotoxic in the human. Differences have been seen in cerebrospinal fluid 5-HT and metabolite concentrations of MDMA users compared to that of controls that never used

MDMA[88]. Additionally, PET imaging techniques have demonstrated changes in 5-HT uptake sites in ex-MDMA users compared to controls. These results have been controversial because of the small sample size, large variation and signal to noise found in such studies[89]. Recent studies seem to confirm previous findings, with two additional aspects to be considered: changes in 5-HT uptake are related to MDMA intensity of use and that recovery over time is observed in these alterations[90]. Even so, studies in humans are littered with methodological problems such as the confounder of polydrug abuse and the possibility of pre-existing differences between users and controls.

Neurotoxic effects of amphetamines on monoaminergic systems have been associated with the formation of reactive oxygen species (ROS)[5]. The administration of antioxidants such as ascorbic acid, selenium and vitamin E can attenuate MDMA-mediated neurotoxicity while the inhibition of superoxide dismutase (SOD) may potentiate toxicity[91-94]. In transgenic mice expressing human SOD, methamphetamine induced neurotoxicity in the dopaminergic and serotonergic systems is attenuated when compared to control animals[95] suggesting that superoxide radicals contribute to these adverse effects. However, there is a debate as to what is the source of these ROS. In recent years, a broad amount of evidence appears to indicate that reactive oxygen species generation responsible for 5-HT depletion is dependent upon the prolonged and excessive release of DA elicited by MDMA[8]. It is known that DA can enter the 5-HT terminal by means of the 5-HT transporter[96-98] and once inside the 5-HT terminal, be deaminated by MAO-B resulting in an elevated intracellular level of hydrogen peroxide that is reduced by iron to produce hydroxyl radicals and a subsequent terminal degeneration[8,96,99]. Explaining why MDMA-induced 5-HT depletions also occur in regions such as the hippocampus or frontal cortex, which are sparsely innervated by DA, is the main drawback of this hypothesis. This important issue has been expanded upon recently[100]. These authors showed that MDMA

increases the concentrations of tyrosine in the brain to cause a long-term depletion of 5-HT via the nonenzymatic, tyrosine hydroxylase-independent, hydroxylation of tyrosine to DOPA and subsequently to DA via amino acid decarboxylase. However, this latter hypothesis assumes the pre-existence of hydroxyl free radicals responsible for the oxidation of tyrosine to DOPA.

There is also a close relationship between hyperthermia and neurotoxicity engendered by MDMA[6,7,101]. The blockade of MDMA-induced hyperthermia is the only common link among drugs with disparate mechanisms of action known to prevent MDMA toxicity[33,102-109]. Furthermore, small changes in ambient temperature cause large changes in MDMA-induced 5-HT neurotoxicity and core body temperature in the rat[6,110]. Similarly, other non-pharmacological manipulations that prevent the acute hyperthermic response caused by MDMA also provide substantial protection against the serotonergic neurotoxic effects of MDMA[111]. However, the free radical trapping agent, α-phenyl-*N*-tertbutyl nitrone and the serotonin uptake inhibitor fluoxetine, partially prevent MDMA-induced serotonergic changes without modifying hyperthermia[112,113]. Indeed, MDMA aside, free radical formation in the brain is influenced by body temperature and lowering the body temperature results in decreased free radical formation[114,115]. Taking all this evidence together it appears that hyperthermia has more of a modulating role in MDMA-induced neurotoxicity. Other hypotheses of MDMA-induced neurotoxicity have also been proposed, including dysregulation of energy metabolism[9,10] and the induced release of glutamate and nitric oxide[86].

Not only does MDMA induce DA secretion but its own metabolism generates a catechol in the form of HHMA, which can further oxidise to its corresponding quinone, N-methyl-α-methyldopaquinone, that may participate in MDMA induced neurotoxicity (Figure 1.4)[116]. Similarly, the metabolism of MDA gives rise to α-methyldopaquinone. The quinone products of HHMA and HHA are further conjugated to glutathione and or cysteine[116]. The formation of thioethers of these

quinones lowers their redox potential and increases their ability to redox cycle and to generate ROS[35]. Such electrophilic redox-active quinones may contribute to the damage to serotonergic and DA nerve terminals, their transporters and to the inhibition the tyrosine-hydroxylase. This inhibition may be achieved by modifying proteins at the post-translational level, by converting them to quinoproteins[117]. A neurotoxic metabolite of this kind has been identified in the rat brain[11]. Further evidence that the metabolism of MDMA contributes to its neurotoxicity is evidenced by the fact that the neurodegeneration of the serotonergic system is only evident when the drug is administered systemically but not when administered directly to the brain[118,119].

When discussing MDMA-induced neurotoxicity, a variable that must be taken into account is the prevalence and pattern of use as seen in humans. Data in humans has shown an association between intensity of use and SERT loss[90]. Indeed, in many studies the heavier users are those that differ from controls. Furthermore, repeated dosing is the preferred procedure in animal studies to induce serotonergic changes[120-122]. In terms of MDMA metabolism and neurotoxicity,

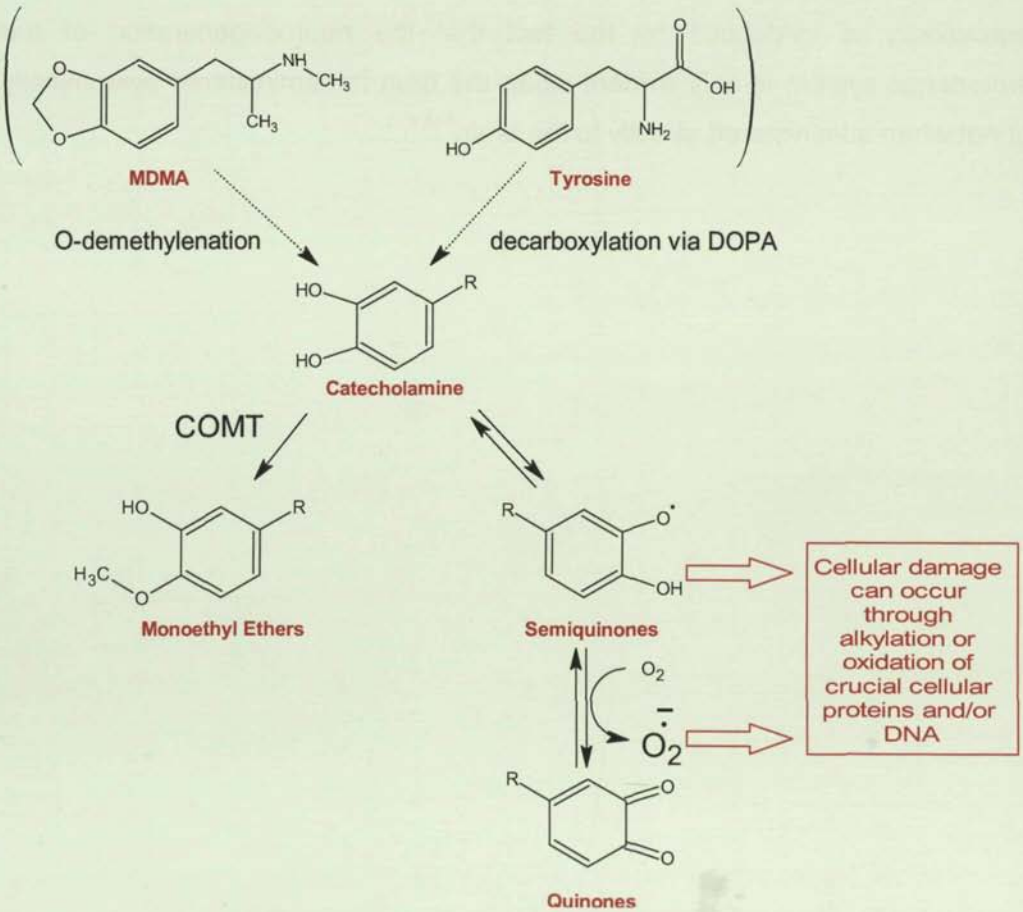


Figure. 1.4 Proposed mechanism for free radical (ROS) formation that leads to neurotoxicity from the metabolism of MDMA and dopamine. When R = -CH_2-CH_3CH-NH-CH_3 it corresponds to the metabolites of MDMA and when R = -CH_2-CH_2-NH_2 it corresponds to metabolites of dopamine.

intense MDMA use or binging therefore could result in an almost permanent inhibition of CYP2D6 in an EM subject, rendering any functional differences due to genetic polymorphisms redundant. Indeed, interindividual differences in other enzymes such as CYP1A2 and COMT could play a more important role in neurobiological changes noted for humans.

1.3.5.1. Problems with relating animal and human data

It has often been asserted in forums of recreational ecstasy users that data on adverse effects of MDMA obtained in experimental animals are not relevant to human use, as the doses administered have been much higher than those used by humans. While it is undoubtedly true that many experimental studies have used high doses, it now appears that different strains have different susceptibilities to the drug. Furthermore, recent studies have demonstrated neurotoxicity in rats following doses that are a fraction of those used in the earlier studies[123]. It is always difficult to make direct comparisons between results obtained in animal and human studies, since small mammals tend to eliminate drugs at a faster rate than large mammals. However, it has been suggested that the technique of interspecies scaling[124] enables prediction of drug elimination in different species based upon the underlying anatomical, physiological, and biochemical similarities among most land mammals. Thus, to achieve a similar effect to that seen in humans, smaller animals require higher doses of drug, estimated according to the relationship:

$$D_{human} = D_{animal} \left(\frac{W_{human}}{W_{animal}} \right)^{0.7}$$

Where D = dose of drug in milligrams and W = body weight in kilograms. According to this equation, a single neurotoxic dose of MDMA (5 mg/kg) administered to a 1 kg monkey can be calculated to equate to a dose in a 70 kg

human of 98 mg or 1.4 mg/kg[125]. A dose of MDMA of 10 to 15 mg/kg that produces substantial damage in the brain of Dark Agouti rats is thus equivalent to a human dose of 140 to 190 mg in a 70 kg human. However, the validity of interspecies dose scaling in relation to MDMA has been argued against by other authors who state that calculations of dose equivalency should be based on the minimal dose which causes a pharmacological effect. In the case of animal models, doses as little as 1-2 mg/kg cause distinct behavioural effects in rats[126], yet no long-term serotonergic changes have been observed as these doses. Furthermore, there are many differences in in administration route, pharmacodynamic response, metabolism and pharmacokinetics between animal studies and MDMA user practice. Although ecstasy is taken orally in tablet form, the majority of animal studies are conducted using other routes of administration usually intra-peritoneal. It is also interesting to note that according to interspecies scaling the dose which causes a notable hyperthermic response in animals does not cause such a drastic response in humans. On a metabolic and pharmacokinetic level, following administration of MDMA to mice, MDMA is the main chemical species observed in both plasma and brain. By contrast, although MDMA is observed in rats and humans following its administration, HHMA and HMMA, which are produced at different rates in rats and humans, are also present in high concentrations in plasma. In fact, MDMA metabolism, except in mice, is qualitatively similar for its major metabolic reactions in most animal species and humans; nevertheless, there are relevant quantitative differences. In rats, the N-demethylation of MDMA leading to the formation of MDA is one of the main metabolic pathways at low doses[127], whereas in humans the O-demethylenation of MDMA to HHMA predominates at any dose tested[45]. In rats, the rate of N-demethylation is dose dependent and at doses higher than 10 mg/kg there is saturation of hepatic clearance[127]. In monkeys given 10 mg/kg of MDMA twice daily for four consecutive days, MDMA plasma concentrations increased by 30% and MDA plasma concentrations increased by 200%,

compared with levels present following the first dose of MDMA; MDA concentrations were as high as 18% of the concentration of MDMA[122]. By contrast, MDA plasma concentrations are usually <5% of the concentration of MDMA in humans following administration of MDMA[45].

In rats, the enzyme involved in O-demethylenation of MDMA is CYP2D1. Although this enzyme is homologous to human CYP2D6, the enzymes are not functionally identical[128]. Interestingly, although there are no major differences among animal species in the affinity of substrate probes for this enzyme (CYP2D1/CYP2D6), there is an approximate tenfold difference in its V_{max} for bufuralol 1-hydoxylase activity. The enzyme in humans has lower V_{max} for bufuralol than the enzyme in rats and monkeys. Furthermore, this activity is inhibited by quinidine in humans but not in mouse or rat[129]. Although in humans a single dose of MDMA can fully inhibit CYP2D6, in non-human primates several doses of MDMA are required for full inhibition of the enzyme[122]. Rats and to more of extent, mice, express many CYP2D isozymes whose contribution towards MDMA metabolism has not been investigated. Thus, differences in both enzyme kinetics and the inhibition of CYP2D6 by MDMA through the formation of an enzyme–metabolite complex suggest that different rates of formation of metabolic neurotoxic species and different susceptibilities to the development of neurotoxicity exist among species. Indeed, in mice, in which MDMA predominates in tissues following its administration, MDMA-induced neurotoxicity is mainly dopaminergic.

1.4. Assessment of the Activities of CYP2D6 and CYP1A2

Previous investigations have shown that: (1) MDMA users are known to take more than one pill per session. (2) CYP2D6 activity is impaired by MDMA through MBI and (3) other isozymes of cytochrome P450 with lower affinity for MDMA contribute to its metabolism once MDMA accumulates in the body. These findings

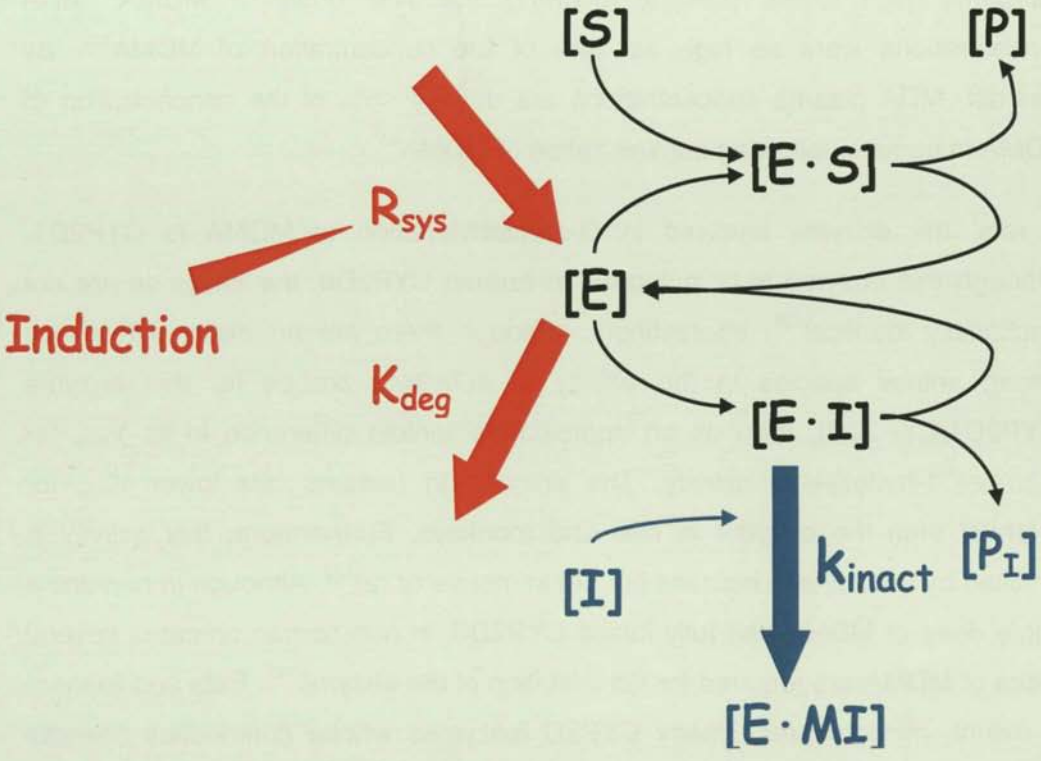


Figure 1.5. Mechanistic scheme for mechanism-based inhibition. R_{sys} = rate of enzyme synthesis; K_{deg} = degradation rate of the enzyme; [S] = substrate concentration; [P] = metabolite concentration; [E·S] = enzyme substrate complex concentration; [E] = enzyme concentration; [I] = inhibitor concentration; [E·I] = inactivated enzyme inhibitor complex; [E·I] = enzyme inhibitor complex; [P_I] = inhibitor metabolite.

have implications for both acute and long-term toxicity of MDMA. Although *in vitro* and animal studies give much information about the metabolism of MDMA, for a full understanding of the system of CYPs involved, human studies are required.

1.4.1. Mechanism-based inhibition

As previously mentioned CYP2D6 is inhibited by MDMA in a quasi-irreversible fashion in a phenomenon otherwise referred to as mechanism-based inhibition

(MBI). MBI is also known as suicidal inhibition, irreversible inhibition or time-dependent inhibition and although it is an unusual occurrence in most enzymes it is more frequently observed in cytochrome P450 catalysed reactions. A substrate undergoes a catalytic transformation by the enzyme to a species that, prior to release from the active site, inactivates the enzyme[130]. There are three kinds of MBI: covalent binding to the apoprotein, covalent binding and destruction of the prosthetic heme group and chelation by alkylation or arylation of the prosthetic heme group or binding (non-covalently) to the apoprotein (quasi-irreversible). MBI is generally of greater concern than reversible/competitive inhibition as it can result in a more profound and prolonged effect than the dose or exposure might suggest. This is illustrated by a measurable change (1.21-fold) in midazolam AUC following administration of a low dose oral contraceptive preparation containing 30 mg/day ethinylestradiol and 75 mg/day gestodene[131], both compounds being mechanism-based inhibitors of CYP3A4. An example of greater clinical significance relates to mibefradil, a potent mechanism-based inhibitor of CYP3A4, which was withdrawn from use because of unmanageable drug interactions. For example, at a relatively low dose of 50 mg/day, mibefradil increased the plasma AUC of triazolam by 9-fold[132]. The extent of these interactions can not be anticipated based on the competitive inhibitory potencies of the compounds involved. Mechanism-based inhibitors that bind covalently to CYPs and potentially other proteins via formation of reactive metabolites are of additional concern due to the risk of idiosyncratic toxicity. For example, tienilic acid, which is metabolised to an electrophile that binds covalently to CYP2C9, was withdrawn from use due to hepatotoxicity mediated by an auto-immune response[133]. However, MBI is not always associated with the formation of covalently bound species and the binding may be specific to a CYP. For example, macrolide antibiotics such as erythromycin are metabolised by CYP3A4 to reactive nitrosoalkanes, which form metabolite intermediate complexes through co-ordination to the heme of the enzyme[134].

The formation of P450 metabolite complexes upon oxidation of drugs containing tertiary amine or methylenedioxy functions involves oxidation leading to inhibition of P450 catalytic activities. Evidence for complex formation by methylenedioxy compounds was explained by findings from *in vitro* studies that showed a decrease in CYP content when microsomal fractions were incubated with piperonyl butoxide and NADPH. Unusual absorbances near 427 and 455 nm were detected in microsomal difference spectra during such incubations. Thus, it was suggested that methylenedioxy compounds undergo CYP-dependent oxidation and generate complexes with CYP that contribute to the observed decline in drug and xenobiotic oxidation[135]. MDMA has the particularity of having on the same molecule both the tertiary amine and the methylenedioxy functions. In the case of MDMA the amine moiety may oxidise to a nitroso metabolite which readily reacts with the Fe (II) of the heme, conversely the methylenedioxy group can be metabolised to a carbene, forming stable Fe (II) and Fe(III) complexes[40]. However, the nature of the metabolite that is involved in the complexation of CYPs remains unclear and other possible intermediates have been suggested, including benzodioxolium ions, homolytic radicals, and carbanions, formed by oxidation at the methylenic carbon[135]. Recovery of activity of the enzyme *in vivo* depends on *de novo* synthesis of protein.

1.4.1.1. *In vitro* assessment of MBI and enzyme activity recovery

Many *in vitro* studies exist which try to predict the *in vivo* outcome of MBI[134]. Figure 1.5 presents a mechanistic scheme for MBI showing the kinetic parameters of CYP inactivation, namely the maximum inactivation rate constant (k_{inact}) and the inhibitor concentration that produces half-maximal rate of inactivation (K_I). Two alternative experimental procedures for investigating the time and concentration dependent effects of MBI's *in vitro* have been proposed[130].

1. Preincubation of the enzyme and co-factors with different inhibitor concentrations for varying times, followed by further incubation of a dilution (at least 1:50) of the reaction mixture with a probe substrate to assess the degree of enzyme inactivation.

2. As in (1) but with further incubation after direct addition of the probe substrate to the preincubation mixture, without a dilution step.

An apparent rate of inactivation (k_{obs}) is estimated graphically from the initial slope of a plot of the natural log of enzyme activity remaining after pre-incubation against pre-incubation time, corrected for any loss of activity in the absence of inhibitor. The following equation describes the relationship between the parameters:

$$k_{obs} = \frac{[I] \times k_{inact}}{[I] + K_I}$$

Values of k_{inact} and K_I are then obtained from a double reciprocal plot of k_{obs} against inhibitor concentration. In the case of MDMA a K_I of 0.01 $h^{-1}$and k_{inact} of 0.29 min^{-1} have been calculated[2]. A physiologically-based pharmacokinetic model has been used to calculate a value of 260 h for 90% recovery of CYP2D6 activity following recreational doses of MDMA[136]. However, this is based on a number of assumptions and since the degradation rate constant (K_{deg}) or turnover half-life of a particular CYP in the liver is expression dependent its calculation must be conducted *in vivo*.

1.4.1.2. *In vivo* assessment of MBI and enzyme activity recovery

To monitor the activity of any CYP, the typical experimental approach *in vivo* is to use a probe drug, as *in vitro*. Probe drugs have been used for many years to distinguish phenotypes of various CYPs. A subject is administered a substrate

which is specific to a certain CYP and concentrations of the substrate and its metabolites are measured in plasma and/or urine over a period of time. The individual is then assigned a phenotype based on the amount of substrate metabolised. This is usually expressed in a molar metabolic ratio (MR) of substrate versus metabolite(s) and then compared to a previous decided cut-off value, or antimode, to assign the phenotype. The change in enzyme activity over time can be monitored by administering repeated doses of the probe drug and calculating the MR at each time interval. These values can then be compared to a basal or control condition, where the individual has not been administered anything that may alter the activity of a CYP.

As when k_{inact} and K_I are calculated *in vitro* an apparent rate of recovery (K_{cyp}) is estimated graphically from the initial slope of a plot of the log of enzyme activity remaining after administration over time. The following equation defines the rate of recovery *in vivo:*

$$K_{cyp} = K_{deg} + \left[\frac{(f_u \times [I]) \times k_{inact}}{(f_u \times [I]) + K_I} \right] = K_{deg} + K_e$$

Where K_{deg} is the degradation rate constant of the enzyme, f_u is the fraction unbound in blood plasma and [I] is the inhibitor concentration. As seen in the above equation the elimination rate constant of the inhibitor is a function of k_{inact}, K_I and unbound plasma inhibitor concentration.

This equation implies that when the unbound plasma concentration of the inhibitor is much higher than its K_I value and its k_{inact} value is much larger than the degradation rate constant of the enzyme, CYP activity loss would be 100% irrespective of the kinetic parameter values and is associated with almost

complete abolition of drug probe clearance. In this case, the relationship in the above equation becomes linear, whereby $K_{cyp} \approx K_{deg}$. Analysis of data on some of the compounds that cause MBI of CYP3A (Figure. 1.5) indicated that, depending on inhibitor strength, variability in K_{deg} estimates have a large effect on the predicted net loss of enzyme activity[134]. Thus, in situations where unbound plasma concentrations are lower than the k_{inact} and K_I the apparent rate of recovery of CYP2D6 activity to baseline must factor in the mean residence time of the inhibitor in the system and should reflect two processes: the CYP K_{deg} and inhibitor elimination rate constant (K_e). The CYP degradation rate constant can thus be determined using a noncompartmental deconvolution (component analysis) approach, whereby the mean-residence time ($1/K_e$) is subtracted from the mean recovery time ($1/K_{cyp}$) resulting in a mean degradation time for the CYP enzyme ($1/K_{deg}$).

A half-life estimate of 50 h (range 25–80 h) has been reported for human CYP2E1 by measuring the time course of return of chlorzoxazone 6-hydroxylation clearance to baseline following administration of the rapidly cleared CYP2E1 inactivator disulfiram[137]. A time course study with three SSRI's that inhibit CYP2D6 has been conducted[138]. Following SSRI discontinuation, calculation of a CYP2D6 inhibition half-life ($t½_{inh}$) revealed the time course of fluoxetine inhibition ($t½_{inh}$ = 7.0 +/- 1.5 days) to be significantly longer than either paroxetine ($t½_{inh}$ = 2.9 +/- 1.9 h) or sertraline ($t½_{inh}$ = 3.0 +/- 3.0 h) ($p < 0.01$), but the latter were not significantly different from each other ($p > 0.05$). Significant differences from DEX MR at baseline were measured successfully for up to 35 days in this study. Using the former data for paroxetine[139] used *in vivo-in vitro* extrapolation to estimate a CYP2D6 degradation half-life of 51 h, but this

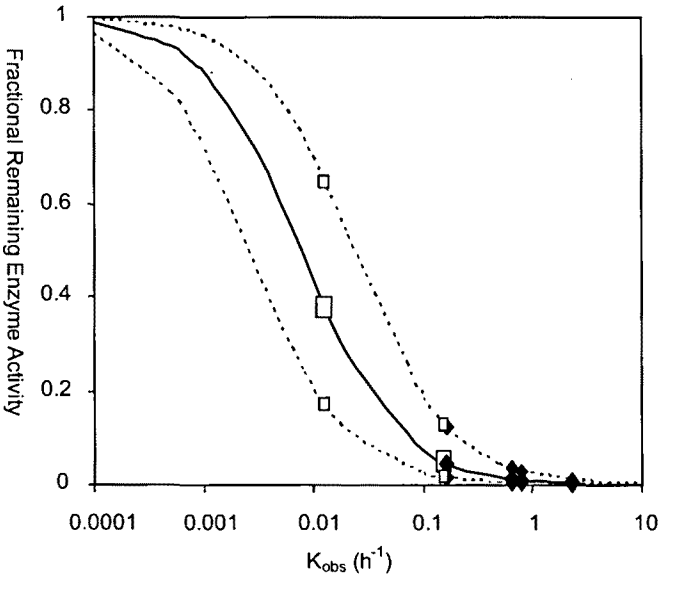


Figure 1.5. Illustration of the insensitivity (erythromyicin; -♦-) or sensitivity (clarithromycin; -□-) of the predicted decrease in enzyme activity to differences in reported values k_{obs}. The simulations were carried out under three different assumptions for the K_{deg} value of CYP3A, corresponding to half-lives of 30, 90 and 270 h, respectively. Source: Ghanbari et al., 2006.

calculation had many assumptions including the assumption that paroxetine steady-state concentrations are lower than that its K_i for CYP2D6.

1.4.1.3. Probes for assessing CYP2D6 activity *in vivo*

The three best known CYP2D6 drug probes are dextromethorphan[140] (DEX), debrisoquine[51] and sparteine[52]. Debrisoquine and sparteine have been used to phenotype CYP2D6 since the late 1970s, but concerns about availability and safety have limited their use. Debrisoquine may be the preferred probe for subjects with high levels of CYP2D6 activity because of its lower turnover compared with DEX. However, debrisoquine can cause hypotension. On the other hand, sparteine may be preferred in subjects with impaired renal function because of its lack of glucuronidation and the fact that it is unaffected by changes in creatinine clearance. On the other hand, DEX is often used because of its wide safety margin and ready availability. Some pharmacokinetic studies have demonstrated that the DEX metabolic ratio is closely correlated with the partial clearance of DEX to dextrorphan[141]. In other words, DEX metabolism depends on CYP2D6 activity and not on renal elimination[142]. However, the use of the DEX has not been short of its controversy and its robustness due to differences in hepatic and renal function, gender and urinary pH has been evaluated[143-146].

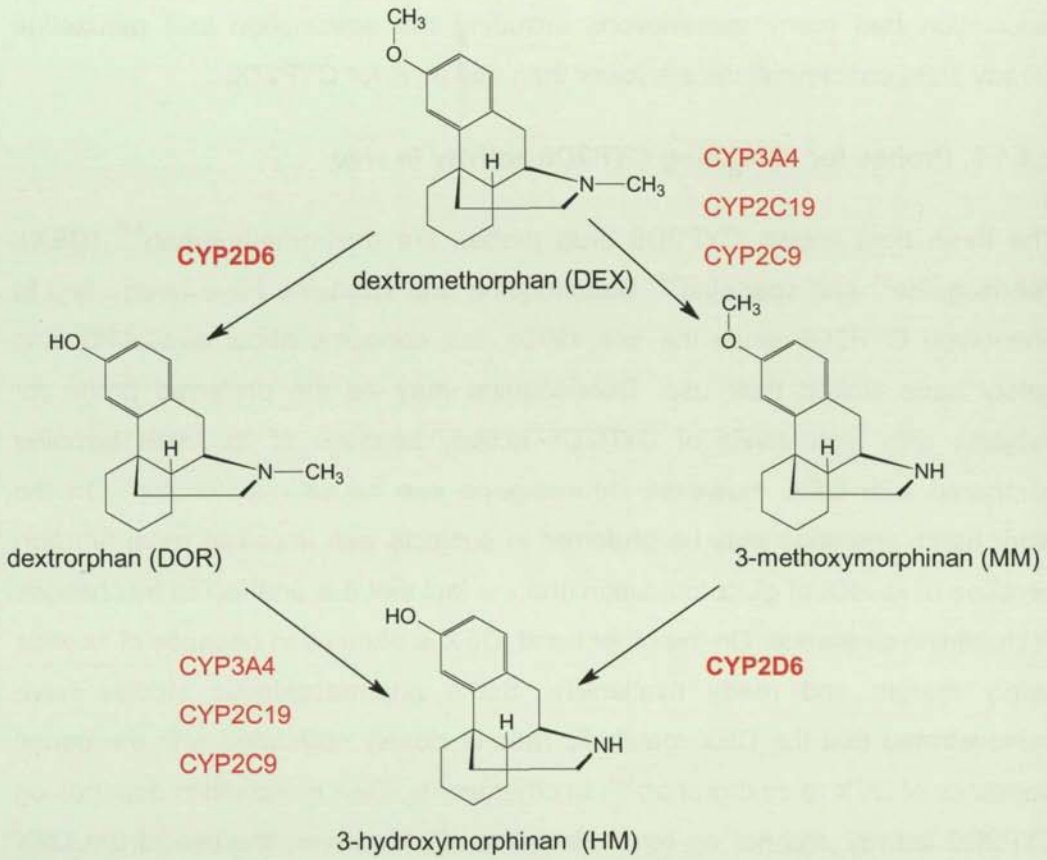


Figure 1.6. Pathways of dextromethorphan (DEX) metabolism in the human.

DEX is a synthetic opioid used as an antitussive. It is primarily metabolised by CYP2D6 forming dextrorphan (DOR, Figure 1.6). It is also metabolised by CYP3A4 forming 3-methoxymorphinan (MM). DEX is metabolised to a lesser extent by CYP3A4 producing 3-hydroxymorphinan (HM), which is also the metabolic product of MM. *In vitro* studies from *Baculovirus* expression systems suggest that the O-demethylation of dextromethorphan is carried out predominantly by CYP2D6, with minor contributions from CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2C9 and CYP3A4[147-150]. During its use as a probe DEX is usually administered at a sub-clinical dose. DEX and its metabolites are primarily found in human plasma and urine as glucuronide conjugates. Following

a dose of 30 mg p.o. dextromethorphan hydrobromide to CYP2D6 EM, total plasma concentrations of DEX reach a maximum of approximately 2.0 μg/L at 2.4 h, displaying a t½e of 2.4 h[141]. DOR is the main metabolite found in plasma reaching maximum concentrations of approximately 400 μg/L, followed by HM with a C_{max} of 104 μg/L, with MM being barely detectable in CYP2D6 EM subjects. In CYP2D6 PM subjects, pharmacokinetics of DEX is quite different, with DEX being the main compound found, reaching a C_{max} of 23.0 μg/L, followed by DOR with a C_{max} of 9.1 μg/L, HM with a C_{max} of 4.6 μg/L and MM with a C_{max} of 2.5 μg/L. Following the administration of the CYP2D6 inhibitor, quinidine, plasma concentrations of DEX in EM subjects are comparable to those of PM subjects. Concentrations of DEX and its metabolites in urine reflect those in plasma with 27% and 16% of the dose recovered as DOR and HM in CYP2D6 EM, respectively. In PM, 26% of the dose is recovered as DEX[141]. The urinary MR of DEX/DOR has been preferred to plasma concentrations when probing for CYP2D6 activity because it is less invasive. Various time intervals have been used for urine collection from 0-4 h up to 24 h. The standard urine collection has been from 0 to 8 h since the test can be completed in one day and the majority of the dose would be excreted by an EM individual within this interval. The antimode for PM phenotype assignment following a dose of DEX is 0.3. In a study of 229 individuals of mixed decent in the U.S. population the mean DEX MR ranged from 0.001 to 5.980. Within the subpopulation of EM the MR differed some 3000 fold, with two individuals classed genotypically as CYP2D6 EM having a value greater than the antimode[151]. Although the DEX MR was able to distinguish EM from PM well, it was not able to distinguish between those individuals with decreased activity *CYP2D6* alleles (IM) and those who possess *CYP2D6* gene duplications (UM).

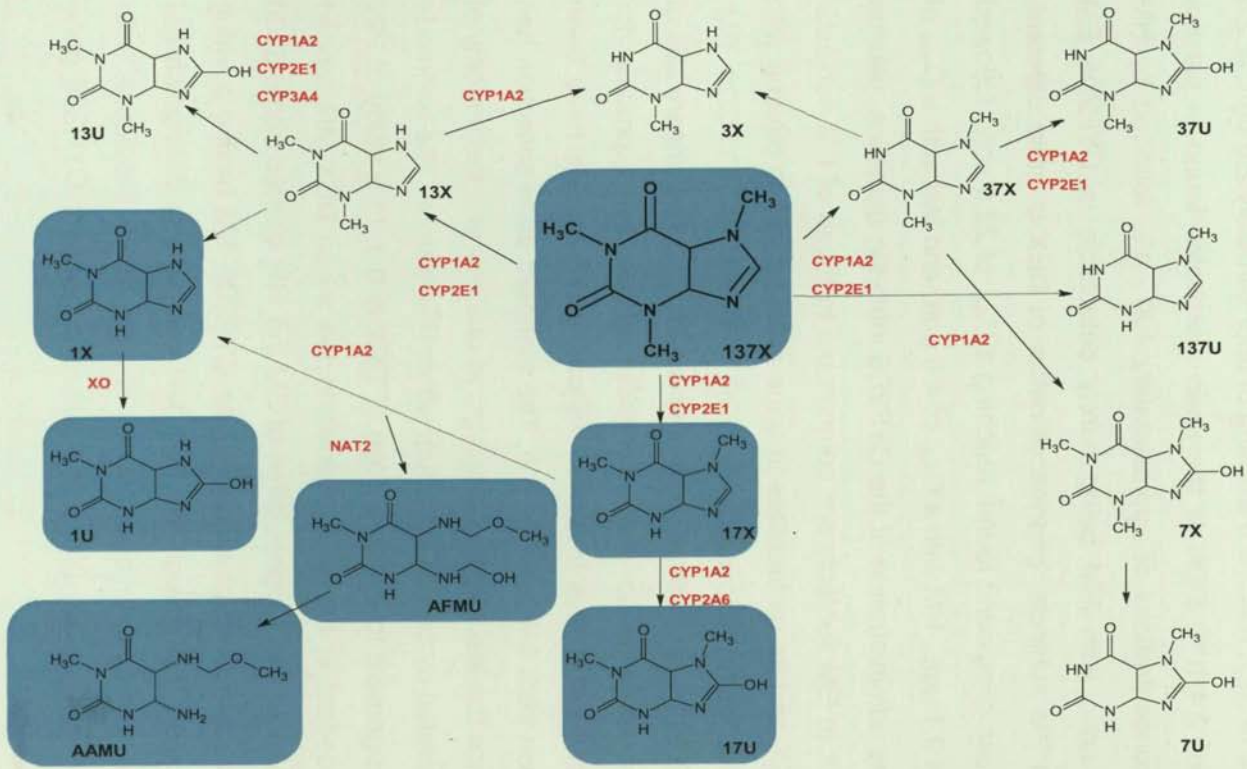


Figure 1.7. Principle pathways of caffeine metabolism in the human. Compounds frequently used in urinary metabolic ratios are highlighted in blue. 137X = 1,3,7-trimethylxanthine (caffeine); 17X = 1,7-dimethylxanthine (paraxanthine); 17U = 1,7-dimethyluric acid; 1X = 1-methylxanthine; 1U = 1-methyluric acid; AAMU = 5-acetylamino-6-amino-3-methyluracil; AFMU = 5-Acetylamino-6-formylamino-3-methyluracil; 13X = 1,3-dimethylxanthine (theophyline); 37X = 3,7-dimethylxanthine (theobromine); 3X = 3-methylxanthine; 13U = 1,3-dimethyluric acid; 37U = 3,7-dimethyluric acid; 137U = 1,3,7-trimethyluric acid; 7X = 7-methylxanthine; 7U = 7-methyluric acid.

1.4.2. CYP1A2 activity and methylenedioxy compounds

As mentioned previously, CYP1A2 is involved in the activation of environmental procarcinogens, such as arylamines, heterocyclic amines, and aflatoxin B1. CYP1A2 activity may be considerably increased by xenobiotics, including cigarette smoke[56]. Accordingly, as the expression levels of the enzyme increases, so does the metabolism of certain procarcinogens to DNA-reactive products, leading to a higher risk of cancer development. On the other hand, inhibition of CYP1A2 by quinolone antibiotics[152], serotonin reuptake inhibitors[153], and oral contraceptives[154] may produce adverse reactions when these agents are administered concomitantly. The MBI caused by MDMA is not an exclusive property of this compound. Indeed, the inhibitory properties methylenedioxy compounds have been exploited for some years by using them as pesticide synergists. These compounds are not pesticides in themselves, their purpose being to block the metabolism of a pesticide and so increase its strength. Methylenedioxy compounds are also found as aromatic constituents of oils and spices. As well as inhibiting many cytochrome P450's, methylenedioxy compounds have also been known to induce their activity, including that of CYP1A2[155]. The mechanism of induction is thought to follow a binding of the compound to the aryl hydrocarbon (Ah) receptor which accumulates in the cell nucleus as a heterodimeric complex. The nuclear Ah receptor complex then acts as a ligand-induced transcription factor which binds to genomic dioxin/xenobiotic responsive elements (DREs/XREs) located in the 5'-regulatory region upstream from the initiation start site and this interaction results in activation of gene transcription and an increase in protein expression. On the other hand, evidence suggesting that rat CYP1A1 regulation is modulated by a protein kinase C suggested that a phosphorylation cascade mechanism that activates the Ah receptor may be involved[135]. Indeed, MDMA has been shown to activate protein kinase C within synaptic terminals[156]. Hence, there is reason to hypothesise that

CYP1A2 could be induced by MDMA. This would have consequences for both MDMA pharmacokinetics and toxicity and that of other CYP1A2 substrates.

1.4.2.1. The measurement of CYP1A2 activity *in vivo*

Caffeine is well known as a metabolic probe for CYP1A2[157] and it has a high margin of safety. It has been used extensively in the past to investigate gender and age differences in CYP1A2 activity[158,159] and changes in activity due to medication, smoking and diet[56,152,160]. Following ingestion caffeine is essentially completely absorbed from the gastrointestinal tract. Caffeine is efficiently eliminated, with a mean systemic clearance of approximately 9 L/min and mean terminal half-life of 4 h in healthy adults[161]. Hepatic metabolism is the dominant elimination mechanism, with less than 5% of an ingested dose being eliminated unchanged in urine. Caffeine biotransformation is complex, and at least 17 urinary metabolites can be detected following its consumption. The principle metabolic pathways of caffeine are shown in Figure 1.7. Although CYP2E1, CYP2A6 and CYP3A4 are involved in caffeine metabolism, 90% of its clearance is thought to be controlled by CYP1A2. Its clearance in plasma is considered the "gold standard" for the measurement of CYP1A2 activity in humans. At least six different urinary metabolic ratios for caffeine have been proposed as *in vivo* probes for CYP1A2 activity (Table 1.4.1) and of these the ratio (AAMU+1X+1U)/17U seems to be the most robust seeing as they are the most sensitive to changes in CYP1A2 activity and not affected as much by other enzyme activities or urine flow[157]. The combination of both caffeine and DEX to evaluate the contribution of several metabolic enzymes to the metabolic disposition of a third drug has already been applied successfully[162].

Ratio	Components	Reference
1	17X/137X	[163]
2	(17U+17X)/137X	[164]
3	(AFMU+1X+1U)/17X	[165]
4	(AFMU+1X+1U)/17U	[154]
5	(AFMU+1X+1U+17X)/137X	[166]
6	(13X+17X+37X)/137X	[167]

Table 1.4.1 Urinary metabolic ratios of caffeine and metabolites used to probe CYP1A2 activity *in vivo*. See Figure 1.7 for abbreviations.

2. OBJECTIVES

To investigate the role of MDMA metabolism and its consequences for MDMA acute and long-term toxicity a series of experiments in animals and humans was carried out. Due to differences in human an animal CYPs the MBI of CYP2D6 and contribution of CYP1A2 was investigated in a controlled clinical setting and conversely, due to ethical questions arising from physiological and pharmacological manipulations of MDMA metabolism, the former studies were carried out in Wistar rats.

Consequently a series of experiments were designed with the following objectives to test the following hypotheses:

Hypothesis I

MDMA affects the activities of the cytochrome P450 enzymes responsible for its phase I metabolism and this has consequences for the acute and long-term toxicities of the drug.

1) Evaluation of the duration of CYP2D6 induced inhibition in humans by MDMA by using the probe drug dextromethorphan (DEX) in humans.

2) Calculation of the *in vivo* turnover half-life for CYP2D6 in humans.

3) Investigation the role of CYP1A2 in the metabolism of MDMA in humans by using the probe drug caffeine.

Hypothesis II

Interindividual differences in the activity of catechol-o-methyltransferase (COMT) are relevant in the development of long-term toxicity of the drug.

4) Inhibition of MDMA phase II metabolism in the rat using the COMT inhibitor, entacapone and investigation of its neurotoxic consequences.

Hypothesis III

MDMA systemic metabolism and not MDMA-induced hyperthermia is the limiting step in the development of long-term neurotoxicity.

5) The administration of single and multiple doses of MDMA at various temperatures in rats.

6) The study of the effect of direct application of MDMA into the striatum or entacapone into the hippocampus in combination with a toxic dosage regimen of MDMA.

Hypothesis IV

MDMA peripheral metabolism and not the formation of free radicals from tyrosine/dopamine is the limiting step in the development of long-term serotonergic changes.

7) Administration of a toxic dose of MDMA or a non-toxic dose of MDMA in rats and measurement of tyrosine in plasma.

8) The effect of direct application of MDMA into the striatum in combination with an injection of tyrosine.

3. METHODS

3.1. Clinical trial design

3.1.1. Recruitment of the subjects

3.1.1.1. Study sample size and selection

Not knowing the exact effects of the interactions between MDMA and DEX, it was difficult to calculate the sample size of the study, for this reason a pilot study was planned. Based on the methodology of bioequivalence studies, with a risk of alpha value 0.05, power of 80%, variability of 30% and a possible increase in the plasma concentrations of DEX in the presence of MDMA of at least 40%, it seemed sufficient to include at least 10 subjects in the final study. The possibility of retirement or withdrawal from the study of approximately 10% was also considered and for this reason it was proposed to sample between 12 and 16 subjects (2-4 for the pilot study, 10 for the final and 2 for possible retirement or withdrawal).

The selection of the volunteers was accomplished three weeks before the beginning of the trial. Volunteers were recruited by word of mouth. The subjects were informed as to the characteristics of the study, and they were given an information leaflet. If they decided to participate they signed a consent form. A medical examination was then arranged for them which included medical history, a complete physical exam, psychiatric interview structured according to DSM-IV guidelines (PRISM), a basic analysis (glucose, creatinine, hepatic enzymes and bilirubin, CPK, LDH, uric acid, urea, cholesterol, triglycerides, total protein and albumin, potassium and sodium), haemogram (haemoglobin, red blood cells, leucocytes), coagulation (platelets, PTT and TP), VSG, basic urine analysis (glucose, protein, red blood cells, leucocytes, ketone bodies), serology (Hepatitis B and C, HIV), and 12 lead ECG. The same analysis was carried out at the end of the study. The CYP2D6 phenotype was determination by means of the DEX

test (administration of 30 mg DEX hydrobromide and urine collection during the following eight hours). With the intention of confirming the results from the previous test, blood samples were collected (1 mL) *a posteriori* for DNA extraction and subsequent *CYP2D6* genotyping (DrugMEt®, Jurilab Ltd., Finland).

The clinical information from the study was then required to fulfil the following criteria:

3.1.1.2. Criteria of Inclusion

1. Male volunteers between the ages of 18 and 45.
2. A history or results from a physical exam that demonstrated that volunteers do not present physical or psychiatric problems.
3. The 12 lead ECG, general blood and urine analyses taken before the trial should be normal. Small variations within the limits of the norm were admitted, those that the investigators consider to be without clinical relevance.
4. The weight of the subject will not exceed 15% of the ideal weight which corresponds to the subject's height and was between 50 and 100 kg.
5. The acceptance of trial procedures and signature of the consent form.
6. A history of recreational use of 3,4-methylenedioxymethylamphetamine, having taken "pills" of ecstasy at least 10 times throughout their lives and at least twice in the last year. No presentation of severe adverse effects during the use of MDMA.
7. Normal criteria in the psychiatric review (structured interview according to the DSM-IV).
8. Possession of a phenotype of extensive metabolizer for CYP2D6.

3.1.1.3. Criteria of Exclusion

1. Non-compliance with the criteria of inclusion.

2. History of abuse or dependence on pharmaceutical or other drugs (except nicotine) or habitual consumption of psychoactive substances (with the exception of recreational use of MDMA).
3. Having suffered from any major illness or underwent major surgery in the last three months before the study.
4. History of possession of psychiatric illness or schizophrenia amongst close family members.
5. Alcohol consumption exceeding 4 units or 40 g per day for male subjects and 2 units or 20 g per day for female subjects.
6. Smokers of more than 20 cigarettes per day.
7. Subjects presenting intolerance or adverse reactions to coffee.
8. Regular ingestion of medication in the month preceding the study. Single doses of symptomatic medication were accepted up to the week preceding the trial (it can be expected that the drug would have been completely eliminated on the day of the trial).
9. Having donated blood or participated in clinical trials with drugs in the 4 weeks leading up to the trial.
10. History of allergic or adverse reactions to medication.
11. History of gastrointestinal, hepatic or renal problems or any other that may suspect alterations in the processes of absorption, distribution, metabolism, or excretion of a drug.
12. Subjects that or not capable of understanding the nature and consequences of the trial and the procedures that they being asked to follow.
13. Subjects who are serological positive for Hepatitis B and/or C and/or HIV.

3.1.2. Development of the study

3.1.2.1. Study Preliminaries

Medication

DEX (Romilar®, Roche Farma, SA, 15 mg tablets) was supplied by the pharmacy of the Hospital del Mar, Barcelona, Spain. Caffeine anhydride was prepared by the pharmacy of Hospital del Mar, Barcelona, Spain as identically appearing opaque white, soft, gelatine capsules. (*R,S*)-MDMA was supplied by the Spanish Ministry of Health and prepared by the pharmacy of the Hospital del Mar, Barcelona, Spain as identically appearing opaque, white, soft gelatine capsules. Capsules also contained lactose. As an internal validation the contents of the MDMA capsules prepared by the pharmacy of the Hospital del Mar were checked for concentration and purity. Capsules were analysed according to a modified method following two previously published protocols (Military standard 105D, UNE 66020; Recommended methods for testing Illicit Ring-Substituted amphetamine deriviatives, UN, 1987). Briefly, the contents of a capsule of MDMA were weighed and samples of approximately 10 mg were dissolved in 10 mL ultra pure H_2O (MilliQ). The solution was the agitated and sonicated until full dissolution. An aliquot of 1 mL was taken and 100 µL 2M NaOH was added followed by 10 mL dichloromethane. Samples were agitated for 30 min and centrifuged at 3500 rpm for 5 min. The aqueous phase was then removed and 50 µl of methanol:HCl (9:1, v/v) was added. Samples were evaporated at 30°C under a nitrogen stream and reconstituted in 10 mL methanol (solution A). An aliquot of 1 mL solution A was evaporated at 30°C under a nitrogen stream and reconstituted in 1 mL 0.1 M H_2SO_4. Concentrations of MDMA were calculated by UV spectroscopy using an extinction coefficient of 189.7 with maximum absorbance of 284 nm, comparing against a standard solution of MDMA treated with the same procedure. The purity of the sample was checked by an aliquoting

1 mL solution A into a glass vial and adding 50 µl methylephedrine as internal standard. 3 µl were then injected into a GC-NPD system and compared against a standard solution 10 µg/mL MDMA. No other amphetamines or impurities were found in the capsules apart from lactose. Results of the quantification analysis are shown in table 3.1.1.

Design

The clinical trial conformed to the declaration of Helsinki (2000), the recommendations of the OMS, the deontological code and the derivates of Spanish laws concerning clinical trials (Ley de Medicamiento 25/1990, Real Decreto 561/1993). The protocol of the clinical trial (IMIMFTCL/MDMA/6) was submitted and approved by the institutional review board of the IMAS group (CEIC-IMAS) and authorized by the Dirección General de Farmacia y Productos Sanitarios (98/112) of the Spanish Ministry of Health (No. aut. AEMPS 04-0013). Furthermore, as part of a project studying the role of metabolism in the toxicity of MDMA, the protocol was submitted to the National Institute of Health in the United States for funding. The project was approved and funding was granted ("Drug metabolism and pharmacogenetics as contributing factors to MDMA-induced toxicity in humans." Reg. No. 1 R01 DA017987. 01/08/2005).

Capsule sampled (mg)	Weight (mg)	MDMA.HCl (mg)	Precision (%)	Accuracy (%)
100	510.1	95.2	4.8	6.1
S.D.	*42.6*	*5.8*		
90	522.1	82.6	8.3	5.0
S.D.	*21.9*	*4.1*		
80	518.2	75.5	5.7	4.9
S.D.	*6.8*	*3.7*		
75	600.1	68.2	9.1	6.6
S.D.	*32.8*	*4.5*		

Table 3.1.1 Analysis of MDMA capsule content

The design of the study was open since the principal variable is objective (concentrations of drugs and their respective metabolites). The order of the sessions was not randomised since it was assumed that the quantity of both enzymes does not vary in the same subject with time and for this reason was not necessary.

Subjects were requested to refrain from consuming any drug two weeks before and throughout the duration of the study and asked to follow a xanthine free diet 48 h before the beginning of each session. At each session and before drug administration, urine samples were collected to check for the use of drugs of abuse (opiates, cocaine metabolite, amphetamines, and cannabinoids) by immunological methods (FPIA, Abbott Laboratories, Chicago, IL, USA). Before beginning the study sessions, the subjects were familiarised with the subjective evaluation tests and the questionnaires concerning subjective effects.

Any subjects who presented adverse reactions to the treatment that represented a danger to their health, in the judgement of the investigator; was retired from the study. Those who demonstrated a lack of cooperation or who transgress from the

norms of the study were also retired. The participants could retire from the study at any moment they wished; in this case it was planned that new subjects could substitute those abandoned or retired so that the preferred number of inclusions was obtained for each group treated. Only the results of the volunteers who concluded the study were considered.

If any subject was taking any other regular medication then a minimum of 30 days was allowed to elapse following the termination of the medication before the subject could enter the trial. Single doses of symptomatic medication were accepted up to one week before the trial (it was expected that the ingested drug was eliminated by day 1 of the trial).

During the trial additional medication was not permitted. It could only be taken following the prescription of the principal medical investigator or his/her collaborators (dose, time and cause were noted in a corresponding individual questionnaire).

3.1.2.2. Pilot phase of study

A pilot study was conducted in which a determined single dose of caffeine and numerous determined doses of DEX were administered following a dose of MDMA. The study was conducted with 3 healthy male volunteers and served to profile the dose of each drug with regards to the intervals of administration between them.

The design of the study is shown in Figure 3.1. The volunteers participated in 2 sessions, separated by a minimum of 3 days. At the beginning of each session, subjects arrived at the laboratory at 07.30 h following an overnight fast. An indwelling intravenous catheter was inserted into a subcutaneous vein in the forearm of the non-dominant arm and 0.9% sodium chloride solution was infused at a rate of 20 mL/h. Thereafter, they remained seated in a quiet room throughout

the session. Drugs were administered at 8:30 AM. A light meal was provided 6 h after drug administration. In the first session all volunteers received a single 30 mg dose of DEX and a single 100 mg dose of caffeine together with 250 mL of water. With respect to the safety of DEX, the recommended dose for the treatment of cough in adults is 15-30 mg for a maximum of 4 times daily. Hence, the dose administered in this study was less than those used to treat acute cough and has been used in the past to probe CYP2D6 activity. The dose of caffeine was equivalent to an intake of 1-1.5 cups of coffee and was not expected to cause any adverse effects. Urine was collected for 0-8 h and its pH was measured and subsequently acidified and aliquots were stored at –20°C until further analysis. Blood samples of 8 mL were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 25 h after DEX and caffeine administration. After centrifugation at 4°C, four 1 mL aliquots of plasma were stored at -20°C for further analysis. Tobacco smoking was permitted 6 h after drug administration.This session served to determine basal activities of CYP2D6 and CYP1A2.

From the previous experiences with MDMA at single doses (from 50 to 150 mg), in combination with ethanol (100 mg), multiple doses (50 to 100 mg), interaction with paroxetine (100 mg), a single dose of 100 mg clearly inhibits the metabolism of a subsequent dose of MDMA between 2 and 24 h. A dose of 100 mg produces

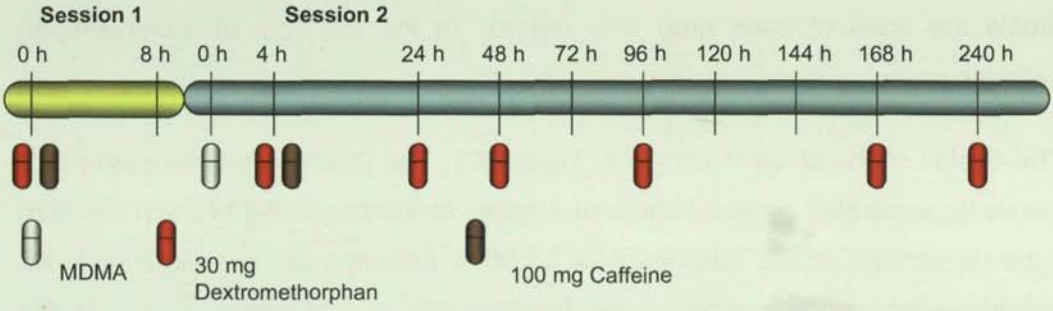


Figure 3.1. Scheme of the clinical trial to measure the duration of inhibition of CYP2D6 by MDMA showing dose intervals.

evident physiological and subjective effects in male subjects between the weights 63 and 82 kg and is not accompanied by relevant adverse effects and has not caused health and safety problems in a laboratory setting. It was proposed to administer a single dose of 1.5 mg/kg (maximum dose 100 mg). In this manner, a participant weighing 50 kg received 75 mg, those weighing 60 kg received 90 mg and finally those who weigh 66.7 kg or more received a dose of 100 mg. Thus, in the second session the subjects received a single dose of MDMA together with 250 mL of water and a single dose of caffeine four hours later. Repetitive oral doses of 30 mg DEX were administered at fixed intervals to investigate the intensity and duration of CYP2D6 inhibition. With respect to the interval between the doses of DEX, it was initially proposed that in the pilot study, the administration of DEX should be 30 mg at 4 h, 24 h, 48 h, 96 h and 168 h after the dose of MDMA. Urine samples were collected from 0-4 h, 4-12 h, 24-32 h, 48-56 h, 96-104 h, 168-176 h following the administration of MDMA. Blood samples of 8 mL were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 25 h after DEX and caffeine administration. After centrifugation at 4°C, four 1 mL aliquots of plasma were stored at -20°C for further analysis. Tobacco smoking was permitted 6 h after drug administration.

Upon completion of the pilot study it was observed that the MR following the final dose of DEX (168 h) had not recovered to basal levels. Therefore, the final study was modified to include a dose of DEX at 240 h following MDMA administration. Apart from this minor change, no other changes in dose or interval of administration were affected and the results from the pilot study were added to the final results.

3.1.2.3. Final phase of study

12 healthy male volunteers were selected. The volunteers participated in 2 sessions separated by a minimum of 3 days. The development of the sessions

was the same as the pilot trial with the exception that DEX was administered at 4 h, 24 h, 96 h, 168 h and 240 h after the dose of MDMA. The demographics and history of drug use of the male volunteers that participated in both phases of the trial are shown in table 3.1.2. Genotype and DEX urinary MR are shown in table 3.1.3.

Study	Code	Age (years)	Weight (kg)	B.M.I.	Height (cm)	Tabacco	GHB	BDZ	MDMA	AL	CA	CO	Others
pilot	1	28	83,5	23,1	190,0	CU	CU	PU	CU	CU	CU	CU	PU
pilot	2	31	89,0	28,7	176,0	N	CU	N	CU	CU	PU	CU	PU
pilot	3	28	73,5	21,9	183,0	CU	N	N	CU	CU	CU	CU	CU/PU
final	11	22	65,7	22,6	170,5	CU	N	N	CU	CU	CU	CU	PU
final	12	19	54,2	18,6	170,5	CU	N	N	CU	CU	CU	PU	CU/PU
final	13	23	69,0	18,3	194,0	CU	N	N	CU	CU	CU	CU	PU
final	14	22	91,2	27,6	181,5	PU	N	N	CU	CU	CU	CU	CU/PU
final	15	26	59,5	17,2	185,5	CU	N	N	CU	CU	CU	CU	CU
final	16	27	73,0	19,2	195,5	CU	CU	PU	CU	CU	CU	PU	CU/PU
final	18	33	78,6	23,6	182,5	CU	PU	N	CU	CU	CU	CU	PU
final	20	27	69,6	20,5	184,0	CU	CU	N	CU	CU	CU	CU	CU/PU
final	21	24	67,3	20,8	180,0	PU	N	N	CU	CU	CU	CU	CU/PU
final	22	22	67,8	22,2	174,5	CU	N	N	CU	CU	CU	CU	PU
final	23	29	59,0	20,2	171,0	N	N	PU	CU	CU	CU	CU	CU/PU
final	24	26	62,2	19,7	177,4	CU	N	N	CU	CU	CU	CU	CU/PU
Mean		25,8	70,9	21,6	181,1	CU	N	N	CU	CU	CU	CU	CU
S.D.		3,8	10,9	3,2	8,0								
Max.		33,0	91,2	28,7	195,5								
Min.		19,0	54,2	17,2	170,5								

Table 3.1.2 Demographics of male volunteers that participated in the study. B.M.I = Body mass index; GHB = γ-hydroxybutiric acid; BDZ = benzodiazepines; AL = Alcohol; CA = Cannabis; CO = Cocaine; Others = LSD, psychoactive mushrooms; CU = current use; N = no use; PU = previous use.

Code	MR (DEX/DOR)	*CYP2D6*	*CYP3A5*	*CYP2C9*	*CYP2C19*	*CYP2B6*	*COMT*
1	0,0007	*1/*1	*3/*3	*1/*2	*1/*1	*1/*4	val/val
2	0,0062	*1/*17	*3/*3	*1/*2	*1/*1	*1/*1	met/met
3	0,0193	*1/*4	*3/*3	*1/*3	*1/*1	*1/*1	val/met
11	0,0268	*1/*4	*1/*3	*1/*1	*1/*2	*1/*5	val/val
12	0,0163	*1/*10	*3/*3	*1/*1	*1/*2	*1/*4	val/met
13	0,0018	*1/*2	*3/*3	*1/*1	*1/*1	*1/*1	met/met
14	0,0115	*1/*9	*3/*3	*2/*3	*1/*1	*1/*4	val/met
15	0,0013	*2/*41	*3/*3	*1/*1	*1/*1	*1/*5	val/met
16	0,0168	*1/*2	*3/*3	*1/*2	*1/*1	*1/*1	met/met
18	0,0265	*9/*10	*3/*3	*1/*2	*1/*1	*1/*1	val/met
20	0,0022	*2/*4	*3/*3	*1/*1	*1/*2	*1/*5	val/met
21	0,0034	*1/*2	*3/*3	*1/*3	*1/*1	*1/*5	met/met
22	0,0124	*1/*2	*3/*3	*1/*2	*1/*1	*1/*5	val/val
23	0,0039	*1/*2	*1/*3	*1/*1	*1/*1	*1/*1	val/met
24	0,0010	*1/*5	*3/*3	*1/*1	*1/*1	*1/*1	val/met

Table 3.1.3 DEX phenotype and genotype of cytochrome P450 enzymes involved in MDMA and DEX metabolism of male volunteers who participated in the study.

3.2. Validation of analytical methods

In preparation for the analysis of the samples generated from the clinical trial, methods for the quantification of the markers of CYP activity were validated. A calibration curve was chosen which represented the expected concentrations range of each analyte. Each analytical procedure was validated following the conduction of four assays (table 3.2.1). The first assay was conducted to calculate the limits of detection, limits of quantification, recovery as well as the linearity of the calibration curve. The second was carried out to monitor the stability of freeze/thaw actions on the analyte. The third monitored contamination between samples and the fourth checked for stability in case of dilution of the sample. The four assays were then compared and inter assay precision and accuracy was calculated.

Type	Level	1st assay		2nd assay			3rd assay	4th assay	
		M	W	M			M	M	
				F/T 0	F/T 1	F/T 2		V	V_r
Control	0	4 (2is)	2	2			5	2	
Calibrator	1	4	4	2			2	2	
Control	CI			3	3	3	3	3	
Calibrator	2	4		2			2	2	
Control	CM			3	3	3	3	3	
Calibrator	3	4		2			2	2	
Calibrator	4								
Control	CS			3	3	3	3	3	3
Calibrator	5	4	4	2			2	2	
Total		24	14	21	9	9	24	21	3
Total per assay		38		39			24	24	

Table 3.2.1 Scheme of validation procedure for quantification of substances in biological matrices. CI = inferior control; CM = medium control; CS = superior control; M = Matrix; W = Without Matrix; F/T = Freeze/Thaw cycles; V = volume; V_r = reduced volume, assay volume divided by 2.

3.2.1. Validation of the quantification of DEX and metabolites in plasma and urine

Standard solutions of 1000 mg/L DEX and metabolites and internal standard (levalorphan) were prepared in methanol. Serial dilutions were then made of each standard solution for final concentrations of 100, 10 and 1 mg/L. Concentrations of standard solutions were confirmed by UV spectroscopy. The concentrations of the calibrators for each analyte were 40, 250, 500, 800 and 2000 µg/L for DEX, DOR, MM and HM in urine and 10, 50, 100, 200 and 300 µg/L for DEX, DOR, MM and HM in plasma. Controls had a concentration of 60, 400 and 1000 µg/L in urine and 20, 125 and 250 µg/L in plasma.

Aliquots of urine and plasma were analyzed by HPLC/FLD using levalorphan as an internal standard. Samples were hydrolyzed for 3 h at 55°C or overnight at 37°C using 1 mL β-glucuronidase solution (Type H-1 from *Helix pomatia*) in 0.1 M sodium citrate (pH 5.0). Extraction was carried out after the addition of 1 mL (NH_4Cl/NH_3 (pH 9.5) on mixed-mode solid reversed phase columns (Bond Elut Certify™, Varian, Palo Alto, CA, USA) pre-conditioned with 2 mL methanol and 2 mL H_2O. Columns were then washed with 2 mL H_2O (MilliQ, ultrapure), 1 mL 1 M acetic acid and 2 mL methanol. Columns were dried for 10 min at room temperature and analytes are eluted with 2 mL chloroform:isopropanol 80:20, ammonium hydroxide 2%. The eluent was dried at 40°C under a nitrogen stream for 20 min (Turbovap®, Zymark®, Kopkinton, MA, USA). Samples were reconstituted in 100 µL mobile phase and 15 µL (urine) and 25 µL (plasma) were injected into the chromatographic system.

Chromatographic separation was carried out on an Ultrasphere ODS (4.6 cm x 7.5 cm x 3 µm) (Beckman Coulter ™, Palo Alto, USA) connected to a 1090 II high performance liquid chromatograph (Hewlett Packard, Palo Alto, CA) coupled to a fluorescent detector model 1100 at excitation and emission wavelengths of 280 and 310 nm, respectively. The mobile phase used was acetonitrile: 22 mM sodium acetate buffer, 0.05% TEA (pH 3.3) (20:80, v/v) applied as an isocratic gradient with a flow rate of 1 mL/min for a total run of 21 min.

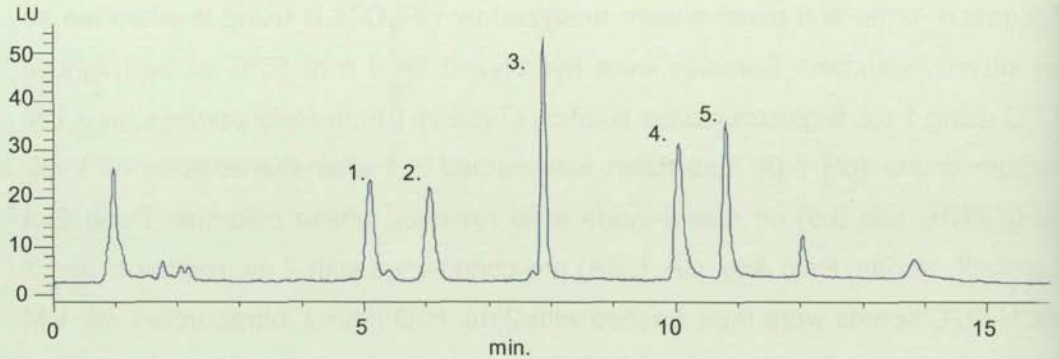


Figure 3.2. Chromatogram of dextromethorphan and metabolites analysis by HPLC/FLD. 1. = dextrorphan; 2. = hydroxymorphinan; 3. = levalorphan (internal standard); 4. = dextromethorphan; 5. = methoxymorphinan.

A chromatogram showing the retention times of DEX and metabolites is shown in Figure 3.2. Calibration curves for the HPLC method were linear over the chosen concentration ranges for DEX, DOR, MM and HM. Peak ratios between compounds and internal standard were used for calculations. A weighted (1/concentration) least square regression analysis was used (SPSS for Windows, version 12.0). Recovery was >70% for all compounds. Mean determination coefficients were 0.994 ± 0.002 for DEX, 0.991 ± 0.002 for DOR, 0.992 ± 0.002 for MM, and 0.991 ± 0.001 for HM. In urine, the inter-assay precision and accuracy were 11.4% and 10.1% for DEX, 9.1% and 8.5% for DOR, 11.9% and 12.3% for MM and 12.0 and 9.0% for HM with limits of detection of 13 μg/L, 7 μg/L, 12 μg/L and 13 μg/L for DEX, DOR, MM and HM, respectively. In plasma, the inter-assay precision and accuracy was 14.6% and 12.3% for DEX, 12.0% and 9.5% for DOR, 15.0% and 12.6% for MM and 10.2% and 11.8% for HM with limits of detection of 1.5 μg/L, 2.5 μg/L, 1.5 μg/L and 4.0 μg/L for DEX, DOR, MM and HM respectively. No cross-contamination of samples was observed in the chromatograms of various blank samples. The assay was stable to a dilution factor of two and up to two freeze and thaw actions for all compounds in both plasma and urine.

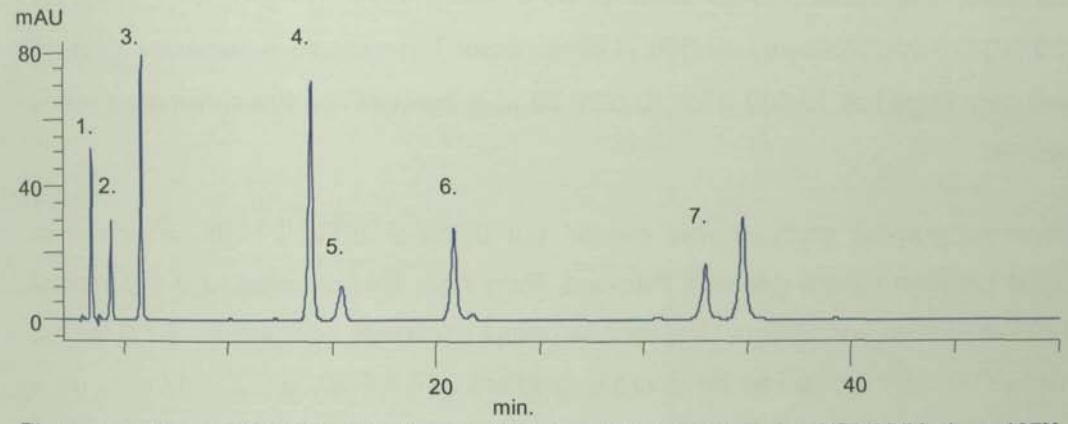


Figure 3.3 Chromatogram of caffeine and metabolites analysis by HPLC/UV. 1. = 137X (caffeine); 2. = 17X (paraxanthine); 3. = 1X (1-methyl xanthine); 4. = 17U (1,7-dimethyluric acid); 5. = AAMU (5-acetylamino-6-amino-3-methyluracil); 6 = 1U (1-methyluric acid); 7 = 9U (9-methyluric acid, internal standard).

3.2.2. Validation of the quantification of caffeine and metabolites in urine

Standard solutions of 1000 mg/L of caffeine (137X), paraxanthine (17X), 1-methylxathine (1X), 1-methyluric acid (1U), 1,7-methyluric acid (17U), 5-acetylamino-6-amino-3-methyluracil (AAMU) and internal standard, 9-methyluric acid (9U) were prepared in water or 10 mM sodium hydroxide. Serial dilutions were then made of each standard solution for final concentrations of 100, 10 and 1 mg/L. Concentrations of standard solutions were checked by UV spectroscopy. The concentrations of the calibrators for each analyte were 2, 10, 20, 30 and 60 mg/L for 137X, 17X, 1X, 1U, 17U and AAMU. Controls had a concentration of 5, 15 and 40 mg/L.

Caffeine and metabolites were analysed in urine by a modified procedure previously published[168]. Briefly, 30 µl of 0.5M NaOH is added to 150 µl aliquots of urine and vortexed. The solution is left at RT for 30 min to oxidise AFMU to AAMU and then 40 µl of 0.5 M HCl is then added followed by 50 µL of internal

standard. The solution is vortexed for 30 s. 100 µl dimethylfluorene:ethylacetate (30:70) is added followed by 800 µl acetonitrile. The mixture is vortexed for 30 s and centrifuged at 10,000 g for 10 min. 25 µl is injected into the chromatographic system.

Chromatographic analysis was carried out using a 1090 II high performance liquid chromatograph (Hewlett Packard, Palo Alto, CA) coupled to a UV model 1100 at 260nm and 280nm. The mobile phase used was acetonitrile: 0.7% formic acid (91:9, v/v) applied as an isocratic gradient with a flow rate of 1 mL/min for a run of 62 min. The HPLC column used was a Grom-sil 120 (4.6cm x 25cm x 5 µm) (GROMAnalytik, Rottenburg-Hailfingen, Germany).

A chromatogram showing the retention times of 137X and metabolites is shown in Figure 3.3. Calibration curves for the HPLC method were linear over the chosen concentration ranges for 137X, 17X, 1X, 1U, 17U and AAMU. Peak ratios between compounds and internal standard were used for calculations. A weighted (1/concentration) least square regression analysis was used (SPSS for Windows, version 12.0). Since the procedure was extractionless recovery was considered to be 100% for all compounds. Peak ratios between compounds and internal standard were used for calculations. Mean determination coefficients were 0.999 ± 0.001 for 137X, 0.999 ± 0.001 for 17X, 0.998 ± 0.001 for 1X, 0.998 ± 0.001 for 17U, 0.999 ± 0.002 for AAMU and 0.998 ± 0.002 for 1U. Limits of detection and quantification as well as inter-assay precision and accuracy are shown in table 3.2.2. No contamination of samples was observed in the chromatograms of various blank samples. The assay was stable to a dilution factor of two and up to two freeze and thaw actions for all compounds in both plasma and urine.

Analyte	Detection Limit (mg/L)	Quantification Limit (mg/L)	Inter-assay	
			Precision (RSD,%)*	Accuracy (%)
137X	0.3	0.9	1.9	3.3
17X	1.3	4.0	1.7	4.6
1X	0.7	2.2	6.7	7.4
17U	1.1	3.2	5.7	7.3
AAMU	0.4	1.3	6.1	10.3
1U	1.6	4.9	6.1	5.2

Table 3.2.2 Limits of detection and quantification for caffeine and metabolites in urine. 137X = 1,3,7-trimethylxanthine (caffeine); 17X = 1,7-dimethylxanthine (paraxanthine); 1X = 1-methylxanthine; 17U = 1,7-dimethyluric acid; AAMU = 5-acetylamino-6-amino-3-methyluracil; 1U = 1-methyluric acid. * Relative Standard Deviation of the control samples.

3.2.3. Validation of the quantification of caffeine and paraxanthine in plasma

Standard solutions of 1000 mg/L of caffeine (137X) and paraxanthine (17X) were prepared in water, and internal standard diphylline in methanol. Serial dilutions were then made of each standard solution for final concentrations of, 100, 10 and 1 mg/L. Concentrations of standard solutions were checked by UV spectroscopy. The concentrations of the calibrators for each analyte were 50, 200, 500, 1000 and 2000 μg/L for 137X and 17X. Controls had a concentration of 100, 600 and 1500 μg/L.

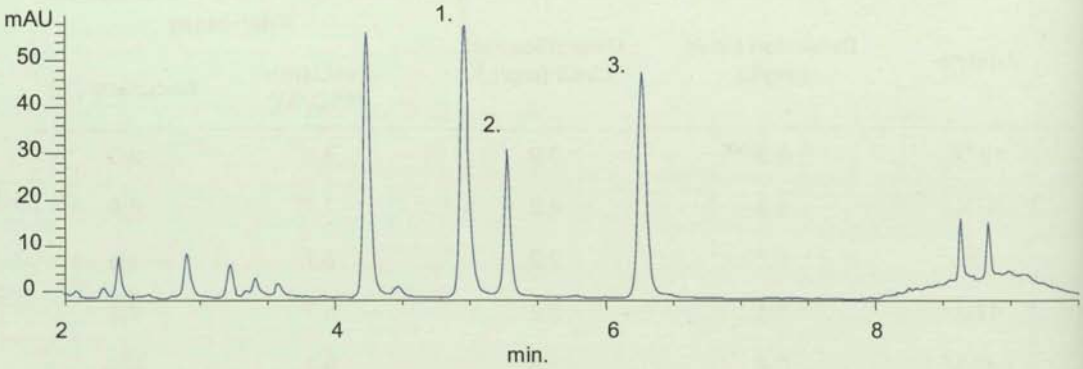


Figure 3.4. Chromatogram of caffeine and paraxanthine analysis by HPLC/UV. 1. = 17X (paraxanthine); 2. = diphyline (internal standard); 3. = 137X (caffeine).

Caffeine and paraxanthine are extracted from 500 mL of plasma using a liquid/liquid extraction procedure by adding 4 mL of ethylacetate and 200 mg of potassium sulphate. The mixture was vortexed for 30 s. Samples were agitated for 20 min and then centrifuged at RT for 5 min. The organic layer was separated and evaporated to dryness at RT at 40ºC using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 100 μl mobile phase and 25 μl was injected into the HPLC system.

Chromatographic analysis was carried out using a 1090 II high performance liquid chromatograph (Hewlett Packard, Palo Alto, CA) coupled to a UV model 1100 at 274 nm. The mobile phase used was acetonitrile: acetic acid (0.05%) (99:1, v/v) applied as an isocratic gradient with a flow rate of 1 mL/min for a run of 15 min. The HPLC column used was an Ultrasphere ODS (4.6cm x 7.5cm x 3 μl) (Beckman Coulter ™, Palo Alto, USA).

A chromatogram showing the retention times of 137X and 17X is shown in figure 3.4. Calibration curves for the HPLC method were linear over the chosen concentration ranges for 137X and 17X. Peak ratios between compounds and internal standard were used for calculations. A weighted (1/concentration) least square regression analysis was used (SPSS for Windows, version 12.0).

Recovery was 100% for 137X and 93% for 17X. Mean determination coefficients were 0.994 ± 0.003 for 137X and 0.997 ± 0.001 for 17X. Limits of detection were 3 μg/L and 12 μg/L for 137X and 17X respectively. Inter-assay precision and accuracy were 10.0% and 7.8% for 137X and 4.2% and 5.9% for 17X. No contamination of samples was observed in the chromatograms of various blank samples. The assay was stable to a dilution factor of two and up to two freeze and thaw actions for all compounds in both plasma and urine.

3.3. Animal Studies

3.3.1. Animals and MDMA treatments

In all cases, the doses of MDMA used refer to the hydrochloride salt. All the procedures followed in the present work were in compliance with the European Community Council Directive (86/609/EEC) and were approved by an Ethical Committee.

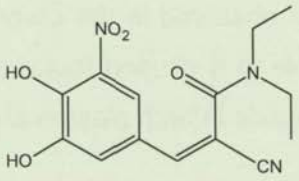
3.3.1.1. COMT inhibition and MDMA-induced hyperthermia and metabolism

Male Wistar rats (290-340 g) were housed in plastic cages in a temperature controlled room (21.5 ± 1° C) with free access to food and water and maintained on a 12/12 h light/dark cycle (lights on at 07.00 h). Three sets of experiments were performed:

1. Saline or the catechol-O-methyltransferase (COMT) inhibitor entacapone (30 mg/kg i.p.) was administered 30 min before MDMA (3 x 5 mg/kg i.p.) in order to prevent the O-methylation of HHMA or HHA. Rats were sacrificed 1 h after MDMA administration for the measurement of MDMA, its metabolites, including HHMA and HHA, and serum concentrations of L-tyrosine. In parallel, a different batch of rats was sacrificed one week after MDMA to measure brain indole content. The dose of entacapone was chosen based on previous findings

indicating that rat liver COMT activity is completely inhibited 30 min after injection and remains reasonably inhibited (~75%) six hours later[169].

entacapone



2. To ascertain the influence of different ambient temperatures on MDMA metabolism and long-term neurotoxicity. Rats were treated intraperitoneally with either saline or MDMA (final dose equivalent to 15 mg/kg) with two different dosing regimens: 1 x 15 mg/kg; or 3 x 5 mg/kg every 2 hours at ambient temperatures of 15, 21.5 or 30 ± 1°C. Trunk blood was withdrawn by saphenous vein puncture to measure plasma concentrations of MDMA and its main metabolites at different time points. Rats were sacrificed at 1 week and brain indole content was measured.

3. The effect of direct application of MDMA into the striatum or entacapone into the hippocampus in combination with a toxic dosage regimen of MDMA (3 x 5 mg/kg i.p., given 2 h apart) was assessed. For this, 12 h before dialysis experiments the obturators were removed from the guide cannulae and a microdialysis probe (CMA/11) was inserted slowly through each cannula into the brain of the awakened rat such that the membrane protruded its full length (4 mm) from the end of the probe. The probes were connected via spring-covered FEP-tubing (CMA, Sweden) to a dual channel swivel (Instech, Plymouth Meeting, PA, USA) that allowed for relatively unrestrained movement of the animal. Probes were perfused overnight with artificial cerebrospinal fluid (aCSF; NaCl: 120 mM; KCl: 1.4 mM; $CaCl_2$: 1.2 mM; $MgCl_2$: 0.83 mM and D-glucose 10 mM pH 7.4) at a flow rate of 1 µl/min. In the following morning, fresh aCSF was perfused at a flow

rate of 2 μl/min for an additional 1.5 h equilibration period. The perfusion medium of the probe of each animal was then switched to one containing MDMA (100 μM) or entacapone (100 μM). At this time point rats also received the first of three injections of saline or MDMA (5 mg/kg i.p.) given every 2 h. The perfusion continued for an additional 5 h in the case of MDMA or 2 h in the case of entacapone. The concentration of MDMA in the perfusate was chosen based upon previous studies showing that the extracellular concentration of MDMA under these experimental conditions is similar to that found after systemic neurotoxic doses of MDMA[100,119,170]. On the other hand, the concentration of entacapone in the perfusate was chosen based upon a previous study showing an effective inhibition of brain COMT activity[171].

Seven days after reverse dialysis experiments were completed, animals were sacrificed by decapitation, their brains were rapidly removed and one 1 mm thick tissue section was taken (approximately 0.5 mm to either side of the probe). Striatal tissue was dissected out from the side where the probe was implanted and 5-HT, dopamine and their respective metabolites were quantified using HPLC-ED.

3.3.1.2. The role of L-tyrosine in the development of MDMA-induced neurotoxicity in rats

Male Wistar rats (290-340 g) were housed in plastic cages in a temperature controlled room (21.5 ± 1° C) with free access to food and water and maintained on a 12/12 h light/dark cycle (lights on at 07.00 h). Three sets of experiments were performed:

1. Rats were treated intraperitoneally with saline, a toxic dose of MDMA (15 mg/kg i.p.) or a non-toxic dose of MDMA (7.5 mg/kg). In this case, hyperthermia, serum tyrosine levels, metabolic disposition of MDMA and long-term 5-HT

depletions were compared among groups. All the parameters analyzed differed between both doses of MDMA. For this reason, using the non-toxic dosing regimen of MDMA different experiments were performed aimed at modifying serum tyrosine levels and/or MDMA metabolic disposition to ascertain the real contribution of both factors in the mechanism underlying MDMA-induced 5-HT neurotoxicity. Accordingly, MDMA was administered alone or in combination with L-tyrosine methylester·HCl (equivalent to 0.2 or 0.5 mM/kg free L-tyrosine). The tyrosine/MDMA combination was given in one sole intraperitoneal injection at a standard ambient temperature (21.5±1°C), or at a low ambient temperature (15±1°C) to reduce the metabolic rate of MDMA. In one experiment rats were also treated with a non-toxic dose of MDMA and had their rectal temperature kept elevated to near that seen in rats given a toxic intraperitoneal injection of MDMA (15 mg/kg). This was achieved by placing the rats in a cage with a homeothermic blanket (Homeothermic System HB121S402, Panlab, Spain) covering the base. The blankets were switched on 2 hours after the administration of the non-toxic dose of MDMA (once the hyperthermia of the animals started to decrease) and were turned off six hours later so that rats were kept hyperthermic for 8 hours. In this experiment, control animals were heated 1 h before saline injection to cause an overall increase in body temperature similar to that found in the MDMA-treated group.

2. The effect of direct application of MDMA into the striatum in combination with an injection of tyrosine (0.5 mmol/kg) was assessed. For this, 12 h before dialysis experiments the obturators were removed from the guide cannulae and a microdialysis probe (membrane length: 4.0 mm x 240 µm; CMA/11, Sweden) was inserted slowly through each guide cannula into the striatum of the awake rat such that the membrane protruded its full length from the end of the probe. The probes were connected via spring-covered FEP-tubing (CMA, Sweden) to a dual channel swivel (Instech, Plymouth Meeting, PA, USA) that allowed for relatively

unrestrained movement of the animal. Probes were perfused overnight with artificial cerebrospinal fluid (aCSF; NaCl: 125 mM; KCl: 2.1 mM; $CaCl_2$: 1.2 mM; $MgCl_2$: 1.2 mM and D-glucose 0.5 mM pH 7.4) at a flow rate of 1 µL/min. On the following morning, the flow rate was increased to 2 µL/min for an additional 1.5 h equilibration period. The perfusion medium of the probe of each animal was then switched to aCSF containing MDMA (100 µM). At this time point rats received an injection of saline or tyrosine (0.5 mmol/kg i.p.). The perfusion continued for an additional 5 h. In these experiments, rats had their rectal temperature kept elevated for 8 hours according to the procedure described above. Seven days after reverse dialysis experiments were completed, animals were killed by decapitation their brains were rapidly removed and one 1-mm thick tissue section was taken (approximately 0.5 mm to either side of the probe).

3. The catechol-O-methyl transferase (COMT) inhibitor entacapone (30 mg/kg i.p.) was given 30 min before a single injection of MDMA (7.5 mg/kg i.p). The dose of entacapone was chosen based on previous findings indicating that rat liver COMT activity is completely inhibited 30 min after injection and remains reasonably inhibited (~75%) six hours later[169].

3.3.2. Surgical procedures

Rats were anaesthetized with a combination of ketamine (70 mg/kg i.p.) and xylazine (7 mg/kg i.p.) and placed in a Kopf stereotaxic frame, with the incisor bar set at 3.3 mm below the interaural line. The skull was exposed, and holes were drilled to allow implantation of a guide cannula (CMA/11, Sweden) into the right striatum (0.2 mm anterior, 3.0 mm lateral from bregma) or the right ventral hippocampus (5.6 mm posterior, 5.0 mm lateral from bregma)[172]. The guide cannulae were secured to the skull with two bone screws, cyanoacrylate glue and dental acrylic cement. After surgery, the animals were housed individually with

free access to food and water. The rats were allowed to recover from surgery, and the reverse dialysis experiments were carried out 4-5 days later.

Seven days after reverse dialysis experiments were completed, animals were sacrificed by decapitation, their brains were rapidly removed and one 1 mm thick tissue section was taken (approximately 0.5 mm to either side of the probe). Striatal tissue was dissected out from the side where the probe was implanted and 5-HT, dopamine and their respective metabolites were quantified using HPLC-ED. In all cases samples were frozen in dry ice and stored at -80°C until analysis.

3.3.3. Temperature measurements

Rectal temperature of the rats was measured at an ambient temperatures of 15, 21.5 or 30 ± 1°C with a lubricated digital thermometer probe (pb 0331, Panlab, Barcelona) inserted 3 cm into the rectum, the rat being lightly restrained by hand. Rats were exposed to low (15°C) or high (30°C) ambient temperatures from 60 min prior to the first MDMA administration up to one hour after the last rectal temperature measurement. At this time point, rats were put back in the colony room set at ambient temperature of 21.5 ± 1°C. Temperature was recorded before any MDMA treatment and thereafter every 60 min up to 6 or 8 h. Probes were re-inserted from time to time until the temperature stabilized.

3.3.4. Blood Sampling

In the experiments pertaining to metabolism and hyperthermia, rats were separated in two different groups. In one group blood was withdrawn at 1, 3 and 5 h while in the other group of animals blood was withdrawn at 2, 4 and 6 h after MDMA. In the experiments involving L-tyrosine blood was withdrawn at 2, 4 and 6 h after MDMA.

Saphenous vein puncture for blood sampling was used as previously described[173]. Blood drops were collected into Microvette® CB 300LH tubes and centrifuged immediately. After centrifugation, plasma was transferred to sterile Eppendorf tubes containing metabisulphite (2 µL, 0.001% w/v). Samples were frozen and stored at -20°C until analysis.

3.3.5. Pharmacokinetic analysis

MDMA, MDA, HMMA and HMA concentrations in plasma were determined following a previously described method[174]. Briefly, aliquots of 100 µL of plasma were hydrolyzed enzymatically with β-glucuronidase at pH 5.2 (incubation for 16 h at 37°C). After hydrolysis, samples were adjusted to pH 6 and a solid-phase extraction using mixed-mode solid reversed phase columns (Bond Elut Certify™, Varian, Palo Alto, CA, USA) was carried out. The extracts were evaporated to dryness (40°C) and the dried extracts were derivatized with 50 µL of MBTFA for 45 min at 70°C. Once cooled, the samples were transferred to autosampler vials and analyzed using gas chromatography-mass spectrometry (GC-MS, MSD5973, Agilent, Palo Alto, CA).

The presence of HHMA and HHA in plasma was determined by using a previously published method[175]. An acidic hydrolysis was performed before a solid-liquid extraction using strong cation exchange (SCX, Varian, Palo Alto, CA, USA) columns followed by HPLC-ED.

Serum L-tyrosine concentrations were measured by HPLC-ED as previously described by Bongiovanni et al.[176] with minor modifications. Briefly, the HPLC system consisted of a Waters Spherisorb ODS2 column 4.6 x 150 mm (Waters, Milford, Massachusetts, USA), an ED operated at a relative potential of 0.75 V to a Ag/AgCl reference electrode. The mobile phase consisted of 75% aqueous 0.133 mM Na_2HPO_4 and 25% methanol adjusted to pH 6.8 with o-phosphoric

acid. Serum (50 µL) was added to a series of 1.5 mL Eppendorf tubes followed by 450 µL of ice-cold ethanol containing 20 nmol/mL of norvaline (internal standard). Samples were then vortexed for 30 s and centrifuged for 45 min at 8000 x g. To detect tyrosine, a derivatizing agent was used (OPA-S; 10 mg o-phthaldehyde and 30 mg sodium sulphite diluted to 5.0 mL with 0.1 M sodium carbonate pH 10.4) for the reaction media. To a series of 1.5 mL Eppendorf tubes was added 10 µL of sample, standards (1.25–20 nmol/mL) or blanks, and 5 µL OPA-S, reacted for 5 min and brought to a final volume of 100 µL with HPLC mobile phase. A 20 µL sample was injected onto the column. Data for L-tyrosine is presented in nmol/mL.

3.3.6. Neurochemical measurements

Concentrations of 5-HT, 5-HIAA, DA, DOPAC and HVA in the brain regions of the rats were determined by high performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described[10].

It has been recently suggested that measurement of tissue 5-HT concentration may overestimate neurotoxic damage caused by MDMA. For this reason [^{3}H]paroxetine binding studies to the 5-HT transporter were performed according to the procedure described by Aguirre et al.[177]. These experiments were only carried out in those groups of rats showing long-term 5-HT deficits.

3.4. Data Analysis

3.4.1. Clinical trial

Values of the maximum plasma concentration (Cmax) and the time to reach Cmax (tmax) were noted directly from the plasma concentration – time profiles of DEX, DOR, MM and HM. Area under curve values (AUC(0,t) were determined from 0-8 h (session 1 data) and 4-12 h (session 2 data) using the trapezoidal

rule. Elimination rate constants (k) were estimated by log-linear regression of terminal data points. Values of pharmacokinetic parameters were compared between sessions by the paired Student t test (Cmax and AUC) and the Wilcoxon test (tmax). Differences were considered to be significant at $p < 0.05$. Relationships between urinary DEX/DOR metabolic ratio (MR) and Cmax;DEX/Cmax;DOR, MR and urine pH, and MR and urine creatinine concentration were assessed by the Pearson correlation coefficient. The fold-change in MR values at the mean of each urine sample period in session 2 relative to the MR value in session 1 were plotted against time after MDMA dosage for each volunteer. A monoexponential equation was fitted to the individual profiles by non-linear regression, with weighting by the standard deviation of the MR in the respective urine sampling interval, to estimate a value of CYP2D6 recovery half-life ($\ln(2)/K_{cyp}$):

$$A(t) = A_1 e^{-K_{cyp}.t}$$

Where A(t) is the fold change in DEX urinary MR following MDMA administration expressed by $MR_{inh}/MR_{control}$ at the mid-point of time interval t, A(0) is the baseline value, and K_{cyp} is the first-order rate constant for CYP2D6 recovery. Goodness of fit of each equation to the observed values was evaluated by visual examination of residual and observed versus predicted plots. A biphasic model was also considered:

$$A(t) = A_1 e^{-K_{cyp_1}.t} + A_2 e^{-K_{cyp_2}.t}$$

Equations were fitted to observed data by minimising the weighted sum of the squares through change of the parameters A and K_{cyp} in the above equations using the Newton method in the "Solver" tool in Excel®, Microsoft (1000 iterations, tolerance of 1%, precision of 1×10^{-6} and convergence of 1×10^{-4}).

Weighted sum of the squares (WSS) were calculated for the model by the following equation:

$$WSS = \sum_{t=1}^{t=n} \left(Y_{observed,t} - Y_{predicted,t} \right) \cdot W_i$$

Where *t* is the urine recovery time interval, *n* is the number of intervals and W_i is the standard deviation of A ($MR_{session\ 2,t}/MR_{session\ 1}$) at time interval *t*.

The goodness of fit of each equation to the observed values was evaluated by Akaike's Information Criterion (AIC) whereby:

$$AIC = N \cdot \ln(WSS) + 2 \cdot M$$

Where *N* is the number of data points and *M* is the number of parameters. Goodness of fit was also assessed visually by plotting observed versus predicted values and time interval versus residuals.

A degradation constant K_{deg} of CYP2D6 was calculated as described previously[139]. The half-life of return of CYP2D6 activity to baseline should reflect the CYP2D6 turnover half-life and MDMA steady-state half-life. The CYP2D6 turnover half-life was thus determined using a non-compartmental deconvolution of the mean residence time of MDMA ($1/K_e$) from the mean time of return of CYP2D6 activity to baseline ($1/K_{cyp}$) giving a mean CYP2D6 degradation time ($CYP2D6_{MDT}$), first-order degradation rate constant K_{deg} ($1/CYP2D6_{MDT}$) and degradation half-life ($\ln(2)/K_{deg}$).

3.4.2. Animal Studies

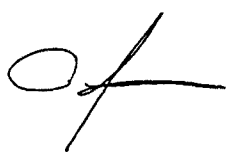
Time course of temperature changes was analyzed by two-way ANOVA for repeated measures. Treatment was used as the between-subjects factor and

time as the repeated measure. Temperature measures were also converted to a composite measure (TAUC) as previously described by[178]. The TAUC was calculated for each rat by the application of Simpson's Rule to temperatures measured at times –30 min and every hour up 6 or 8 h following MDMA administration. This composite measure represents the area under the curve of a plot of temperature (°C) versus time (h), and has units of °C x h. Differences in TAUC and biochemical concentrations were analyzed either by unpaired Student t-test or one-way ANOVA. Multiple pair wise comparisons were performed using the Tukey's test. Differences in MDMA and metabolites, brain monoamine concentrations and 5-HT transporter density (B_{max}) were analyzed either by unpaired Student t-test or one-way ANOVA. Treatment differences were considered statistically significant at $p < 0.05$. Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 11.0).

DEPARTAMENT DE CIÈNCIES EXPERIMENTALS I DE LA SALUT
PROGRAMA DE DOCTORAT EN CIÈNCIES DE LA SALUT I DE LA VIDA
UNIVERSITAT POMPEU FABRA (UPF)

Clinical and toxicological significance of the involvement of the cytochrome P450 system in the metabolism of 3,4-Methylenedioxymethamphetamine (MDMA, ECSTASY)

Memòria presentada per Brian O'Mahony per optar al títol de doctor per la Universitat Pompeu Fabra. Treball realitzat sota la direcció del Dr. Rafael de la Torre Fornell, en el Grup de Recerca clínica en farmacologia humana i neurociències, IMIM-Hospital del Mar, Parc de Recerca Biomèdica de Barcelona. Programa de Doctorat de la Universitat Pompeu Fabra.



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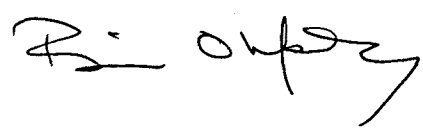

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4. RESULTS

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Caffeine Pharmacokinetics and Urinary Metabolic Ratios following a recreational dose of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) in humans

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INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is a widely abused substituted amphetamine. Although there is a growing consensus that MDMA is toxic in humans, the mechanisms underlying its toxic effects remain to be elucidated. MDMA systemic metabolism has been postulated as a major contributing factor to drug neurotoxicity[1]. The majority of MDMA hepatic metabolism in humans occurs via the O-demethylenation route and is carried out by isoforms of cytochrome P450 forming 3,4-dihydroxymethamphetamine (HHMA). The minor active N-demethylated MDMA metabolite MDA (3,4-methylenedioxyamphetamine), is also O-demethylenated giving rise to 3,4-dihydroxyamphetamine (HHA). The O-demethylenation step *in vitro* shows biphasic kinetics with a high and a low affinity component. The high affinity component is regulated mainly by the cytochrome P450 2D6 isoenzyme (CYP2D6) and the low affinity component is regulated by CYP1A2 and to a lower extent by CYP2B6 and CYP3A4[2]. Both HHMA and HHA are subsequently O-methylated by the enzyme catechol-O-methyltransferase (COMT) mainly to 4-hydroxy-3-methoxy-methamphetamine (HMMA) and 4-hydroxy-3-methoxy-amphetamine (HMA), respectively. These metabolites are known to be excreted in the urine as glucuronide or sulfate conjugates[3].

It has been observed *in vivo* that MDMA inhibits its own metabolism[4]. This is due to the quasi-irreversible mechanism based inhibition (MBI) of CYP2D6 [5;6]. Hence, a short time following the administration of MDMA the high affinity component of its first pass metabolism is

completely and irreversibly inhibited and the demethylenation of the drug is entirely dependent on the activities of the enzymes involved in the low affinity component, in part regulated by CYP1A2. If MDMA oxidative metabolism contributes to neurotoxicity then it would be prudent therefore to study the *in vivo* activity of CYP1A2 after CYP2D6 has been inhibited by MDMA.

CYP1A2 is involved in the activation of environmental procarcinogens, such as arylamines, heterocyclic amines, and aflatoxin B1 and its activity is considerably increased by xenobiotics, including cigarette smoke[7]. It has been observed that the methylenedioxy compound isosafrole inhibits CYP1A2[8] and methylenedioxy compounds have also been known to induce cytochrome P450 activity, including that of CYP1A2[9]. The mechanism of induction is thought to follow a binding of the compound to the aryl hydrocarbon (Ah) receptor which upon accumulation in the cell nucleus as a heterodimeric complex acts as a ligand-induced transcription factor resulting in an increase in protein expression. On the other hand, evidences suggest that rat CYP1A1 regulation is modulated by a protein kinase C and that a phosphorylation cascade mechanism that activates the Ah receptor may be involved[10]. Indeed, MDMA has been shown to activate protein kinase C within synaptic terminals[11]. Hence, there is reason to hypothesize that CYP1A2 could be modulated by MDMA. This would have consequences for both MDMA pharmacokinetics and toxicity and that of other CYP1A2 substrates.

Caffeine is well known as a metabolic probe for CYP1A2[12] and it has a high margin of safety. It has been used extensively in the past to investigate gender and age differences in CYP1A2 activity[13;14] and changes in activity due to medication, smoking and diet[7;15;16]. Although CYP2E1, CYP2A6 and CYP3A4 are involved in caffeine metabolism, 90% of its clearance is thought to be controlled by CYP1A2. Its clearance in plasma is considered the "gold standard" for the measurement of CYP1A2 activity in humans. At least six different urinary metabolic ratios for caffeine have been proposed as in vivo probes for CYP1A2 activity and of these the ratio (AAMU+1X+1U)/17U seems to be the most robust seeing as it is the most sensitive to changes in CYP1A2 activity and not affected as much by other enzyme activities or urine flow[12].

In an attempt to monitor the activity of CYP1A2 following a recreational dose of MDMA a clinical trial was designed whereby caffeine pharmacokinetics and urinary metabolic ratios were measured in subjects who had previously been administered MDMA.

MATERIALS AND METHODS

The study was conducted in accordance with the Declaration of Helsinki, approved by the local Institutional Review Board (CEIC-IMAS), and authorized by the Direcciòn General de Farmacia y Productos Sanitarios (98/112) of the Spanish Ministry of Health. All volunteers gave their written informed consent before inclusion in the

study and were compensated for their participation in the experimental sessions.

Volunteers were recruited by word of mouth. Eligibility criteria required the recreational use of MDMA on at least ten occasions and twice in the previous year. Each eligible subject was initially interviewed by a physician to exclude concomitant medical conditions, and underwent a general physical examination, routine laboratory tests, urinalysis, and 12-lead ECG. Volunteers who fulfilled the inclusion criteria were then interviewed by a psychiatrist (Psychiatric Research Interview for Substance and Mental Disorders for DSM-IV, PRISM-IV) to exclude individuals with history or actual major psychiatric disorders (schizophrenia, psychosis, and major affective disorder) (21). Volunteers had a mean age of 26 years (range 19–33), mean body weight of 69.6 Kg (range 54.2–91.2), and mean height of 181 cm (range 171–196). They referred an average of 26 previous experiences (range 6–100) with MDMA. All but four subjects were current smokers. None met criteria of abuse or drug dependence (except for nicotine dependence). All had previous experience with other psychostimulants, cannabis or hallucinogens. None had history of adverse medical or psychiatric reactions after MDMA consumption.

The design of the study has been published previously[17]. Briefly, in the first session of the study subjected received 100 mg caffeine anhydride together with 30 mg dextromethorphan orally. Blood and urine samples were taken over 24 hours to determine the concentrations of caffeine, dextromethorphan and their respective metabolites (control). In a second session subjects were administered a 1.5 mg/kg oral dose of MDMA and four hours later 100 mg caffeine anhydride together with 30 mg dextromethorphan (active).

At the beginning of each session, subjects arrived at the laboratory at 07.30 h following an overnight fast. An indwelling intravenous catheter was inserted into a subcutaneous vein in the forearm of the non-dominant arm and 0.9% sodium chloride solution was infused at a rate of 20 mL/h. Thereafter, they remained seated in a quiet room throughout the session. Drugs were administered at 8:30 AM (dextromethorphan and caffeine in session 1 and MDMA in session 2). A light meal was provided 6 h after drug administration. Urine was collected for 0-8 h in session 1 following caffeine administration and 4-12 h in session 2 following MDMA administration. Urine pH was measured and subsequently acidified and aliquots were stored at –20°C until further analysis. Blood samples of 8 mL were collected at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 25 h after caffeine administration in session 1 and at 0, 0.5, 1, 2, 4, 4.5, 5, 6, 8, 10, 12, 25 h after MDMA administration in session 2. After centrifugation at 4°C, four 1 mL aliquots of plasma were stored at -20°C for further analysis. Tobacco smoking was permitted 6 h after drug administration. Subjects were requested to refrain from consuming any drug two weeks before and throughout the duration of the study and asked to follow a xanthine free diet 48 h before the beginning of each session. At each session and before drug administration, urine samples were collected to check for the use of

drugs of abuse (opiates, cocaine metabolite, amphetamines, and cannabinoids) by immunological methods (FPIA, Abbott Laboratories, Chicago, IL, USA).

Caffeine and paraxanthine are extracted from 500 mL of plasma using a liquid/liquid extraction procedure. 4 mL of ethylacetate and 200 µL of saturated potassium sulfate are added to 500 µL of plasma. The mixture was vortexed for 30 s. Samples are agitated for 20 min and then centrifuged room temperature for 5 min. The organic layer was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 100 µL mobile phase and 25 µL were injected into the HPLC system. Chromatographic analysis was carried out using a 1090 II high performance liquid chromatograph (Hewlett Packard, Palo Alto, CA) coupled to a UV model 1100 at 274nm. The mobile phase used was acetonitrile: acetic acid (0.05%) (99:1, v/v) applied as an isocratic gradient with a flow rate of 1 mL/min for a run of 15 min. The HPLC column used was an Ultrasphere ODS (4.6cm x 7.5cm x 3µL) (Beckman Coulter ™, Palo Alto, USA). The inter-assay precision and accuracy was 10.0% and 7.8% for caffeine and 4.2% and 5.9% for paraxathine with limits of detection of 3.5 µg/L and 12 µg/L for caffeine and paraxanthine respectively. Caffeine (137X), paraxanthine, (17X), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U), 1-methyluric acid (1U) and 5-acetylamino-6-amino-3-methyluracil (AAMU) were analyzed in urine by a previously published procedure[18]. The procedure was validated in our laboratory giving an inter-assay precision and accuracy of less than 7.0% and 10.0% for all analytes with limits of detection less than 1.5 µg/mL.

For caffeine and paraxanthine plasma concentrations, peak concentration (C_{max}), time to reach peak concentration (t_{max}) and area under the concentration-time curve from 0-8 h and 0-21 h in session 1 and 4-12 h and 4-25 h in session 2 in session 2 (AUC_{8h}; AUC_{21h}) were calculated. The AUC values were calculated by the trapezoidal rule. The elimination rate constant for caffeine and paraxanthine was calculated by log-linear regression and converted to half-life of elimination ($t½_e$). AUC's were brought to infinity and the systemic oral clearance (Cl_o) was calculated by dividing the dose in mg/Kg body weight by AUC_{21h}. The following urinary metabolic ratios were calculated for caffeine and metabolites:

17X/137X	:MR1
(AAMU+1X+1U)/17U	:MR2
(AAMU+1U+1X+17U+17X)/137X	:MR3

Values for urinary metabolic ratios and caffeine and paraxanthine pharmacokinetics in session 1 and session 2 were compared using a paired Student's t test (MR's, C_{max}, AUC, $t½_e$ and Cl_o) and the Wilcoxon test for non-parametric values (t_{max}). Differences associated with p values lower than 0.05 were considered to be statistically significant. Spearman correlations were also carried out for the following variables in the current population: urinary metabolic ratio (MR)

versus caffeine AUC_{8h} and the ratio of 17X to 137X plasma concentrations at 8 h.

TABLE 1. Caffeine and paraxathine pharmacokinetics before (session 1) and after (session 2) a 1.5 mg/Kg oral dose of MDMA (n = 15, standard deviations in parentheses).

Session	1	2
Caffeine		
C_{max} (μg/L)	2345.6	1965.4*
	(±618.3)	(±536.3)
t_{max}[a] (h)	4.5	5.0
	(±1.6)	(±1.7)
AUC_{8h}[b] (μg/L.h^{-1})	5557.6	5212.9
	(±1799.9)	±1802.2)
AUC_{21h} (μg/L.h^{-1})	11249.6	11752.7
	(±4929.6)	(±6611.6)
$t_{½e}$ (h)	3.0	3.9
	(±1.1)	(±1.8)
$AUC_{0-\infty}$ (μg/L.h^{-1})	11482.4	12499.7
	(±5367.6)	(±7852.3)
Cl_{oral} (L/h^{-1}.Kg^{-1})	0.146	0.151
	(±0.057)	(±0.071)
Paraxanthine		
C_{max} (μg/mL)	764.7	759.0
	(±102.9)	(±144.2)
t_{max} (h)	8.0	8.0
	(±2.4)	(±2.0)
AUC_{21h} (μg/L.h^{-1})	9627.6	10129.7
	(±2193.3)	(±3243.4)
$17X/137X_{8h}$	0.77	0.82
	(±0.32)	(±0.38)

[a] t_{max} values are given as medians

[b] AUC values in session 1 refer to 0-8 h and in session 2 refer to 4-12 h

[c] Paraxathine and caffeine plasma concentrations at 8 h post MDMA dose

*$p < 0.05$ by paired Student t-test

RESULTS

Caffeine and paraxanthine concentration profiles before and after a 1.5 mg/kg dose of MDMA are shown in Figure 1, pharmacokinetic parameters are shown in table 1 and, caffeine urinary MR's are shown in table 2. Although there were significant differences in caffeine C_{max} following MDMA ($p = 0.025$; $t = 2.515$), no other differences were found in caffeine and paraxathine pharmacokinetics between the first and second session. No correlation was found between the three MR's and caffeine AUC_{8h} or 17X/137X at 8 h in plasma. There are no significant differences in the three metabolic ratios of caffeine and metabolites in urine between session 1 and session 2.

DISCUSSION

In session 1, caffeine and paraxanthine C_{max} (2345.6 μg/L and 764.7 μg/L) and AUC_{21h} (11249.6 μg/L.h-1 and 9627.6 μg/L.h-1) values were similar to previous studies following a dose of 100 mg[19-21]. Caffeine C_{max} decreased significantly from 2345 μg/L to 1883 μg/L ($p = 0.025$; $t = 2.515$) following 1.5 mg/Kg MDMA, however no other pharmacokinetic parameters differed significantly suggesting that MDMA does not affect caffeine pharmacokinetics, at least to the extent that CYP1A2 activity is affected. This anecdotal observation may be related to changes in gut pH following MDMA administration which would affect caffeine absorption, or indeed a disruption of the active transport of caffeine by MDMA. A pharmacokinetic interaction between caffeine

and MDMA has been observed[22]. When MDMA (5 mg/Kg) and caffeine (20 mg/Kg) are administered together in the rat there is significant decrease in brain MDMA C_{max} and $AUC_{0\text{-}300min}$ compared to when MDMA was administered alone, suggesting an interaction between the two substances. However, recent results show that MDMA's transport does not appear to be P-gp mediated[23]. Another explanation could come from the fact that both caffeine and MDMA are known to increase blood pressure[24;25] and this may affect the increase of availability of the substrate for first pass metabolism by an increase in the blood flow through the hepatic portal vein. However, these conclusions are purely speculative and beyond the scope of results presented herein.

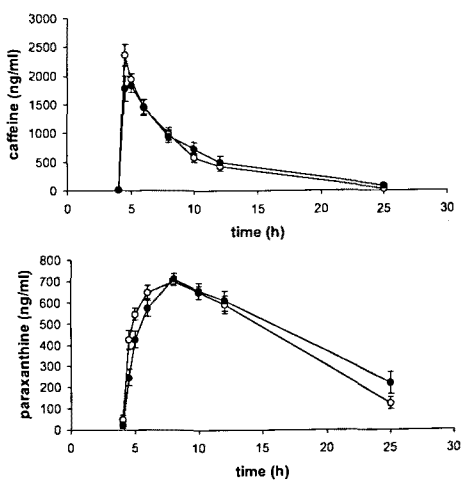

FIGURE 1. Mean plasma concentrations of caffeine and paraxthine before (-o-) and after (-•-) a 1.5 mg/kg dose of MDMA (n = 15: error bars = S.E.)

TABLE 2. Caffeine and metabolite urinary metabolite ratios for eight recovery intervals before (session 1) and after (session 2) a 1.5 mg/Kg oral dose of MDMA (n = 15, standard deviations in parentheses).

Session	1	2
MR1	10.0	11.2
	(±5.7)	(±17.7)
MR2	8.7	8.4
	(±4.6)	(±4.9)
MR3	65.5	51.0
	(±34.7)	(±40.8)

Caffeine was chosen as a probe for CYP1A2 following an MDMA administration because being a compound which is normally present in just about everyone, it had a high safety index especially following a single dose of a drug of abuse. Other probes that can be used for the evaluation of CYP1A2 in humans are fluvoxamine and phenacetin. Fluvoxamine in this case cannot be used due to a possible pharmacodynamic interaction with MDMA. Phenacetin is as an analgesic and would not be suitable for such a study with a drug of abuse and is no longer commercially available as it was withdrawn because of its induction of metahemoglobinemia in some patients. At least six different urinary MR's of caffeine have been proposed as probes for in vivo CYP1A2 activity. Notarianni et al.[26] reported very poor correlations between most of these ratios in a study of 237 healthy subjects, and concluded that, since the ratios clearly do not mark the

same thing, some may be better than others at indicating CYP1A2 activity. More recently, the sensitivity of each ratio to change in the intrinsic activity of CYP1A2 and of other confounding variables, such as the activities of CYP2A6, CYP2E1, xanthine oxidase, NAT2 and changes in urine flow was further explored [27]. In support of Notariani et al., it was found that the ratio (AFMU + 1X + 1U)/17U in urine was the best ratio to use. It also showed that cumulative urine samples are preferable to spot or slice-of-time samples. Further simulations endorsed the view of Fuhr and Rost[28] that the plasma or saliva ratio of 17X/137X measured at 5 ± 7 h is a much more robust CYP1A2 marker than any of the urinary metabolite ratios. Hence in this study, caffeine and six of its respective metabolites were analyzed in urine, so as to calculate and compare many ratios.

MR ratios measuring CYP1A2 activity fell within the ranges of previous studies[29;30]. Interestingly, none of the ratios from session 1 correlated with caffeine AUC_{8h} in this study sample. No significant changes were observed in any urinary caffeine MR's following a single dose of 1.5 mg/kg MDMA. Considering that no correlation was found between the urinary caffeine MR's and caffeine clearance and the high variance in these parameters, it seems that caffeine and paraxanthine plasma concentrations are better markers for CYP1A2 activity, at least for this sample size.

In any case, although an inhibitive interaction with CYP1A2 could have been observed here, it is debatable that any induction could be observed following the administration of a single dose of MDMA. The induction of CYP1A2 is usually observed following multiple doses of a putative inducer with the CYP1A2 probe being administered after the inducer has been cleared from the body [31]. The design of the studied is therefore limited, although a multiple dose study using MDMA would be highly unethical and currently impossible to conduct.

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*Authors name in Irish, co-author

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Goñi-Allo, B. Puerta, E. *Ó Mathúna, B. Hervias, I. Lasheras, B. de la Torre, R. Aguirre, N. **On the role of tyrosine and peripheral metabolism in 3,4-methylenedioxymethamphetamine-induced serotonin neurotoxicity in rats.** Neuropsychopharmacology 2008; 54(5): 885-900.

*Authors name in Irish

Goni-Allo B, Puerta E, Ó Mathúna B, Hervias I, Lasheras B, de la Torre R, et al. On the role of tyrosine and peripheral metabolism in 3,4-methylenedioxymethamphetamine-induced srotonin neurotoxicity in rats. Neuropharmacology. 2008; 54(5): 885-900.

5. DISCUSSION

5.1. Human Studies

Hypothesis I

MDMA affects the activities of the cytochrome P450 enzymes responsible for its phase I metabolism and this has consequences for the acute and long-term toxicities of the drug.

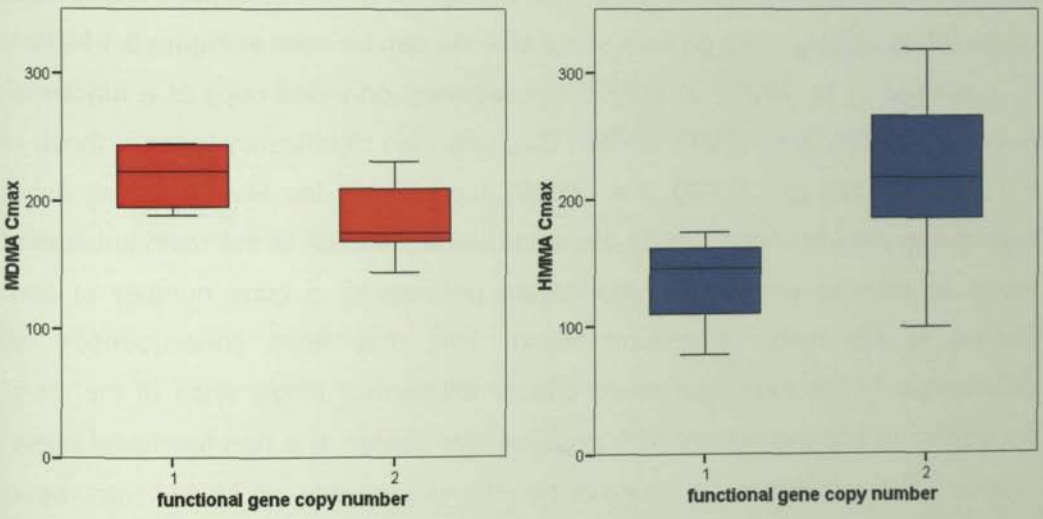

Figure 5.1. Box-plots of A. MDMA C_{max} and B. HMMA C_{max} following an administration of 1.5 mg/kg MDMA by CYP2D6 functional copy number (lines represent median, boxes represent the first and third quartile, whiskers represent the range).

5.1.1. MDMA pharmacokinetics

MDMA pharmacokinetics were similar to those previously published following a similar dose[45]. It was hoped that pharmacokinetic parameters could be compared by genotype *a posteriori,* however the frequency of each allele found and the small sample size hindered any possible statistical analysis. Nonetheless, the frequencies of each genotype within the sample were similar to those found in a Spanish population[179]. It is worth commenting that any analysis

of MDMA pharmacokinetics from a controlled clinical trial according to *CYP2D6* genotype would require a very large sample size to capture all possible genotypes, which considering the difficulty in recruiting subjects for such a study, would be currently implausible. In an attempt to bypass this inconvenience subjects were given a score according to functional gene copy number. Thus, individuals possessing alleles which cause the complete loss of CYP2D6 activity were given a score of 1 and those possessing alleles which cause either partial or complete activity were given a score of 2. As can be seen in Figure 5.1 MDMA C_{max} tended to be higher in subjects possessing only one copy of a functional allele ($p = 0.078$; $z = -1.828$). HMMA C_{max} was also significantly lower in those in these individuals ($p = 0.040$; $z = -2.089$) according to the Mann-Whitney exact test. It can also be seen that in these individuals MDMA is the main substance found in plasma whereas in individuals possessing a copy number of two, HMMA is the main compound found. This may have consequences for differences in the individual acute effects following a single dose of the drug, especially in PM individuals who possess two copies of a non-functional allele. Indeed, this difference in pharmacokinetic parameters of MDMA has been observed previously[4]. However, from the dextromethorphan data presented here and data from the previous study, concentrations of MDMA and metabolites following subsequent doses of MDMA would not differ according to *CYP2D6* genotype, nullifying any differences in risk of toxicity. No differences were found in dextromethorphan and metabolite plasma concentrations according to *CYP2D6* functional copy number.

5.1.2. DEX and metabolite pharmacokinetics

A single recreational dose of MDMA caused a dramatic increase in the systemic exposure to the model CYP2D6 substrate DEX, with a concomitant decrease in exposure to its primary metabolite DOR. There is a 10 fold increase in plasma concentrations of DEX following a recreational dose of MDMA. Indeed, there is

an accumulation effect seen in the concentrations of DEX and DOR following the second dose of DEX which can be seen by an increase in expected concentrations of DEX and DOR at 25 h. A similar increase in DEX plasma concentrations has been observed following a dose of another CYP2D6 inhibitor, quinidine[141,180]. Furthermore, the pharmacokinetics of DEX and its metabolites are comparable to those for PM in the studies cited above, although multiple dosing of quinidine is needed to phenocopy EMs to PMs. The mechanisms of inhibition of both compounds are distinct. MDMA administration results in the MBI of CYP2D6 which has been observed *in vitro*[2,40], this causes an irreversible inhibition, the recovery of which is dependent on *de novo* expression of the enzyme. These observations, which appear to reflect potent mechanism-based inhibition of CYP2D6, confirm implications suggested by previous investigations[136].

5.1.3. DEX urinary MR

5.1.3.1. Phenocopying

When considering the results in urine, 67% of the volunteers had MR values in greater than the antimode for phenotype assignment[140], suggesting that following a single dose of MDMA, individuals are phenocopied to the PM. There was no distinction in genotype between those who did not phenocopy and those that did. This result explains the absence of correlations between *CYP2D6* genotype and MDMA acute toxicity in the literature, since following the first dose of MDMA the difference in phenotype is rendered obsolete. This observation also differs from previous studies with other CYP2D6 MBI's such as paroxetine[138] where none of the subjects attained an MR greater than the antimode for PM assignment. Since MDMA and paroxetine have similar K_I and k_{inact}[181,182], this difference may be due to differences in dosing (20 mg for paroxetine and 1.5 mg/kg for MDMA) or due

to high plasma protein binding of paroxetine which would limit its access to hepatic tissue.

5.1.3.2. Sample variance and DEX MR as a marker for CYP2D6 activity

The use of urinary MR as a measure for CYP2D6 activity has been debated in the literature. The use of DEX urinary MR has also been questioned due to contradictory data attempting to correlate it with DEX and DOR plasma levels[183]. This ratio is also sensitive to any changes in the renal clearance of DEX. In this study, using creatinine as a measure of renal function, no correlation was found between session 1 urinary MR and urine creatinine concentrations. Additionally, there was no relationship found between urinary pH and DEX urinary MR as suggested in previous studies[145,146]. Nevertheless, since the volunteers in this study were from a homogeneous sample and their dietary intake was not significantly variable, differences in renal function and pH values were not significant enough to allow observation of these effects within the small sample size.

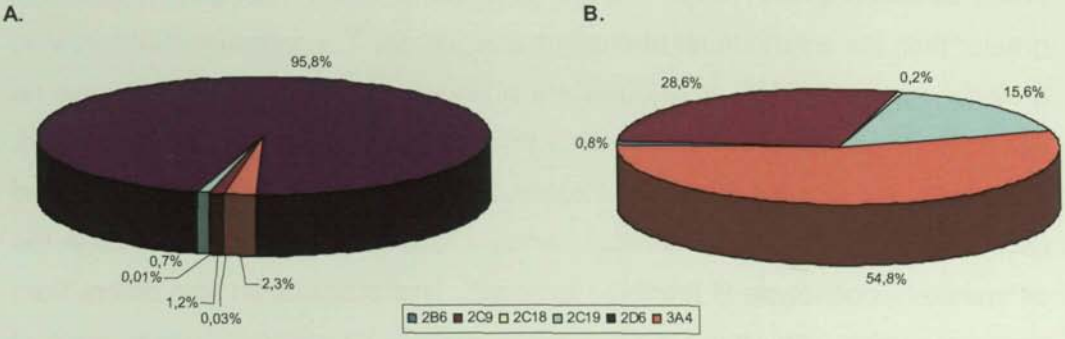

Figure 5.2. Percentage contribution of each CYP enzyme to dextromethorphan O-demethylation intrinsic clearance (V_{max}/Km) before (panel A.) and after (panel B.) CYP2D6 inhibition.

In vitro studies from *Baculovirus* expression systems suggest that the O-demethylation of dextromethorphan is carried out predominantly by CYP2D6, with minor contributions from CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2C9 and CYP3A4[147-150]. An expression that estimates the total net intrinsic metabolic clearance by the whole liver ($Clu_{H,int}$) from data obtained with recombinant expressed CYP enzymes is given by the following equation:

$$Clu_{H,\,int} = \left[\sum_{j=1}^{n}\left(\sum_{i=1}^{n} ISEF_{ij} \times \frac{V_{max\,i}(rhCYP_i) \times CYP_j abundance}{K_{mi}(rhCYP_j)}\right)\right] \times MPPGL$$

Where there are *i* metabolic pathways for each of *j* CYPs, *rh* indicates recombinant expressed enzyme, V_{max} is the maximum rate of metabolism by an individual CYP, K_m is the Michaelis constant, MPPGL is the amount of microsomal protein per gram of liver and ISEF is a scaling factor that compensates for any difference in the activity per unit of enzyme between recombinant systems and hepatic enzymes[184]. The contribution of each CYP to a particular pathway can then be calculated as a percentage. For DEX O-demethylation, it can be seen that when CYP2D6 is inhibited, CYP3A4 and CYP2C9 are responsible for 55% and 29% of DEX O-demethylation, respectively (Figure 5.2). It should be noted that according to these data the total intrinsic clearance would decrease substantially following CYP2D6 inhibition, and this has been observed here *in vivo*. CYP3A4 is subject to induction and inhibition by substances found in the diet[185-187] and CYP2C9 activity is polymorphic. Hence, interindividual differences in these enzyme activities could explain the variance found in the MR in this study following CYP2D6 inhibition by MDMA. Indeed, in the time interval following the administration of MDMA the recovery of DEX in urine and the DEX C_{max} tends to be greater in individuals possessing the PM allele *CYP2C9*3*. Both these alleles have been associated with higher concentrations of CYP2C9 substrates in the past[188]. However, due to

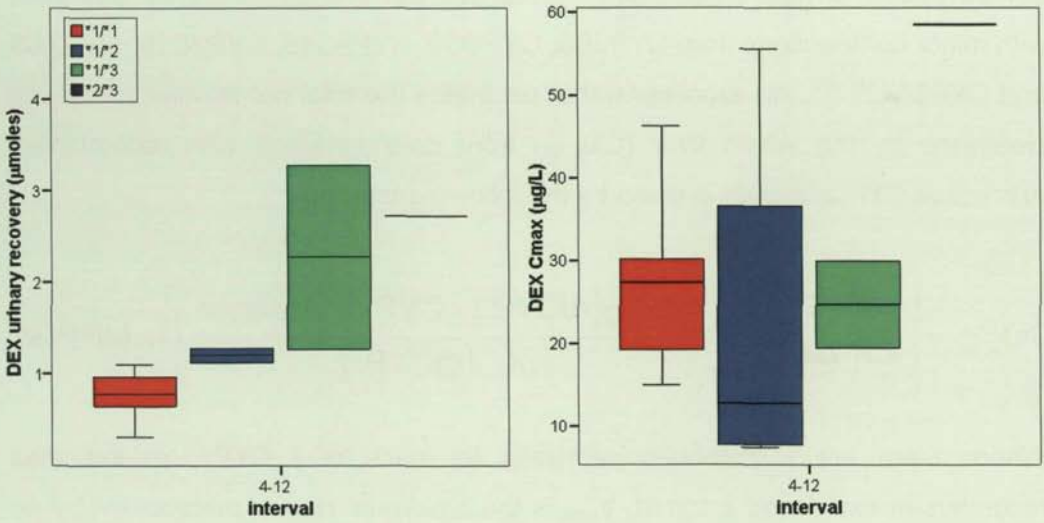

Figure 5.3. Box-plots of DEX urinary recovery and DEX C_{max} in the first eight hours following administration of 1.5 mg/kg MDMA by CYP2C9 genotype (lines represent median, boxes the first interquartile, whiskers represent the range).

the small sample size, the statistical power is not great enough to draw solid conclusions about the contribution of CYP2C9 to *in vivo* DEX clearance when CYP2D6 is inhibited.

5.1.3.3. The CYP2D6 turnover half-life

The half-life of inhibition was first estimated by non-linear regression according to a monoexponential equation. However, this model does not fit the observed data in this study and underestimates the half-life of inhibition. It is clear from the log transformed data that the recovery of inhibition displayed biphasic kinetics. A biphasic model was not considered in previous studies measuring K_{deg} of cytochrome P450 isoenzymes[137,138]. Liston et al. followed the recovery of inhibition after discontinuation of paroxetine, fluoxetine and sertraline. In the case of a known MBI paroxetine[181], recovery was only monitored for 7 days and although no statistical differences were seen between the last study day and

basal values, the inhibition does not appear to have fully recovered after this time and assumption of first-order kinetics may be premature. Furthermore, upon considering the data for fluoxetine it appears that the data better fits a biphasic model. The biphasic recovery of CYP2D6 activity, as marked by serial measurement of the DEX/DOR MR, may reflect differences between CYP2D6 turnover rates in the gut and liver or a dominant initial competitive inhibition of the enzyme followed by a greater impact of MBI. The mean value of CYP2D6 half-life of 94 h that was estimated by the biphasic model is greater than the value of 51 h previously estimated using paroxetine as an inhibitor[138,139]. Although this may reflect differences in the subjects studied, this value is within the 95% confidence interval of that reported previously. Importantly, large differences in urinary MR found in both studies hamper the accurate calculation of an inhibition half-life. Additionally, gender and ethnic differences in hepatic and gut abundance values and distinct *CYP2D6* genotype frequencies may be a reason for differences in estimation. Indeed, in this sample both allele *CYP2D6*10* and *CYP2D6*17* were found. Not only these alleles have been associated with decreased intrinsic clearance of DEX *in vitro* but allele *CYP2D6*17* has also been associated with decreased MDMA intrinsic clearance[55,189]. Furthermore, it has been observed recently that allele *CYP2D6*10* has an impact on the DEX MR[190]. At the very least, the sample in the present study was all male and came from a more homogenous Spanish population which could account for a more realistic K_{deg} value for this population. A faster degradation half-life of 50 ± 19 h was also calculated for an isoenzyme similar to CYP2D6, CYP2E1[137]. However, CYP2E1 is inducible by ligand stabilization[191] which could cause for the underestimation of the half-life. The present study's results agree with a previous model of MDMA induced CYP2D6 inhibition[136].

5.1.4. Caffeine as a marker for CYP1A2 activity

Caffeine was chosen as a probe for CYP1A2 following an MDMA administration because being a compound which is normally present in just about everyone, it had a high safety index especially following a single dose of a drug of abuse. Other probes that can be used for the evaluation of CYP1A2 in humans are fluvoxamine and phenacetin. Fluvoxamine in this case cannot be used due to a possible pharmacodynamic interaction with MDMA. Phenacetin is as an analgesic and would not be suitable for such a study with a drug of abuse and whatsmore, is no longer commercially available as it was withdrawn because of its induction of metahemoglobinemia in some patients. At least six different urinary MRs of caffeine have been proposed as probes for *in vivo* CYP1A2 activity (Table 1.4.1). Notarianni et al.[166] reported very poor correlations between most of these ratios in a study of 237 healthy subjects, and concluded that, since the ratios clearly do not mark the same thing, some may be better than others at indicating CYP1A2 activity. More recently, further explored the sensitivity of each ratio to change in the intrinsic activity of CYP1A2 and of other confounding variables, such as the activities of CYP2A6, CYP2E1, xanthine oxidase, NAT2 and changes in urine flow[192]. In support of Notariani et al., it was found that the ratio (AFMU + 1X + 1U)/17U in urine was the best ratio to use. It also showed that cumulative urine samples are preferable to spot or slice-of-time samples. Further simulations endorsed the view of Fuhr and Rost[193] that the plasma or saliva ratio of 17X/137X measured at 5 ± 7 h is a much more robust CYP1A2 marker than any of the urinary metabolite ratios. Hence in this study, caffeine and six of its respective metabolites were analyzed in urine, so as to calculate and compare many ratios. Furthermore, the pharmacokinetics of caffeine and paraxanthine in plasma were monitored.

In session 1, caffeine and paraxanthine C_{max} (2345 μg/L and 750 μg/L) and $AUC_{0\text{-}24h}$ (11090 $\mu g/L.h^{-1}$ and 9499 $\mu g/L.h^{-1}$) values were similar to previous studies

following a dose of 100 mg[194-196]. MR ratios measuring CYP1A2 activity also fell within the ranges of previous studies[197,198]. Interestingly, none of the ratios from session 1 correlated with caffeine clearance in this study sample. No significant changes were observed in either urinary caffeine MR's following a single dose of 1.5 mg/kg MDMA. Considering that no correlation was found between the urinary caffeine MR's and caffeine clearance and the high variance in these parameters, it seems that caffeine and paraxanthine plasma clearance are better markers for CYP1A2 activity, at least for this sample size.

Caffeine C_{max} decreased significantly from 2345 μg/L to 1883 μg/L ($p = 0.025$; $t = 2.515$), no other pharmacokinetic parameters differed significantly suggesting that MDMA does not affect caffeine pharmacokinetics, at least to the extent that CYP1A2 activity is affected. This anecdotal observation may be related to changes in gut pH following MDMA administration which would affect caffeine absorbance, or indeed a disruption of the active transport of caffeine by MDMA. A pharmacokinetic interaction between caffeine and MDMA has been observed[199]. When MDMA (5 mg/kg) and caffeine (20 mg/kg) are administered together in the rat there is significant decrease in brain MDMA C_{max} and $AUC_{0\text{-}300min}$ compared to when MDMA was administered alone, suggesting an interaction between the two substances. However, recent results show that MDMA's transport does not appear to be P-gp mediated[200]. Another explanation could come from the fact that both caffeine and MDMA are known to increase blood pressure[60,201] and this may affect the increase the availability of the substrate for first pass metabolism by an increase in the blood flow through the hepatic portal vein. However, these conclusions are purely speculative and beyond the scope of results presented herein.

In any case, although an inhibitive interaction with CYP1A2 could have been observed here, it is debatable that any induction could be observed following the administration of a single dose of MDMA. The induction of CYP1A2 is usually

observed following multiple doses of a putative inducer with the CYP1A2 probe being administered after the inducer has been cleared from the body[202]. The design of the studied is therefore limited, although a multiple dose study using MDMA would be highly unethical and currently impossible to conduct.

5.1.5. Clinical implications

Considering the clinical implications for MDMA users, following a single dose of MDMA a CYP2D6 EM are converted to PM according to DEX phenotyping. Users are known to on average take more than one pill per session (range 1.8 to 2.9 pills) reaching a maximum of 6-7 pills depending on the type of user , with doses averaging 70 mg (range 50-100 mg) [18]. It has also been documented that many users binge on ecstasy, and these binges may last several days[23]. Users must be warned that the risk of acute toxicity due to these practices is drastically increased. Indeed, it has been shown in a previous study that the concentrations of MDMA are significantly increased even when the second dose is taken twenty-four hours later beyond what is expected from accumulation of concentration[3]. Evidence presented here suggests that a second dose of MDMA taken up to one week later would present higher plasma concentrations and higher risk of acute toxicity. It can even be suggested that MDMA users who use the drug on a frequent basis have a permanently compromised CYP2D6 activity.

To date the most important CYP2D6 mechanism-based inhibitors marked for possible toxic drug interactions are SSRI antidepressants such as paroxetine. Differences in doses taken and the way in which the drugs are administered have repercussions for their respective toxicities. Paroxetine for instance is a well studied, prescribed commercial drug and continuing medical education in psychiatry has increased awareness by prescribers of the potential for drug-drug interactions[203]. MDMA is an illegal substance taken at higher but inexact doses outside the scope of medical supervision. A single recreational dose of MDMA

rapidly and completely inhibits CYP2D6 whereas several prescription doses of paroxetine are needed to achieve a similar inhibition[46,138].

It is known that MDMA users are not exclusive users of the drug and belong to a poly-drug abusing population. Dextromethorphan itself is abused at much higher doses than those given in this study[204] and its abuse in combination with MDMA would cause a toxic interaction. Any other substituted amphetamine that is a substrate of CYP2D6 would also potentially interact with MDMA in this manner. Other possible interactions would be with SSRI previously mentioned or other substrates of CYP2D6 such as codeine, tramadol, risperidone and metoprolol among others. However, the only fatal interaction reported to date has been with the antiretroviral and CYP3A4 substrate ritonavir[57]. On a pharmacodynamic level, SSRI's such as citalopram and paroxetine block the positive effects of MDMA[69,205]. Indeed it has been suggested that MDMA users may take common SSRI's to modify the effects of MDMA[113]. It should be noted however that while the neuropharmacodynamic effects of the drug would be changed, individuals using SSRI's in conjunction with multiple doses of MDMA may not be aware that the metabolism of both substances is compromised leading to possible increase of the risk of acute adverse effects already reported for CYP2D6 PM[206]. Similar pharmacodynamic interactions may occur with any abused substance, such as cocaine and cannabis, even if its metabolism does not have an important CYP2D6 component. Disproportionately increased MDMA plasma concentrations in combination with any similar monoaminergic drug would elevate the risk of toxic episodes associated with the effects of all these drugs of abuse.

In the context of the general population, the rate of degradation of CYP2D6 and other cytochrome P450 enzymes of high clinical importance is of relevance since the knowledge of substrate affinities and/or inhibition and inactivation constants is not sufficient to accurately predict the pharmacokinetics of potentially new

drugs at the preclinical level. The half-lives of CYPs calculated can be used along with intrinsic clearance values, chemical properties and physiological values from a population to create an *in vivo-in vitro* extrapolation of the pharmacokinetics of novel drugs before they are assayed in an animal or human[184]. This results in faster and more cost effective drug discovery especially in the high-throughput pharmaceutical industry of today.

5.2. Animal Studies I

Hypothesis II

Interindividual differences in the activity of catechol-o-methyltransferase (COMT) are relevant in the development of long-term toxicity of the drug.

5.2.1. COMT inhibition by entacapone in rats

In an attempt to further investigate the role of the catechol type metabolites of MDMA in the neurotoxic process, entacapone was administered 30 min before the binge dosing regimen of MDMA. Entacapone is a COMT inhibitor, approved for human use in the treatment of Parkinsons disease. In this case it acts by increasing L-dopa availability through its inhibition of its metabolism. The action of entacapone is mainly thought to be in the periphery, at least in animal models at doses of up to 10 mg/kg. At higher doses (30 – 100 mg/kg) central COMT activity is seen to decrease and truly "peripherally selective" inhibition seems to between doses of 2.5 and 5 mg/kg. However, entacapone does not modify the effects of clorgylline, selegiline and nomifensine on striatal dopamine metabolism in the rat brain[207]. Here, entacapone was administered at 30 mg/kg i.p. and was chosen based on previous findings indicating that rat liver COMT activity is completely inhibited 30 min after injection and remains reasonably inhibited (~75%) six hours later[169]. It was speculated that this dose would be sufficient to

inhibit the peripheral metabolism of HHMA and HHA regulated by COMT to HMMA and HMA respectively and would increase their plasma concentration and this would, in turn, lead to larger 5-HT depletions. Indeed, entacapone did exacerbated MDMA-induced long-term 5-HT depletions. In a recent report by Breier et al.[100], it has been suggested that increased tyrosine concentrations after MDMA and its eventual conversion to DA within 5-HT terminals could play an important role in MDMA neurotoxicity. According to previously mentioned data, at the dose used here (30 mg/kg) entacapone will necessarily interfere with DA turnover and by reducing its metabolism by this pathway, and could enhance its accumulation or metabolism via other pathways, leading to ROS production that could enhance 5-HT neurotoxicity. According to results, however, this possibility seems unlikely since perfusion of entacapone into the hippocampus did not affect 5-HT or 5-HIAA depletions caused by MDMA. Furthermore, entacapone when given systemically caused no change in serum tyrosine concentrations. Taken together, these results suggest that entacapone exacerbates MDMA-induced 5-HT depletions by interfering with peripheral MDMA metabolism.

Noteworthy, plasma concentrations of HMMA and HMA were significantly lower in the entacapone treated animals indicating that the O-methylation pathway was successfully inhibited. By contrast, plasma concentrations of HHMA and HHA were unchanged suggesting that these compounds must have been cleared someway. As stated above, HHMA and HHA are highly unstable catechols which cannot only conjugate with sulphate or glucuronic acid, but can also be rapidly oxidized to their corresponding orthoquinones and form adducts with GSH and other thiol-containing compounds[5,41,116]. Such compounds have been related to MDMA neurotoxicity[11,35,208,209]. So, what at first sight was an unexpected finding may offer an explanation as to why entacapone exacerbated MDMA-induced 5-HT depletions.

The effect of entacapone was not related to an effect on core temperature, since pre-treatment with entacapone caused a marked hypothermia when injected alone and did not significantly modify MDMA-induced hyperthermia. Studies *in vitro* have been shown that entacapone is a potential uncoupling agent but not at the doses given here[210]. However, entacapone co-administration (400 mg/kg) with L-dopa and carbidopa in rats produced a marked hypothermia similar to that observed here[210]. Interestingly, dopamine agonists are known to induce a decrease in body temperature. The decrease in body temperature seen here could be due to the increase in dopamine concentrations following COMT inhibition. If this is the case, then the further increase in dopamine following MDMA administration should lead to an exacerbated hypothermic response. Indeed, the opposite is the case, leading to the speculation that it may be the MDMA-induced hyperthermic response may indeed be 5-HT mediated and not DA mediated as some studies would suggest[79].

5.2.2. Clinical implications

According to these data COMT activity plays an important role in MDMA metabolism and toxicity in rats. It is known that the level of COMT enzyme activity is genetically polymorphic in human tissues with a trimodal distribution of low ($COMT^{LL}$), intermediate ($COMT^{LH}$), and high ($COMT^{HH}$) activities and is caused by a mutation in position 108/158 of the COMT gene leading to a Val→Met substitution. This polymorphism, which according to segregation analysis of family studies is caused by autosomal co-dominant alleles, leads to 3- to 4-fold differences in COMT activity in human erythrocytes and liver[207]. This in theory would lead to interindividual differences in the concentrations of the reactive catechol metabolites, HHMA and HHA and could therefore be relevant for human users in terms of susceptibility to MDMA neurotoxicity. Indeed, after analysing the pharmacokinetic data for MDMA and HMMA it was found that the ratio of MDMA to HMMA has a tendency to be higher in val/val individuals

(Figure 5.4). In otherwords, upon correcting for interindividual differences in MDMA concentrations (taking the ratio MDMA/HMMA) due to variance in CYP activity, it can be inferred that subjects possessing the val/val genotype metabolise the reactive catechol HHMA slower and may be more susceptible to its neurotoxic effects.

Animal Studies II

Hypothesis III

MDMA systemic metabolism and not MDMA-induced hyperthermia is the limiting step in the development of long-term neurotoxicity.

5.2.3. MDMA-induced hyperthermia and metabolism

The acute hyperthermia induced by MDMA can strongly influence its long-term neurotoxic effects[76]. The blockade of the acute hyperthermic effect of MDMA is a feature common to many different pharmacological or non-pharmacological manipulations known to protect against MDMA-induced neurotoxicity and such protection is abolished when the temperature of the animals is kept

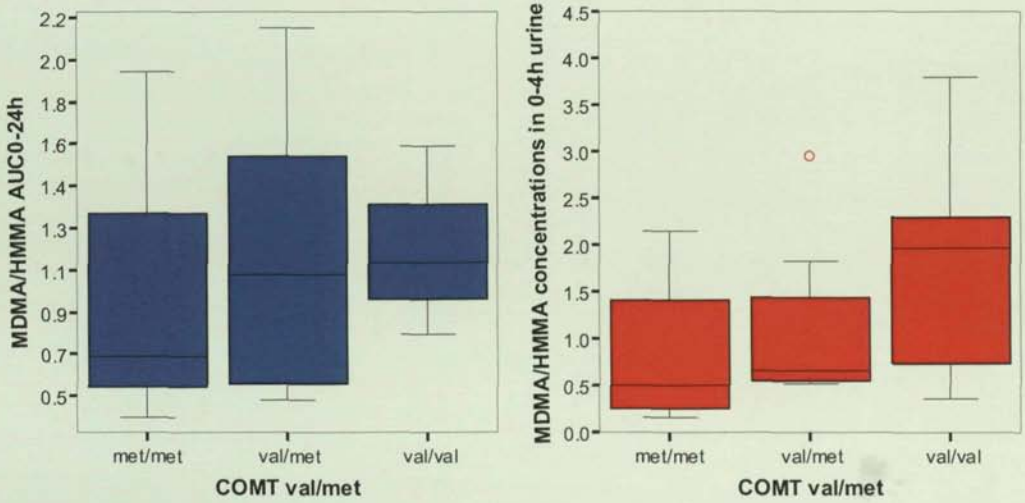

Figure 5.4. Box Plots of the ratios of urinary and plasma concentrations ($AUC_{0\text{-}24h}$) of MDMA to HMMA versus COMT val/met genotype in humans following a single oral dose of 1.5 mg/Kg MDMA (n = 15; (lines represent median, boxes the first interquartile, whiskers represent the range).

elevated[102,103]. Conversely, the degree of long-term damage produced by MDMA appears to be closely related to the magnitude of the hyperthermic response[6]. Furthermore, Capela et al have demonstrated *in vitro* that neuronal cell death caused by MDMA and its metabolites is potentiated at higher temperatures[211]. Indeed, generally the production of ROS is increased during hyperthermia[114,115]. The quinone thioether conjugates of MDMA metabolites, by redox cycle interference, are postulated to generate the ROS which leads to neurotoxicity[212]. The hyperthermia caused by MDMA may increase ROS production even further and so lead to the serotonergic changes observed in animals. On the other hand, hyperthermia may not be a defining factor in MDMA-induced neurotoxicity. The free radical trapping agent, α-phenyl-*N*-tertbutyl nitrone and the serotonin uptake inhibitor fluoxetine, partially prevent MDMA-induced serotonergic changes without modifying hyperthermia[112,113]. The mechanism underlying the relationship between MDMA-induced changes in core body temperature and long-term toxicity is as yet unclear. The results show here that MDMA metabolism is greatly affected by core body temperature, providing new clues to clarify the relationship between changes in core body temperature and 5-HT toxicity induced by MDMA.

5.2.3.1. Single dose of MDMA at different temperatures in rats

In the first set of experiments a single high dose of MDMA (15 mg/kg i.p.) was administered at two different ambient temperatures and the plasma concentrations of MDMA and its metabolites, MDA, HMMA and HMA were analyzed for every hour for a period of six hours. The administration of MDMA at a low ambient temperature (15 ± 1°C) prevented MDMA-induced hyperthermia and resulted in an almost complete protection. The only region where decreases in 5-HT and 5-HIAA were detected was in the hippocampus and the percentage decrease from the saline control was much less than the change at 21°C. Indeed, this result is not surprising since the hippocampus appears to be more

sensitive to MDMA-induced neurotoxicity than the striatum or the frontal cortex. Although consistently reported[111,213], the reasons for such different sensitivity are still unknown. Interestingly, it was also found that hepatic metabolism of MDMA was reduced. MDMA concentrations were higher than those obtained from rats treated at a standard ambient temperature (21.5 ± 1°C). Accordingly, plasma concentrations of MDA, HMMA and HMA in rats treated at 15°C were significantly lower than those found in rats treated at 21.5°C. Because increases in ambient temperature exacerbate MDMA-induced 5-HT neurotoxicity and hyperthermia in the rat[6], we also tried to verify whether increasing the ambient temperature up to 30°C would have consequences on the metabolism of MDMA. However, we could not successfully carry out these experiments because, under such experimental conditions, the mortality rate of the animals was above 90%.

5.2.3.2. Multiple doses of MDMA at different temperatures in rats: a model of "binging"

One of the main objections often raised to MDMA studies is that the doses of MDMA used to cause 5-HT toxicity in laboratory animals are too high and do not resemble those taken by humans. A practice sometimes employed by recreational ecstasy users is binge dosing, comprising the ingestion of several doses on a single occasion[19]. A multiple dosing regimen was administered to examine how such a regimen influences the effect of MDMA on acute hyperthermia and long-term 5-HT toxicity. The results indicate that for the same dose exposure, binge dosing produces an identical acute hyperthermic response and modification of secondary biomarkers of 5-HT neurotoxicity than a similar non-divided dose. An interesting finding was that a single high dose of MDMA (15 mg/kg) resulted in an approximately 20% mortality, while the binge dosing regimen caused no deaths. Data from others (Bagdy et al. personal communication) show that the mortality rate after a single high dose of MDMA (15 mg/kg) increases up to 50-60% in male Dark Agouti rats, an effect that is

almost completely prevented with a repeated dose regimen of MDMA (3 x 5 mg/kg i.p., every 2 h). It is interesting to note that the mortality rate is higher in the former strain than in the Wistar rat, considering that past studies have used the female Dark Agouti rat as a model for CYP2D6 PM and found that the hyperthermic response in was higher females than males[85].

Subsequently, the first set of experiments was reproduced but by administering a binge dosing regimen given at low (15°C), standard (21.5°C) and high (30°C) ambient temperatures. As in the case of a single high dose of MDMA, low ambient temperature prevented MDMA-induced acute hyperthermia and long-term 5-HT toxicity. Although somehow expected, MDMA metabolism was also reduced at 15°C as plasma MDMA concentrations were significantly higher while those of MDA, HMMA and HMA were lower than those found in rats treated at 21.5°C. It is apparent from these results that despite higher plasma concentrations of the serotonergic neurotoxin MDMA at low ambient temperature, hyperthermia and 5-HT depletions induced by MDMA are reduced. These observations would be in agreement with the hypothesis that a reduction in drug metabolism is correlated with reductions of biomarkers of neurotoxicity. The dose regimen used, for an equal total dose exposure, does not substantially modify these conclusions. Interestingly, the converse also occurred. The administration of MDMA (3 x 5 mg/kg) at 30°C potentiated hyperthermia and long-term toxicity, but what is most important, the concentrations of all 3 metabolites of MDMA increased drastically over the 6 h time following the first MDMA injection. Unfortunately, due to an insufficient amount of sample the concentrations of the catechol type metabolites of MDMA, HHMA and HHA could not be measured. However, because the only metabolic pathway leading to HMMA or HMA formation involves the methylation of HHMA and HHA[214], it appears reasonable to suggest that, at some point, the plasma concentrations of

HHMA and HHA (the proposed precursors of neurotoxic species) must have been higher in rats treated at 30°C vs. 21°C.

5.2.3.3. Perfusion of MDMA into the brain of rats

Data from the perfusion experiments confirm and expand on those reported by others indicating that direct perfusion of MDMA into the brain does not elicit any sign of 5-HT toxicity[119]. However, the absence of tissue depletions following the local infusion of MDMA has been suggested to be due to the lack of effect on body temperature[170]. In order to bypass this inconvenience, intrastriatal perfusion of MDMA (100 μM, 5h) was combined with MDMA (3 x 5 mg/kg i.p.) known to increase core body temperature. It should be noted that the concentration of MDMA used in these experiments produces an increase in extracellular DA concentrations similar to that seen after a toxic MDMA dosing regimen[170,215]. Furthermore, such a concentration of MDMA in the perfusate gives rise to the range of extracellular concentrations of MDMA observed following peripheral administration of neurotoxic doses of MDMA[119]. Despite these considerations, the extent of 5-HT and 5-HIAA depletions was similar and independent of whatever was perfused seven days earlier. Again, these data are consistent with the hypothesis that peripheral generation of neurotoxic metabolites contributes to MDMA-induced serotonergic neurotoxicity.

5.2.3.4. Clinical implications

The data suggest that peripheral metabolism of MDMA plays a key role in the whole neurotoxic process. Based on the literature and the results presented herein, it is tempting to speculate that the so far unclear relationship between the acute hyperthermic response and long-term damage produced by MDMA may involve the influence of core body temperature in the metabolic rate of this drug. I should also be pointed out that these findings may be relevant not only for

future animal investigations, but also for recreational MDMA users in spite of the limitations of translating animal data to humans[216,217]. High doses (and or plasma concentrations) of MDMA are associated with acute toxicity and mortality as it has also been observed in humans[84]. The practice of taking low doses of MDMA repeatedly in a short period of time and for the same dose exposure (i.e. a high dose inducing acute toxicity and mortality) might reduce the risk of acute toxicity but does not modify the risk of neurotoxic effects, especially in crowded and hot dance club conditions where MDMA is often ingested.

5.3. Animal Studies III

Hypothesis IV

MDMA peripheral metabolism and not the formation of free radicals from tyrosine/dopamine is the limiting step in the development of long-term serotonergic changes.

5.3.1. The role of L-tyrosine in the development of MDMA-induced neurotoxicity in rats

The contribution of L-tyrosine or MDMA metabolism to the mechanisms underlying MDMA-induced brain 5-HT depletions was investigated. For this, a non-toxic dose of MDMA (7.5 mg/kg i.p.) alone or in combination with L-tyrosine was first administered. Secondly, using the same dosage regimen of MDMA, MDMA metabolic disposition was interfered with. Data suggest that while L-tyrosine can contribute to 5-HT toxicity, MDMA metabolism appears to be the limiting step in the neurotoxic process.

There is a sizeable amount of evidence indicating that increased free radical formation is responsible for MDMA-induced neurotoxicity. However, the source of free radicals responsible for toxicity has been an issue of an intense debate

during the last two decades. Following on from the experiments in hyperthermic response, it was decided to focus on the role of peripheral MDMA metabolism and L-tyrosine.

Since direct injection of MDMA into the brain fails to reproduce the serotonergic neurotoxicity seen following systemic administration[119,170], the first hypothesis postulates that peripheral metabolism of MDMA into toxic compounds is responsible for neurotoxicity. In rats, MDMA is mainly N-demethylated by several cytochrome P450 isozymes to form MDA. MDMA and MDA are further O-demethylenated to HHMA and HHA, respectively. HHMA and HHA are highly redox-unstable catechols and can be rapidly oxidized to their corresponding orthoquinones forming adducts with GSH and other thiol-containing compounds[116]. It has been postulated that thioether adducts of MDMA metabolites cross the blood-brain barrier using glutathione specific transporters[209], and once inside the brain they generate reactive oxygen species in a 5-HT transporter-dependent manner[97,212]. Interestingly, intracerebroventricular administrations of GSH and N-acetylcysteine conjugates of MDMA metabolites resemble not only the acute neurobehavioral effects of this drug but also its neurotoxic pattern[35]. The *in vivo* detection of quinone thioeter adducts in rats after MDMA administration and the evaluation of the neurotoxicity of some of these metabolic species, further strengthens the hypothesis that MDMA metabolic disposition contributes significantly to the induction of neurotoxicity[11].

On the other hand, a broad amount of evidence appears to indicate that reactive oxygen species generation responsible for 5-HT depletion is dependent upon the prolonged and excessive release of DA elicited by MDMA[8]. It is known that DA can enter the 5-HT terminal by means of the 5-HT transporter[96-98] and once inside the 5-HT terminal, be deaminated by MAO-B resulting in an elevated intracellular level of hydrogen peroxide that is reduced by iron to produce

hydroxyl radicals and a subsequent terminal degeneration[8,96,99]. Explaining why MDMA-induced 5-HT depletions also occur in regions such as the hippocampus or frontal cortex, which are sparsely innervated by DA, is the main drawback of this hypothesis. This important issue has been expanded upon recently[100]. These authors showed that MDMA increases the concentrations of tyrosine in the brain to cause a long-term depletion of 5-HT via the nonenzymatic, tyrosine hydroxylase-independent hydroxylation of tyrosine to DOPA and subsequently to DA via amino acid decarboxylase. However, this latter hypothesis assumes the pre-existence of hydroxyl free radicals responsible for the oxidation of tyrosine to DOPA.

5.3.1.1. MDMA metabolism and L-tyrosine plasma concentrations in rats following a toxic and non-toxic dose

In order to investigate these two hypotheses further, the differences in acute hyperthermia, L-tyrosine concentrations and MDMA metabolic disposition between a toxic versus a non-toxic dose of MDMA were analyzed to determine which, if any, of these parameters could account for differences in long-term toxicity. Although somewhat expected, plasma concentrations of MDMA and its main metabolites (MDA, HMMA, HMA) were significantly lower after the non-toxic versus the toxic dose of MDMA.

Increments in core body temperature and serum tyrosine concentrations were also significantly lower in rats treated with the low dose of MDMA despite significance when compared to saline-treated rats. Although hyperthermia caused by MDMA is a rather complex phenomenon, it is worth noting that the time course of sustained temperature elevation parallels the sustained elevation of plasma concentrations of MDA suggesting an important role for MDA in mediating MDMA-induced hyperthermia. To determine whether the rise in serum tyrosine concentrations after the low dose of MDMA was insufficient to cause

toxicity, a non-toxic dose of MDMA (7.5 mg/kg i.p.) in combination with different doses of L-tyrosine was administered. The combination of MDMA (7.5 mg/kg i.p.) and L-tyrosine (0.2 mmol/kg i.p.) yielded similar serum tyrosine concentrations to those found after the toxic dose of MDMA (15 mg/kg i.p.), however, this was not enough to cause long-term 5-HT depletions. It was necessary to increase the dose of L-tyrosine to 0.5 (mmol/kg i.p.) to find reductions of biomarkers of neurotoxicity. Under these experimental conditions the AUC of serum L-tyrosine concentrations were higher (~1.7 fold) than those found after the toxic dose of MDMA. Noteworthy, an excellent correlation has been demonstrated between serum tyrosine concentrations and brain tissues or brain microdyalisates following systemic tyrosine administration in the range of doses used in the present study[176]. Accordingly, these findings already suggest that L-tyrosine alone is not enough to explain MDMA-induced 5-HT depletions as we initially supposed.

5.3.1.2. Perfusion of MDMA into the Striatum in combination with L-tyrosine

The next step was therefore, to interfere with MDMA metabolic disposition. Previous data indicated that there is a direct relationship between the rise in core body temperature induced by MDMA and its metabolism. Accordingly, a non-toxic dose of MDMA (7.5 mg/kg i.p.) in combination with L-tyrosine (0.5 mmol/kg i.p.) at a low ambient temperature (15 ± 1°C) was administered. Lowering ambient temperature prevented MDMA-induced hyperthermia and resulted in a complete protection against 5-HT depletions. To demonstrate that the lack of toxicity was due to a reduction of MDMA metabolism and not to the lack of hyperthermia, an experiment in which MDMA was directly perfused into the striatum in combination with a systemic injection of L-tyrosine (0.5 mmol/kg i.p.) was performed. Core body temperature of these animals was kept around 39.5°C for 8 h by means of heating pads. It is worth noting that rats stay hyperthermic for no longer than 6 h when treated with a neurotoxic dose of

MDMA (15 mg/kg i.p.). It should also be noted that the dose of MDMA used in these experiments produces an increase in extracellular DA concentrations similar to that seen after a toxic MDMA dosing regimen[170,215]. Furthermore, such concentration of MDMA in the perfusate gives rise to the range of extracellular concentrations of MDMA observed following peripheral administration of neurotoxic doses of MDMA[119]. Despite hyperthermia, large amounts of extracellular MDMA and of L-tyrosine no significant reduction of striatal indole content was observed 7 days later. The contralateral striata, hippocampi and frontal cortices were also analyzed for 5-HT and 5-HIAA concentrations but no evidence of 5-HT depletion was found either. Although, hyperthermia is neither necessary nor sufficient to cause MDMA serotonergic neurotoxicity, it can strongly influence not only its long-term neurotoxic effects[76], but also MDMA metabolism. Accordingly, a non-toxic dose of MDMA (7.5 mg/kg i.p.) was administered and once core body temperature began to return to basal levels (2 h later), the heating pads were turned on to maintain rats hyperthermic for 8 h. It should be noted that by this time (2 h after MDMA), plasma L-tyrosine concentrations had returned to basal values. Interestingly, these animals showed significant reductions of 5-HT and 5-HIAA concentrations in all the brain regions examined 7 days later.

5.3.1.3. COMT inhibition and L-tyrosine

A further experiment was performed with entacapone to investigate if COMT inhibition would have the same effect as keeping rats hyperthermic in terms of long-term 5-HT depletions. Noteworthy, the entacapone/MDMA combination decreased 5-HT concentrations, an effect not related to changes on core body temperature, since entacapone caused a marked hypothermia when injected alone but did not significantly alter MDMA-induced hyperthermia. Furthermore, MDMA increased serum L-tyrosine levels to a higher degree than the

entacapone/MDMA combination suggesting that MDMA catechol metabolites and not tyrosine *per se*, instigate neurotoxicity.

5.3.1.4. Clinical Implications

Although the tyrosine/dopamine hypothesis cannot be ruled out from the mechanisms underlying MDMA-induced toxicity, especially when the doses of MDMA are high enough to make tyrosine levels rise sufficiently[100], findings appear to indicate that peripheral metabolism of MDMA may be the limiting step in the chain of biochemical events leading to 5-HT neurotoxicity.

5.4. General Discussion

It is necessary to make a few comments on the aspects of each experiment and the limitations that they present. The first question that presents itself is why certain studies were performed in humans and others in rats. With relation to the duration of inhibition of CYP2D6, the recovery of enzyme activity is based on *de novo* expression of the enzyme and hence studies must be performed *in vivo*. Studies have been performed in animals to estimate the turnover rate of cytochrome P450's but usually differences in values arise when similar studies are performed in humans[137]. Furthermore, in the case of CYP2D6 studies in rodents would be misleading since they do not possess this isozyme. Obviously, ethical questions come to mind when a clinical trial with a drug of abuse is performed and the design of the trial and the relatively strict criteria of inclusion attempt to assuage such matters. In this case, DEX and caffeine were administered 4 hours following the dose of MDMA to avoid any possible adverse reactions but at the same time to assure that CYP2D6 inhibition is optimal. At this time, MDMA peak effects have passed and the substance is entering the elimination phase of metabolism, so any possible pharmacodynamic interaction or synergy is avoided. Furthermore, a single recreational dose of MDMA (1.5

mg/kg) has not caused any serious adverse reactions in a controlled setting to this date. The sample size of the study is also kept minimal, but, however this presents limitations when analysing for the impact of certain infrequent *CYP2D6* alleles, such as *CYP2D6*10* and *CYP2D6*17*, and when considering the variance in the MR's of both DEX and caffeine seen here. Nevertheless, and at least in the case of DEX MR, the fold change in MR is so drastic that it allows for a reasonable calculation of CYP2D6 degradation half-life in this sample. Furthermore, results from caffeine MR's are strengthened by observations in caffeine and paraxanthine concentrations in plasma. An alternative design to calculate the duration of inhibition is possible and has been used in the past[218]. This design consists of a calculation on the basis of change in AUC over time rather than urinary MR. However, the design is more invasive on account of the increase in the amount of blood to withdraw and in the case of CYP2D6 and dextromethorphan the same limits such as variance in genotype and renal clearance would apply.

On the other hand studies involving changes in temperature and the inhibition of the clearance of a putative neurotoxic catechol metabolite of MDMA by entacapone cannot be performed in humans. Indeed, a clinical trial protocol was presented for review to the ethical committee of the Hospital del Mar, Barcelona involving the interaction of the COMT inhibitor, entacapone with MDMA (IMIMFTCL/MDMA/7). Unfortunately, the protocol was not approved [by Americans, not by Spaniards] and hence similar studies were performed in an animal model. However, experiments in animal models involving MDMA present certain difficulties, especially when interpreting results and applying them to the clinical consequences for humans. MDMA metabolism of humans and rodents differs significantly. In rats, the main pathway is N-demethylation to MDA whereas in humans this pathway is minor, with approximately 5% of the dose being cleared as MDA. In contrast, the main pathway in humans is O-

demethylenation to HHMA and subsequent methylation by COMT to HMMA. This difference could have consequences for the development of long-term serotonergic changes in both species. However, on a qualitative level it has been observed here that the inhibition of COMT and temperature changes results in changes in metabolism in the rat which leads to differences in markers of neurotoxicity and this alone can be applied to what occurs in MDMA users.

A further question is dose equivalency. Ecstasy is taken orally, but in the animal studies performed here MDMA was administered intraperitoneally. This would have obvious consequences for the absorbance kinetics of the drug which may lead to suggestions of incomparability of results. Furthermore, whether the so called neurotoxic dose regimen used is comparable to what is taken in humans is debatable. Many authors argue that the doses are equivalent following interspecies scaling calculations. However, others argue that calculations of dose equivalency should be based on the minimal dose which causes a pharmacological effect. In the case of animal models, doses as little as 1-2 mg/kg cause distinct behavioural effects[126], yet no long-term serotonergic changes have been observed as these doses. On the other hand, data suggest here that on a qualitative level MDMA metabolism and the enzymes involved therein have important roles in the development of both the acute and long-term effects of the drug.

6. CONCLUSIONS

The general hypothesis of this thesis is that MDMA metabolic disposition contributes to both acute toxicological effects and mid/long-term neurotoxicity. Studies evaluating the mechanism based inhibition of MDMA metabolism and its implication in acute toxicity have been performed in humans. Studies examining the contribution of COMT (phase II MDMA metabolic pathway), tyrosine (related to dopamine) and ambient temperature to neurotoxicity, for ethical reasons have been performed in animal models.

Conclusion I

This conclusion is related to the hypothesis that MDMA affects the activities of the cytochrome P450 enzymes responsible for its phase I metabolism and this has consequences for the acute and long-term toxicities of the drug.

Key results

- Following a single recreational dose of MDMA, the systemic exposure to the model CYP2D6 substrate dextromethorphan increases, with a concomitant decrease in exposure to its primary metabolite dextrorphan.
- The urinary dextromethorphan MR increased drastically, with the majority of subjects having a value greater than the antimode for assigning the PM phenotype.
- The MR takes more than ten days to recover to basal values. Using MR to model the recovery of inhibition a CYP2D6 degradation half-life of 46 h was calculated.
- A single recreational dose of MDMA does not appear to significantly alter the pharmacokinetics of caffeine, a marker of CYP1A2 activity.

Conclusions

There is rapid phenocopying from the EM phenotype of subjects to apparent PM status after a single recreational dose of MDMA, explaining the **inability to relate acute toxicity with *CYP2D6* genotype**.

There are toxicological implications for potent and prolonged inhibition of the metabolism of other CYP2D6 substrates that might be taken after a single dose of MDMA (including SSRI's, other amphetamines and DEX itself, which is also subject to abuse), and for understanding of the determinants of MDMA kinetics on continuous dose. **MDMA users must be warned that the risk of acute toxicity due to "binging" on ecstasy is drastically increased.** Evidence suggests that subsequent doses of MDMA taken up to one week later would present higher plasma concentrations and higher risk of acute toxicity. **It can even be suggested that MDMA users exposed to the drug on a frequent basis have a permanently compromised CYP2D6 activity.**

Conclusion II

This conclusion is related to the hypothesis that interindividual differences in the activity of catechol-O-methyltransferase (COMT) are relevant in the development of long-term toxicity of the drug.

Key results

- Inhibition of COMT activity in the rat by entacapone given before a neurotoxic dose regimen of MDMA resulted in a significant reduction of plasma concentrations of the downstream metabolic products of reactive catechols implicated in serotonergic changes.

- COMT inhibition exacerbated the long-term effects of MDMA on rat indole content measured in the frontal cortex, hippocampus and striatum 1 week after drug treatment.

Conclusions

The activity of **COMT plays a key role in MDMA metabolic disposition and the development of serotonergic neurotoxicity** caused by the drug in rats.

The COMT polymorphism in humans, which according to segregation analysis of family studies is caused by autosomal co-dominant alleles, leads to 3- to 4-fold differences in **COMT activity in the liver and could therefore be relevant for human users in terms of susceptibility to MDMA neurotoxicity**.

Conclusion III

*This conclusion pertains to the hypothesis that it is MDMA systemic metabolism and **not** MDMA-induced hyperthermia that is the limiting step in the development of long-term neurotoxicity.*

Key results

- The administration of single and multiple doses ("binging" or "stacking" regimen) of MDMA at 15°C blocked the hyperthermic response and long-term 5-HT depletion found in rats treated at 21.5°C. At 15°C, plasma concentrations of MDMA were significantly increased while those of three of its main metabolites were reduced when compared to rats treated at 21.5°C. By contrast, hyperthermia and indole deficits were exacerbated in rats treated at 30°C.

- Perfusion of MDMA into the striatum did not alter the core body temperature of rats. Acute hyperthermia and long-term loss of 5-HT and 5-HIAA content in the striatum were evident only when MDMA was given systemically.

- The effect of entacapone on 5-HT depletions caused by MDMA was independent of any effect of MDMA-induced hyperthermia.

Conclusions

Data suggest that **peripheral metabolism of MDMA plays a key role in the whole neurotoxic process.** It is tempting to speculate that the so far unclear relationship between the acute hyperthermic response and long-term damage produced by MDMA may rely on the large influence of core body temperature in the metabolic rate of this drug.

This finding has implications on both the temperature dependence of the mechanism of MDMA neurotoxicity and human use, as hyperthermia is often associated with MDMA use in humans. **The practice of "stacking" does not modify the risk of neurotoxic effects, especially in crowded and hot dance club conditions where MDMA is often ingested**.

Conclusion IV

This conclusion states that although the tyrosine/dopamine neurotoxicity hypothesis cannot be ruled out, MDMA peripheral metabolism appears to be the limiting step in the development of long-term serotonergic changes.

Key results

- The administration of a non-toxic dose of MDMA in combination with L-tyrosine produced no changes in brain 5-HT content while a similar increase

in serum tyrosine levels to those found after a toxic dose of MDMA was observed.

- The non-toxic dose of MDMA combined with a higher dose of tyrosine, caused long-term 5-HT depletions in rats treated at 21.5°C but not in those treated at 15°C, conditions known to decrease MDMA metabolism.

- Striatal perfusion of MDMA combined with tyrosine in hyperthermic rats did not cause 5-HT depletions. By contrast, rats treated with the non-toxic peripheral dose of MDMA under heating conditions or combined with entacapone or acivicin, which interfere with MDMA metabolism or increase brain MDMA metabolite availability respectively, showed significant reductions of brain 5-HT content.

Conclusions

Data suggest that while **L-tyrosine can contribute to 5-HT toxicity, MDMA metabolism appears to be the limiting step in the neurotoxic process**, although the tyrosine/dopamine hypothesis from the mechanisms underlying MDMA-induced toxicity cannot be ruled out, especially when the doses of MDMA are high enough to make tyrosine levels increase sufficiently.

General Conclusion

Data suggest that although the hyperthermia and increased dopamine release caused following MDMA administration may modulate MDMA-induced neurotoxicity, it is the peripheral metabolism of MDMA, most likely through the generation of reactive catechol metabolites, that plays a key role in the whole neurotoxic process. Interindividual differences in CYP2D6 activity and their correlation with MDMA-induced toxic episodes are rendered insignificant by the autoinhibition of this enzyme. However, users must be warned of the duration

and subsequent consequences of such an inhibition. It appears that differences in COMT activity are relevant in terms of susceptibility to MDMA neurotoxicity.

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8. APPENDICES

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DIAGRAMA DE FLUJO

DETERMINACIÓN: **CUANTIFICACIÓN DE DEXTROMETORFANO, DEXTRORFANO, 3-METOXIMORFINAN Y MORFINAN-3-OL EN ORINA HUMANO**

CÓDIGO DET.: | PNT: | LOTE:

A. PREPARACIÓN DE LAS MUESTRAS

GRADILLA, identificar # lote

1__+TUBO: identificar : CI (control inferior) [LOTE:]
2__+TUBO: identificar : CM (control medio) [LOTE:]
3__+TUBO: identificar : CS (control superior) [LOTE:]

4__+TUBO: identificar BLO1 (blanco de reactivo)
5__ + 2.5 mL de H2O MilliQ [DI:]

6__+TUBO: identificar BLO1 (blanco de orina sin estándar interno)
7__ + 2.5 mL de orina blanco*

8__+TUBO: identificar BLO2 (blanco de orina con estándar interno)
9__ + 2.5 mL de de orina blanco*

10__+10 TUBOS: identificar:
11__ CA11 y CA12: + 2.5 mL orina blanco* + 40 µl de solución 2,5 ug/mL [LOTE:][DI:]
12__ CA21 y CA22: + 2.5 mL orina blanco* + 25 µl de solución 25 ug/mL [LOTE:][DI:]
13__ CA31 y CA32: + 2.5 mL orina blanco* + 50 µl de solución 25 ug/mL [LOTE:][DI:]
14__ CA41 y CA42: + 2.5 mL orina blanco* + 80 µl de solución 25 ug/mL [LOTE:][DI:]
15__ CA51 y CA52: + 2.5 mL orina blanco* + 20 µl de solución 250 ug/mL [LOTE:][DI:]

16__vórtex 10 seg (a todos los tubos)

17__Adjuntar los TUBOS necesarios para las muestras e identificarlos con el código correspondiente.
18__ + 2.5 mL de la muestra correspondiente en cada tubo [DI:]
(Las muestras deben estar previamente homogeneizadas con vórtex)

19__+ 20 µl de la solución ISTD (100µg/mL) [LOTE:] [DI:] a todos los tubos excepto al tubo BLP1.
20__vórtex, 5 seg

21__+1 mL de β-glucuronidasa HP-1 (10mg/mL) en 0.1M citrato de sodio pH 5.0 [LOTE:] [DI:]
22__vórtex, 5 seg.; tapar los tubos

23__baño a 37ºC durante 16 horas/ baño a 55ºC durante 3 horas

24__+1 mL de NH4Cl/NH3 pH 9 [LOTE:] [DI:]
25__vórtex, 5 seg

26__centrifugar 5 minutos a 3500 rpm.

PROCESADOR ESL, identificar # lote

27 __+ COLUMNAS BOND-ELUT CERTIFY [LOTE:]; identificadas como los tubos

28 __+2 mL MeOH [LOTE:] [DI:]
29 __+2 mL H20 MilliQ (evitar secado columna) [LOTE:] [DI:]

DETERMINACIÓN: **CUANTIFICACIÓN DE DEXTROMETORFANO, DEXTRORFANO, 3-METOXIMORFINAN Y MORFINAN-3-OL EN ORINA HUMANO**		
CÓDIGO DET.:	PNT:	LOTE:

30 __decantar muestra
31 __pasar la muestra lentamente

32 __+2 mL H2O MilliQ [LOTE:] [DI:]
33 __+1 mL CH3COOH 1M [LOTE:] [DI:]
34 __+2 mL MeOH [LOTE:][DI: 163]
35 __secar las columnas 10 min. (presión máxima)
36__+ TUBOS DE ELUCIÓN: identificarlos y ponerlos bajo las columnas respectivas
37__+2 mL de CH3Cl:PrOH 80:20, NH3 2% [LOTE:] [DI:]
(preparación extemporanea)
38__evaporar a sequedad bajo N2 (40°C)
39__reconstruir en 100 µl fase móvil
vórtex, 5 seg

40__CAJA SOPORTE VIALES, identificar: #lote
41__+VIALES EPPENDORF con microvial, identificar como los tubos
42__transferir conten. tubos
43__ultracentrifugación (10000 g), 5 min

CAJA SOPORTE VIALES, identificar # lote

44__+ VIALES con microvial, identificar

45__capsular

B. ANÁLISIS INSTRUMENTAL

46__Instrumento: CL/FLD Código instrumento: _____

Método instrumental: _____

* BLANCO DE PLASMA: [LOTE:] [DI:]

FECHA OPERACIONES NOMBRE FIRMA

DIAGRAMA DE FLUJO CÓDIGO:

IMAS Institut Municipal d'Investigació Mèdica. IMIM	UNITAT DE FARMACOLOGIA *LABORATORI D'ANÀLISI*	DIAGRAMA DE FLUJO

c/ Doctor Aiguader, 80. E-08003 Barcelona. NIF: P-5890004-D. Tel. (+34) 93 221 10 09. Fax (+34) 93 221 32 37. E-mail: postmaster@imim.es

DETERMINACIÓN: **CUANTIFICACIÓN DE DEXTROMETORFANO, DEXTRORFANO, 3-METOXIMORFINAN Y MORFINAN-3-OL EN PLASMA HUMANO**

CÓDIGO DET.:	PNT:	LOTE:

A. PREPARACIÓN DE LAS MUESTRAS

GRADILLA, identificar # lote

1__+TUBO: identificar : CI (control inferior) [LOTE:]
2__+TUBO: identificar : CM (control medio) [LOTE:]
3__+TUBO: identificar : CS (control superior) [LOTE:]

4__+TUBO: identificar BLR1 (blanco de reactivo)
5__ + 1 mL de H2O MilliQ [DI:]

6__+TUBO: identificar BLP1 (blanco de plasma sin estándar interno)
7__ + 1 mL de plasma blanco*

8__+TUBO: identificar BLP2 (blanco de plasma con estándar interno)
9__ + 1 mL de de plasma blanco*

10__+10 TUBOS: identificar:
11__ CA11 y CA12: + 1 mL plasma blanco* + 40 µl de solución 0.25 ug/mL [LOTE:][DI:]
12__ CA21 y CA22: + 1 mL plasma blanco* + 20 µl de solución 2.5 ug/mL [LOTE:][DI:]
13__ CA31 y CA32: + 1 mL plasma blanco* + 40 µl de solución 2.5 ug/mL [LOTE:][DI:]
14__ CA41 y CA42: + 1 mL plasma blanco* + 80 µl de solución 2.5 ug/mL [LOTE:][DI:]
15__ CA51 y CA52: + 1 mL plasma blanco* + 120 µl de solución 2.5 ug/mL [LOTE:][DI:]

16__vórtex 10 seg (a todos los tubos)

17__Adjuntar los TUBOS necesarios para las muestras e identificarlos con el código correspondiente.
18__ + 1 mL de la muestra correspondiente en cada tubo [DI:]
(Las muestras deben estar previamente homogeneizadas con vórtex)

19__+ 20 µl de la solución ISTD (10µg/mL) [LOTE:] [DI:] a todos los tubos excepto al tubo BLP1.
20__vórtex, 5 seg

21__+500 ul de β-glucuronidasa HP-1 (10mg/mL) en 0.1M citrato de sodio pH 5.0 [LOTE:] [DI:]
22__vórtex, 5 seg.; tapar los tubos

24__baño a 37°C durante 16 horas/ baño a 55°C durante 3 horas

25__+500 ul de NH4Cl/NH3 pH 9 [LOTE:] [DI:]
26__vórtex, 5 seg

27__centrifugar 5 minutos a 3500 rpm.

PROCESADOR ESL, identificar # lote

28 __+ COLUMNAS BOND-ELUT CERTIFY [LOTE:]; identificadas como los tubos

29 __+2 mL MeOH [LOTE:] [DI:]
30 __+2 mL H20 MilliQ (evitar secado columna) [LOTE:] [DI:]

31 __decantar muestra
32 __pasar la muestra lentamente

33 __+2 mL H2O MilliQ [LOTE:] [DI:]
34 __+1 mL CH3COOH 1M [LOTE:] [DI:]
Secar las columnas 10 sec (presion maxima)
35 __+2 mL MeOH [LOTE:][DI:]
36 __secar las columnas 10 min. (presión máxima)
37__+ TUBOS DE ELUCIÓN: identificarlos y ponerlos bajo las columnas respectivas
38__+2 mL de CH3Cl:PrOH 80:20, NH3 2% [LOTE:] [DI:]
(preparación extemporanea)

39__evaporar a sequedad bajo N2 (40ºC)

40__reconstruir en 50 µl fase móvil
vórtex, 5 seg

41__ CAJA SOPORTE VIALES, identificar: #lote

42__ +VIALES EPPENDORF con microvial, identificar como los tubos
43__ transferir conten. tubos

44__ ultracentrifugación (10000 g), 5 min

CAJA SOPORTE VIALES, identificar # lote

43__ + VIALES con microvial, identificar

45__ capsular

ANÁLISIS INSTRUMENTAL

B. ANÁLISIS INSTRUMENTAL

46__*Instrumento:* CL/FLD *Código instrumento:* _____

Método instrumental: _____

* BLANCO DE PLASMA: [LOTE:] [DI:]

FECHA OPERACIONES NOMBREFIRMA

DIAGRAMA DE FLUJO CÓDIGO:

IMAS
Institut Municipal d'Investigació Mèdica. IMIM

UNITAT DE FARMACOLOGIA
LABORATORI D'ANÀLISI

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DIAGRAMA DE FLUJO

DETERMINACIÓN: **CUANTIFICACIÓN DE CAFEÍNA Y METABOLITOS EN ORINA HUMANA**

CÓDIGO DET.: | PNT: | LOTE:

A. PREPARACIÓN DE LAS MUESTRAS

GRADILLA, identificar # lote

1__+TUBO: identificar : CI (control inferior) [LOTE:]
2__+TUBO: identificar : CM (control medio) [LOTE:]
3__+TUBO: identificar : CS (control superior) [LOTE:]

4__+TUBO: identificar BLR1 (blanco de reactivo)
5__ + 150 µl de H2O MilliQ [DI:]

6__+TUBO: identificar BLO1 (blanco de orina sin estándar interno)
7__ + 150 µl de orina blanco*

8__+TUBO: identificar BLO2 (blanco de orina con estándar interno)
9__ + 150 µl de orina blanco*

10__+10 TUBOS: identificar:
11__ CA11 y CA12: + 150 µl orina blanco* + 30 µl de solución mix 10 ug/mL [LOTE:][DI:]
12__ CA21 y CA22: + 150 µl orina blanco* + 15 µl de solución mix 100 ug/mL [LOTE:][DI:]
13__ CA31 y CA32: + 150 µl orina blanco* + 30 µl de solución mix 100 ug/mL [LOTE:][DI:]
14__ CA41 y CA42: + 150 µl orina blanco* + 45 µl de solución mix 100 ug/mL [LOTE:][DI:]
15__ CA51 y CA52: + 150 µl orina blanco* + 90 µl de solución mix 100 ug/mL [LOTE:][DI:]

16__vórtex 10 seg (a todos los tubos)

17__Adjuntar los TUBOS necesarios para las muestras e identificarlos con el código correspondiente.
18__ + 150 µl de la muestra correspondiente en cada tubo [DI:]
(Las muestras deben estar previamente homogeneizadas con vórtex)

19__+ 30 µl NaOH 0.5 M (conversión de AFMU en AAMU) [LOTE:] [DI:]
20__vortex 5 seg, dejar 30 min a temperatura ambiente
21__+ 40 µl HCl 0.5 M (volver a pH de orina) [LOTE:] [DI:]
22__vortex 5 seg
23__+30 µl solución ISTD (9-metil urico 100µg/mL) [LOTE:] [DI:] a todos los tubos excepto al tubo BLO1.
24__vórtex, 5 seg

25__+100 µl DMF/AcOEt (30:70) [LOTE:] [DI:]
26__vórtex, 5 seg
27__+800 µl ACN [LOTE:] [DI:]
28__vórtex, 30 seg

29__ CAJA SOPORTE VIALES, identificar: #lote

30__ +VIALES EPPENDORF con microvial, identificar como los tubos
31__ transferir conten. tubos

32__ ultracentrifugación (10000 g), 5 min

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DETERMINACIÓN: **CUANTIFICACIÓN DE CAFEÍNA Y METABOLITOS EN ORINA HUMANA**		
CÓDIGO DET.:	PNT:	LOTE:

33__ CAJA SOPORTE VIALES, identificar # lote

34__ transferir contenido tubos
35__ + VIALES con microvial, identificar

36__ capsular

B. ANÁLISIS INSTRUMENTAL

37__Instrumento: CLAE/UV Código instrumento: _____

Método instrumental: _____

* BLANCO DE ORINA: [LOTE:] [DI:]

FECHA OPERACIONES NOMBREFIRMA

IMAS Institut Municipal d'Investigació Mèdica. IMIM	UNITAT DE FARMACOLOGIA *LABORATORI D'ANÀLISI*	DIAGRAMA DE FLUJO

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DETERMINACIÓN: **CUANTIFICACIÓN DE CAFEÍNA Y PARAXANTINA EN PLASMA HUMANO**

CÓDIGO DET.:	PNT:	LOTE:

A. PREPARACIÓN DE LAS MUESTRAS

GRADILLA, identificar # lote

1__+TUBO: identificar : CI (control inferior) [LOTE:]
2__+TUBO: identificar : CM (control medio) [LOTE:]
3__+TUBO: identificar : CS (control superior) [LOTE:]

4__+TUBO: identificar BLR1 (blanco de reactivo)
5__ + 500 µl de H2O MilliQ [DI:]

6__+TUBO: identificar BLP1 (blanco de plasma sin estándar interno)
7__ + 500 µl de plasma blanco*

8__+TUBO: identificar BLP2 (blanco de plasma con estándar interno)
9__ + 500 µl de plasma blanco*

10__+10 TUBOS: identificar:
11__ CA11 y CA12: + 500 µl plasma blanco* + 5 µl de solución 1 [LOTE:][DI:]
12__ CA21 y CA22: + 500 µl plasma blanco* + 20 µl de solución 1 [LOTE:][DI:]
13__ CA31 y CA32: + 500 µl plasma blanco* + 5 µl de solución 2 [LOTE:][DI:]
14__ CA41 y CA42: + 500 µl plasma blanco* + 10 µl de solución 2 [LOTE:][DI:]
15__ CA51 y CA52: + 500 µl plasma blanco* + 20 µl de solución 2 [LOTE:][DI:]

16__vórtex 10 seg (a todos los tubos)

17__Adjuntar los TUBOS necesarios para las muestras e identificarlos con el código correspondiente.
18__ + 500 ul de la muestra correspondiente en cada tubo [DI:]
(Las muestras deben estar previamente homogeneizadas con vórtex)

19__30 µl solución ISTD (diphyline 10µg/mL) [LOTE:] [DI:] a todos los tubos excepto al tubo BLP1.
20__vórtex, 5 seg

21__+ 200 mg sulfato de amoniaco [LOTE:] [DI:]
22__vortex hasta disolución
23__+ 5 mL acetato de etil [LOTE:] [DI:]
24__vortex 5 sec
24__agitador basculante 40mov/min, 20 min
25__centrifugar 3500 rpm, 10 min

26__GRADILLA, identificar # lote

27__+ TUBOS: identificadas como los otros

28__ transferir fase organ.
29__evaporar bajo N2, 40°C [T: °C]
30__+100 µl 0.05% CH3COOH [LOTE:] [DI]

31__ CAJA SOPORTE VIALES, identificar: #lote

DETERMINACIÓN: **CUANTIFICACIÓN DE CAFEÍNA Y PARAXANTINA EN PLASMA HUMANO**		
CÓDIGO DET.:	PNT:	LOTE:

32__ +VIALES EPPENDORF con microvial, identificar como los tubos
33__ transferir conten. tubos

34__ ultracentrifugación (10000 g), 5 min

35__ CAJA SOPORTE VIALES, identificar # lote

36__ transferir contenido tubos
37__ + VIALES con microvial, identificar

38__ capsular

B. ANÁLISIS INSTRUMENTAL

46__Instrumento: CLAE/UV Código instrumento: _____

Método instrumental: _____

* BLANCO DE PLASMA: [LOTE:] [DI:]

FECHA OPERACIONES NOMBREFIRMA

DIAGRAMA DE FLUJO CÓDIGO:

IMAS Institut Municipal d'Investigació Mèdica. IMIM	UNITAT DE FARMACOLOGIA *LABORATORI D'ANÀLISI*	DIAGRAMA DE FLUJO

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DETERMINACIÓN: **CUANTIFICACIÓN DE MDMA, MDA, HMMA Y HMA EN PLASMA HUMANO**		
CÓDIGO DET.: **7018**	PNT: **MF018E01**	LOTE:

A. PREPARACIÓN DE LAS MUESTRAS

GRADILLA, identificar # lote

1__+TUBO: identificar : CI (control inferior) [LOTE:]
2__+TUBO: identificar : CM (control medio) [LOTE:]
3__+TUBO: identificar : CS (control superior) [LOTE:]

4__+TUBO: identificar BLR1 (blanco de reactivo)
5__ + 1 mL de H2O MilliQ [DI:]

6__+TUBO: identificar BLP1 (blanco de plasma sin estándar interno)
7__ + 1 mL de plasma blanco*

8__+TUBO: identificar BLP2 (blanco de plasma con estándar interno)
9__ + 1 mL de plasma blanco*

10__+10 TUBOS: identificar:
11__ CA11 y CA12: + 1 mL plasma blanco* + 25 µl de solución 1 [LOTE:][DI:]
12__ CA21 y CA22: + 1 mL plasma blanco* + 10 µl de solución 2 [LOTE:][DI:]
13__ CA31 y CA32: + 1 mL plasma blanco* + 20 µl de solución 2 [LOTE:][DI:]
14__ CA41 y CA42: + 1 mL plasma blanco* + 30 µl de solución 2 [LOTE:][DI:]
15__ CA51 y CA52: + 1 mL plasma blanco* + 40 µl de solución 2 [LOTE:][DI:]

16__vórtex 10 seg (a todos los tubos)

17__Adjuntar los TUBOS necesarios para las muestras e identificarlos con el código correspondiente.
18__ + 1 mL de la muestra correspondiente en cada tubo [DI:]
(Las muestras deben estar previamente homogeneizadas con vórtex)

19__+ 20 µl de la solución ISTD [LOTE:] [DI:] a todos los tubos excepto al tubo BLP1.
20__vórtex, 5 seg

21__+1 mL de TAS 1M pH=5.2 [LOTE:] [DI:]
22__+ 50µl de β-glucuronidasa HP-2. [LOTE:] [DI:]
23__vórtex, 5 seg.; tapar los tubos

24__baño a 37°C durante 16 horas

25__+1 mL de TFP 0.1M pH 6 [LOTE:] [DI:]
26__vórtex, 5 seg

27__centrifugar 5 minutos a 3500 rpm.

PROCESADOR ESL, identificar # lote

28 __+ COLUMNAS BOND-ELUT CERTIFY [LOTE:]; idenmtificadas como los tubos

29 __+2 mL MeOH [LOTE:] [DI:]
30 __+2 mL TFP 0.1M pH 6 (evitar secado columna) [LOTE:] [DI:]

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DETERMINACIÓN: **CUANTIFICACIÓN DE MDMA, MDA, HMMA Y HMA EN PLASMA HUMANO**		
CÓDIGO DET.: **7018**	PNT: **MF018E01**	LOTE:

31 __decantar muestra
32 __pasar la muestra lentamente

33 __+1 mL de ácido acético 1M [LOTE:] [DI:]
34 __+6 mL MeOH [LOTE:][DI:]
35 __secar las columnas 2 min. (presión máxima)
36__+ TUBOS DE ELUCIÓN: identificarlos y ponerlos bajo las columnas respectivas
37__+2 mL de acetato de etilo al 2% NH3 [LOTE:] [DI:]
(preparación extemporanea)

38__+20 µL de MBTFA [LOTE:] [DI:]

39__evaporar a sequedad bajo N2 (40°C)

40__estufa de vacío 1 hora

41__+50 µL de MBTFA [LOTE:] [DI:]
vórtex 10 seg; tapar

42__baño seco a 70 °C durante 45 min

CAJA SOPORTE VIALES, identificar # lote

43__ + VIALES con microvial, identificar

44__ transferir contenido tubos

45__ capsular

B. ANÁLISIS INSTRUMENTAL

46__Instrumento: CG/EM Código instrumento: _____

Método instrumental: _____

NOTA: Los viales de limpieza del inyector deben contener acetona.

* BLANCO DE PLASMA: [LOTE:] [DI:]

FECHA OPERACIONES NOMBREFIRMA

X