MDMA PHARMACOLOGY IN HUMANS AND SEROTONERGIC EFFECTS

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A la meva família
Al Carlos

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ABSTRACT

3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is one of the most abused recreational drugs in the world. It has been extensively reported that this drug inhibits its own metabolism by inhibiting a polymorphic liver enzyme, CYP2D6, which is responsible for the clearance of one quarter of drugs used in therapeutics. This phenomenon has important clinical implications, since MDMA users display a higher prevalence of psychopathology, particularly of mood disorders, compared to control population. Importantly, these psychiatric diseases are treated with drugs most of them substrate of this enzyme. In addition, it is not elucidated how MDMA is still metabolically cleared even after repeated drug doses. Therefore, the first part of this thesis was focused on studying the metabolic autoinhibition by MDMA assessing several liver enzyme activities in men and women.

Although MDMA pharmacology is well established, is it still not clear which is the mechanism of action of the drug. MDMA interacts with the serotonergic system at several levels, but nowadays is technically difficult to study the serotonergic function in living human brain. Imaging methods are limited by a number of factors. Thus, it would be advantageous to have a reliable peripheral index of the serotonergic activity in the blood. The second part of this thesis presents the development of several approaches aimed to assess whether the serotonin transporter in platelets can be used as a peripheral biomarker for central serotonergic activity, and determine if it plays any role in drug mechanism of action.

ABSTRACT (català)

La 3,4-metilendioximetanfetamina (MDMA, èxtasi) és una de les drogues més consumides al món. Aquesta droga inhibeix el seu propi metabolisme, inhibint un enzim polimòrfic del fetge, el CYP2D6, que és el responsable de l'eliminació d'una quarta part dels medicaments. Aquest fet té implicacions clíniques rellevants, ja que els consumidors de MDMA presenten una prevalença de psicopatologia més alta respecte a la població no consumidora, i moltes de la patologies psiquiàtriques es tracten amb fàrmacs substrats d'aquest enzim. A més, encara no s'ha discernit com aquesta droga pot ser eliminada de l'organisme, inclús després d'haver-ne consumit dosis de manera repetida. Així doncs, la primera part d'aquesta tesi es centra en estudiar l'autoinhibició de la MDMA determinant l'activitat de diferents enzims del fetge, en homes i dones.

Encara que la farmacologia de la MDMA està descrita a fons, no està del tot clar quin és el seu mecanisme d'acció. La MDMA interactua amb el sistema serotonèrgic de diverses maneres, però avui en dia és molt difícil estudiar tècnicament el sistema serotonèrgic en el cervell humà. Les tècniques d'imatge estan limitades per molts factors, i per tant, seria molt útil tenir un índex perifèric a la sang de l'activitat serotonèrgica al sistema nerviós central. La segona part d'aquest tesi s'enfoca en el desenvolupament de tècniques per determinar a diferents nivells si el transportador de la serotonina a les plaquetes podria ser un bon biomarcador perifèric de la seva activitat al cervell, i d'aquesta manera veure si el sistema serotonèrgic està implicat en el mecanisme d'acció de la MDMA.

PREFACE

The present thesis is based on two main chapters:

1) MDMA PHARMACOKINETICS, GENETICS AND GENDER

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a widely abused substituted amphetamine. This drug is a mechanism-based inhibitor (MBI) of the cytochrome P450 isoform 2D6 (CYP2D6) enzyme, which is the main responsible of MDMA first-pass metabolism in humans. This phenomenon is associated with a decrease in the amount of effective enzyme such that recovery of activity depends on its *de novo* synthesis, with a time course dictated by the half-life of the enzyme. Recent clinical data reported that after administering 1.5 mg/kg MDMA to 15 healthy male subjects, MDMA rapidly and completely inhibited CYP2D6. Enzyme recovered 90% of its activity after 10 days with a recovery half-life of 46.6 hours (O'Mathuna et al., 2008).

It is known that MDMA users are not exclusive users of the drug and belong to a poly-drug abusing population. Besides, MDMA use has been associated with psychiatric symptoms and psychological problems that may require psychopharmacological treatment with antidepressant drugs, some of which are known to be CYP2D6 substrates (Schifano et al., 1998). Therefore, recreational MDMA users are exposed to a higher probability of relative overdose and, therefore, an increased risk of suffering adverse effects from CYP2D6 substrates including MDMA (repeated doses in the same session).

The fact that MDMA presents a metabolic autoinhibition raises the question of how MDMA is metabolically cleared from the body once CYP2D6 has been inhibited. Some data report that the contribution of CYP2D6 to MDMA metabolism in humans is not >30%, therefore other CYP isoenzymes may contribute to metabolize MDMA (Segura et al., 2005).

The main goal of the first part of this dissertation was to study MDMA metabolic disposition in the body, once CYP2D6 had been irreversibly inhibited by the drug. Results are reported in the first three articles, in which the activity of three CYPs is assessed through different probe drugs, and a comparison between genders is done.

Data shows that genetic polymorphism of CYP2D6 and coadministration of CYP2D6 inhibitors may have less impact on MDMA disposition than previously thought, whereas the role of metabolism by other cytochrome P450 enzymes and renal excretion assumes greater importance.

2) MDMA PHARMACODYNAMICS AND SEROTONERGIC EFFECTS

Serotonin (5-HT) is an essential central nervous system (CNS) neurotransmitter that modulates neural activity and a wide range of neurophysiological processes such as digestion, energy balance, pain perception, and mood (Berger et al., 2009). Dysregulation of the serotonergic system has been implicated in the pathogenesis of several psychiatric and neurological disorders such as depression, anxiety, and bipolar disorder. 5-HT levels are regulated by the serotonin transporter (5-HTT), which is expressed in presynaptic serotonin nerve terminals, on axons and 5-HT cell bodies in the raphe nuclei, but also in platelets and

lymphocytes. Its main function is to regulate serotonergic transmission, selectively up-taking 5-HT, as well as Na^+ and Cl^- ions from the synaptic cleft into the presynaptic terminal, and releasing K^+ ions (Rudnick, 2006). The regulation of this transporter has become of clinical relevance, since the molecular sites for 5-HT binding and reuptake, and for 5-HTT auto-regulation have proved to be viable targets for pharmacological intervention in various disorders including acute and chronic pain, migraine, anxiety, depression, and hypertension (Berger et al., 2009).

Besides, several drugs of abuse, including MDMA, amphetamines and cocaine are substrates for 5-HTT and increase 5-HT levels both in neurons, and plasma (Rothman and Baumann, 2003, Zolkowska et al., 2006).

Over the years, several reports show that MDMA interacts with the serotonergic system at different levels; (i) inhibiting the enzyme tryptophan hydroxylase, responsible for 5-HT synthesis (Stone et al., 1987), (ii) altering 5-HTT gene expression (Biezonski and Meyer, 2010) (iii), and reducing 5-HTT density and function (Schenk et al., 2007) probably due to an internalization of the 5-HTT from the cell surface to intracellular vesicles (Kivell et al., 2010). Altogether, MDMA administration has been widely proven to cause long-term depletion of 5-HT in rodents (Lew et al., 1996), non-human primates (Banks et al., 2008), and humans (McCann et al., 1998, McCann et al., 2005).

MDMA mechanism of action is not still fully understood. In humans, neuroimaging studies are the best approach to study serotonergic function in the human living brain before and after drug administration. Nevertheless imaging methods are limited by a number of factors, especially the cost and the selectivity of the available radioligands. At the

present time is technically difficult to measure 5-HTT binding and function *in* vivo; although an impressive number of radio ligands have been synthesized and validated, there is still a lack of suitable ligands for a large part of the 5-HT system. However, data from neuroimaging studies globally agrees in that after MDMA administration, 5-HTT levels are lower in active or abstinent human MDMA users compared to controls (McCann et al., 1998, Reneman et al., 2002a, Reneman et al., 2002b, McCann et al., 2005).

Due to the constraints aforementioned in neuroimaging studies, the idea of using a peripheral biomarker of the central serotonergic activity that could overcome the difficulty in obtaining human tissues and the ethical limitations found in human experiments is becoming relevant and potentially useful.

Outside the CNS, 5-HTT is also present in platelets and in lymphocytes. It is generally accepted that the protein is identical in both tissues, since the gene that regulates its expression is the same (Hoffman et al., 1991). Besides, the polymorphisms affecting its expression have been shown to influence function in human platelets and brain in similar manner (Blakely et al., 1991, Hoffman et al., 1991).

Therefore, it would be potentially valuable to use 5-HTT in platelets as peripheral biomarker of 5-HTT activity in the brain.

The main objective of the second part of this work was to develop different techniques that measure 5-HTT activity in platelets, and in this way elucidate whether serotonergic system is implicated in the mechanism of action of MDMA, and therefore, is the responsible for the drug pharmacological effects observed.

In order to solve this question, several techniques were developed an carried out to assess the peripheral serotonergic system activity;

a) Measure 5-HT whole blood concentrations.

In the literature there is no reference method that measures reliably 5-HT levels in blood. We developed and validated a sensitive method to do so, taking into account several factors in sample manipulation that could be the responsible for the differences reported over the years.

b) Determine 5-HTT gene expression in peripheral lymphocytes.

Pre-clinical data referring to 5-HTT gene expression levels after MDMA administration is contradictory, and clinical data is lacking. We developed a straight forward technique to measure 5-HTT gene expression before and after MDMA intake.

c) Determine 5-HTTLPR genotype in peripheral leukocytes.

5-HTTLPR genotype is known to affect 5-HTT level of expression and functionality. Therefore, classifying each subject by the genotype is required in order to better understand the implication of serotonergic system in drug response.

These techniques were used to determine serotonergic activity in two clinical trials:

-Clinical trial performed in 13 healthy recreational users of MDMA. 5-HTTLPR was genotyped, and 5-HTT gene expression was measured before and after drug administration (study performed at the University of Maastricht, Dr. JG Ramaekers).

-Pilot study carried out at IMIM, with 8 healthy recreational users of MDMA. Subjects were exposed to the new drug of abuse mephedrone (MEPH), using MDMA as a reference drug for the study of MEPH

clinical pharmacology. 5-HT blood levels, 5-HTT gene expression and 5-HTTLPR genotyping were determined.

Data reported show a significant change in serotonergic peripheral system after MDMA administration. This alteration is reflected in 5-HT whole blood concentrations and 5-HTT gene expression. 5-HT blood levels are highly correlated to MDMA and MEPH pharmacological effects being different depending on 5-HTTLPR genotype.

Summary

1. BRIEF INTRODUCTION TO MDMA

Ecstasy is the popular "street" name for a substance identified chemically as 3,4-methylenedioxymethamphetamine or N-methyl-3,4-methylenedioxy-amphetamine. The initial letters of the major portions of the latter name (Methylene*d*ioxy-Meth*a*mphetamine) give rise to the acronym MDMA, by which this substance is commonly designated in the clinical and research literature. As the name implies, MDMA is a derivative of methamphetamine (known by such street names as "speed," "crystal" and "meth" among others) and its parent compound amphetamine.

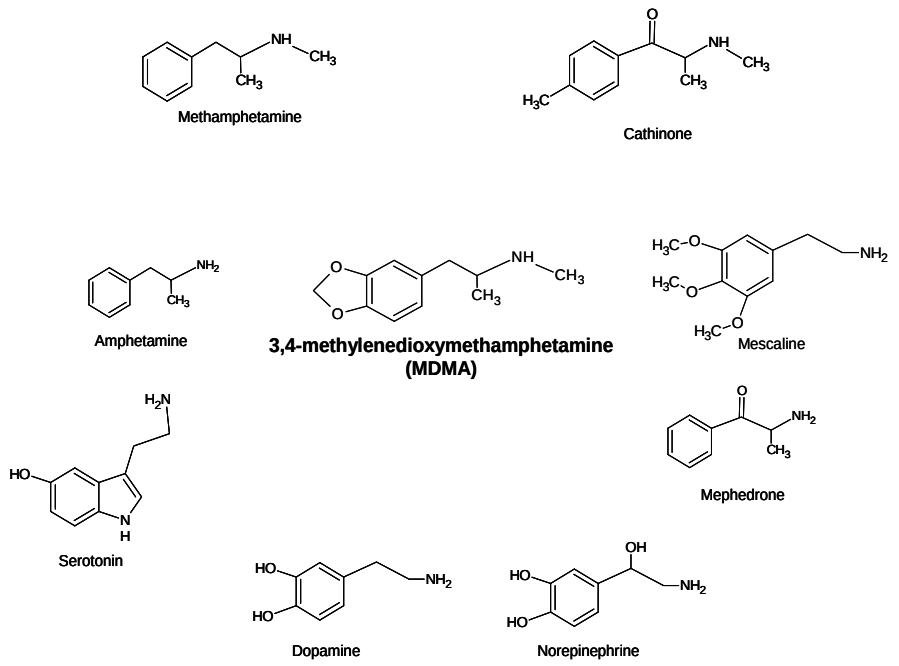

Figure 1. Chemical structures of MDMA and relative compounds.

Ecstasy differs from amphetamine and methamphetamine in one important respect. As shown in figure 1, it has a methylenedioxy (-O-CH2-O-) group attached to positions 3 and 4 of the aromatic ring of the amphetamine molecule (i.e., it is "ringsubstituted").
In this respect, it resembles the structure of the hallucinogenic substance mescaline. As a result, the pharmacological effects of MDMA are a blend of those of the amphetamines and mescaline, as will be described later.

The drug, synthesized in 1912 but never marketed, became popular in the 1970s and 1980s due to the induction of feelings of euphoria, friendliness, closeness to others, and empathy after its use. These properties have been named "entactogen" by some authors (Cami and Farre, 1996) and seem the basis of its use in psychotherapy during the 1980s until it was included in the schedule I psychotropic substances list (Steele et al., 1994). MDMA has become increasingly popular, being primarily consumed by young people in large dance and music environments ("raves") and sometimes in small social settings (Cami and Farre, 1996).

MDMA is almost always taken by mouth and is prepared as single-dose tablets for this purpose. The typical dosage range of MDMA for recreational use varies from 50 mg to 150 mg (Theune et al., 1999) but the amount per tablet in different batches varies 70-fold or more, from almost zero to well over 100 mg (Milroy et al., 1996, Sherlock et al., 1999).

The *desired effects* seek by the users are similar to those of other amphetamines. Physically, it produces a postponement of fatigue and sleepiness, sexual arousal and an increase of energy. Other psychobiological effects are described as well-being, greater sociability, extraversion, and a higher sense of closeness to the other people.

The latter effects have given rise to claim that MDMA could be a potential value in psychotherapy (Bouso et al., 2008).

The *undesired effects* comprehend physical and psychological adverse events. Physically, an increase of tension occurs; in muscles, jaw clenching, tooth grinding (bruxism) and constant movement of the legs. The increased muscle temperature together with a direct action of the drug on the thermoregulatory system in the brain, leads to an increase in body temperature, along with a higher heart rate (HR) and blood pressure. Besides, headache, nausea, loss of appetite and dry mouth are commonly reported during the drug experience and afterwards.
Psychologically, acute effects usually reported are hyperactivity, flight of ideas (difficulty in focusing), and insomnia. Other adverse effects are anxiety, mild hallucinations and agitation. The second day after drug use, the most common mental or mood complaints are difficulty in concentrating, depression, anxiety and fatigue.

In addition to ecstasy acute medical complications, long-term psychiatric disorders and neuropsychological deficits are the major health concerns regarding the misuse of this substance (de Sola et al., 2008, Martin-Santos et al., 2010, Cuyas et al., 2011).

MDMA and other ring-substituted amphetamine derivatives are potent indirect monoaminergic agonists and reuptake inhibitors. Like other transporter substrates, MDMA binds to plasma membrane transporters and are translocated into the cytoplasm where they promote non-exocytotic transmitter release (Rudnick and Wall, 1992, Verrico et al., 2007). An acute dose of MDMA can release around 80% of central 5-HT stores (Green et al., 1995, Huether et al., 1997). The precise mechanism underlying transporter-mediated release is not completely understood but

probably involves drug-induced phosphorylation of cytoplasmic domains on the transporter, which triggers reversal of normal transporter flux (i.e., reverse transport) (Robertson et al., 2009, Sitte and Freissmuth, 2010).

Although MDMA main effects are on 5-HT, it also boost dopamine (DA), noradrenaline (NA), acetylcholine, and histamine (Liechti and Vollenweider, 2001). Many of these neurochemical effects are interlinked. For instance, the MDMA-induced activation of 5-HT_2A receptors facilitates the release of DA (Huether et al., 1997), whereas the activation of 5-HT_2B facilitates the release of 5-HT (Launay et al., 2006, Doly et al., 2008, Doly et al., 2009, Diaz et al., 2012).

MDMA is a mild inhibitor of monoamine oxidase (MAO) and also has some direct actions in several types of receptors including the M1 muscarinic receptor, the α2-adrenergic receptor and the histamine H1 receptor. MDMA inhibits tryptophan hydroxylase (TH), the rate-limiting enzyme of serotonin synthesis, decreasing the formation of 5-HT (Green et al., 1995, Cole and Sumnall, 2003b).

MDMA is a ring-substituted amphetamine widely abused by the youth for its euphoric and empathic effects. Although its mechanism of action is not completely understood, it is probably related to its action as an indirect monoaminergic agonist and reuptake inhibitor on serotonergic and dopaminergic transporters.

2. MDMA PHARMACOKINETICS, GENETICS AND GENDER

2.1. MDMA pharmacokinetics

MDMA is readily absorbed from the intestinal tract and reaches its peak concentration in the plasma about 2 hours after oral administration (Mas et al., 1999, Farre et al., 2004). Doses of 50 mg, 75 mg and 125 mg to healthy human volunteers produced peak blood concentrations of 106 ng/mL, 131 ng/mL and 236 ng/mL respectively. These concentrations are quite low, because a high hepatic metabolic clearance and the fact that drug passes readily into the tissues, and much of it is bound to tissue constituents. The time-course of blood concentrations of MDMA and its pharmacologic effects (eg, hormone secretion, cardiovascular effects) rise and fall with a similar profile. Both peak concentrations and peak effects were obtained between 1 and 2 hours and decreased to return to baseline values 4–6 hours after drug administration for most pharmacologic effects (Mas et al., 1999, de la Torre et al., 2004a).

The drug is broken down metabolically, mainly in the liver, where an enzyme designated the cytochrome P450 2D6 isoenzyme (CYP2D6) is chiefly responsible (Wu et al., 1997). However, several different enzymes are involved in its degradation (Maurer et al., 2000), and some of these appear to be saturated at relatively low concentrations of the drug. Consequently, as the dose is increased and the higher-affinity enzymes are saturated, disproportionately large increases in blood and brain concentrations of the drug occur (de la Torre et al., 2000a). Therefore small increases in dosage may carry the risk of large increases in toxicity. A further aspect to be considered in MDMA pharmacokinetics is its

enantioselective disposition. The O-demethylenation regulated by CYP2D6 exhibits some degree of enantioselectivity toward the S enantiomer. In an experiment in which MDMA racemate (40 mg) was administered to healthy volunteers, plasma concentrations of (R)-MDMA exceeded those of the S enantiomer [ratio R:S of the area under the curve (AUC), 2.4 ± 0.3], and the plasma half-life of (R)-MDMA (5.8 ± 2.2 hours) was significantly longer than that of the S enantiomer (Fallon et al., 1999).

Elimination half-life for MDMA is about 8 hours (Mas et al., 1999, Maurer et al., 2000). Because it takes about 5 half-lives (i.e., about 40 hours for MDMA) for over 95% of the drug to be cleared from the body, this may explain the persistence of troublesome after-effects for one or two days after use. In addition, some of the metabolites of MDMA are still pharmacologically active, especially 3,4-methylenedioxyamphetamine (MDA).

Metabolism of MDMA in humans is rather complex (de la Torre et al., 2000a, de la Torre et al., 2000b, de la Torre et al., 2004b, Wenk et al., 2004). Two main pathways are involved in MDMA metabolic clearance: (1) the O-demethylenation step is the main pathway in human, regulated by several isoforms of cytochrome P450, and gives rise to 3,4-dihydroxymethamphetamine (HHMA) and (2) N-dealkylation leading to the minor metabolite MDA, which is also O-demethylenated giving rise to 3,4-dihydroxyamphetamine (HHA). Both HHMA and HHA are O-methylated in a reaction regulated by catechol-O-methyltransferase (COMT) or form sulphate and glucuronide conjugates. A fraction of HHMA and HHA can undergo further autooxidation to the corresponding ortho-quinones, which are conjugated with glutathione (GSH) to form glutathionyl adducts (Perfetti et al., 2009) (see figure 2).

The O-demethylenation step, which gives rise to HHMA, shows *in vitro* biphasic kinetics with high and low affinity components. The high affinity component is mainly regulated by the CYP2D6, but several other P450 isozymes have the capacity to contribute to the microsomal oxidative metabolism of methylenedioxyamphetamines.
The low affinity component is regulated by the cytochrome P450 1A2 isoenzyme (CYP1A2) and, to a lesser extent, by CYP2B6 and CYP3A4 (Kreth et al., 2000). In a more recent study, CYP2C19 was reported to contribute to the O-demethylenation of MDMA.

MDMA metabolic clearance accounts for about 75% of plasma clearance and 30% of its metabolism is regulated by CYP2D6 (de la Torre et al., 2000b, Segura et al., 2005).

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. Acute MDMA intoxication has been associated with high plasma concentrations but not with non-functional CYP2D6 genotypes that may explain MDMA accumulation in the body (Gilhooly and Daly, 2002). These observations suggest that acute toxicity episodes are related to MDMA exposure at high doses and/or to the fact that MDMA disposition in the body follows nonlinear pharmacokinetics (de la Torre et al., 2000a, Farre et al., 2004, Baumann et al., 2007).

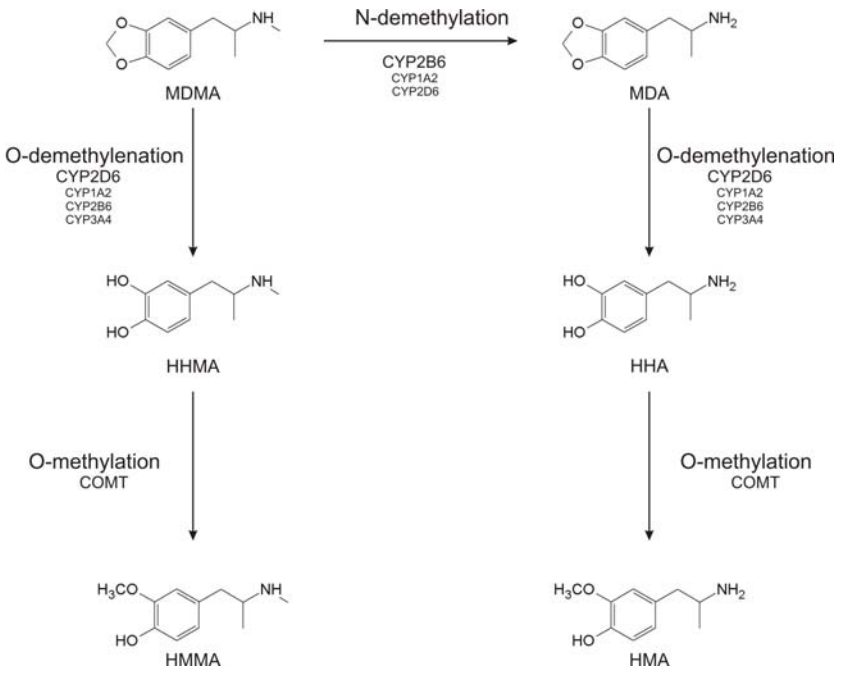

Figure 2. Pathways of 3,4-methylenedioxymethamphetamine (MDMA) metabolism in humans. The parent compound is N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) and O-demethylenated to form 3,4-dihydroxymethamphetamine (HHMA). HHMA is further O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA). Abbreviations: CYP = isozyme of cytochrome P450; COMT = catechol-O-methyl transferase; HMA = 3-methoxy,4-hydroxyamphetamine.

2.1.1. Mechanism-based inhibition (MBI)

MDMA interacts with CYP2D6 both as a substrate and as an inhibitor of its own metabolism by the formation of a complex between CYP2D6 and MDMA (Heydari et al., 2004, Yang et al., 2006), the formation of which has been shown *in vitro* in both rats and humans (Delaforge et al., 1999). It is postulated that the methylenedioxy group present in the chemical structure of MDMA is responsible for the metabolic autoinhibition. Mathematical modelling of MDMA pharmacokinetic data in humans

indicates that a period of 280 hours without dosing is required for the activity of CYP2D6 to return to normal (O'Mathuna et al., 2008, Yubero-Lahoz et al., 2011). In practice, inhibition of CYP2D6 activity is not >30% because of the contribution of alternative CYP isoenzymes, including CYP3A4, following its inactivation (Baumann and Rothman, 2009).

A recent article showed that MBI of CYP2D6 occurs shortly after a single recreational dose, inactivating most hepatic CYP2D6 within 2 h, and returning to a basal level of CYP2D6 activity after at least 10 days (O'Mathuna et al., 2008). MBI is associated with a decrease in the amount of effective enzyme such that recovery of activity depends on its *de novo* synthesis (Liston et al., 2002).

CYP2D6 genotypes are explained below. Briefly, mention that in MBI there is a rapid phenocopying to apparent poor metabolizer (PM) status after a single dose of MDMA, which signifies that within 2 h subjects display the PM phenotype after drug intake, irrespective of their original genotype. Inhibition of CYP2D6 by MDMA and the recovery of its activity, using the dextromethorphan (DEX)/dextrorphan (DOR) metabolic ratio (MR) as a biomarker, have already been reported (O'Mathuna et al., 2008) in male subjects. Moreover, in this study a phenocopying phenomenon was observed in the 67% of male subjects.

Therefore, recreational MDMA users are exposed to a higher probability of relative overdose and an increased risk of suffering adverse effects from CYP2D6 substrates (Farre et al., 2004). Little is known about the pharmacology of MDMA in women and how this drug affects CYP2D6 activity. Preliminary reports suggest marked sex differences in MDMA plasma concentrations (Kolbrich et al., 2008).

MDMA presents non-linear pharmacokinetics due its metabolic autoinhibition; shortly after a single recreational dose of MDMA, most hepatic CYP2D6 is inactivated. Clinical data is lacking regarding the recovery of CYP2D6 over time and the measurement of other CYPs activities while the former is inhibited.

2.2. MDMA Pharmacogenetics

2.2.1. MDMA and CYP2D6 polymorphism

Two of the main enzymes involved in MDMA disposition in humans, CYP2D6 and COMT, exhibit genetic polymorphisms. CYP2D6 exhibits a marked genetic polymorphism over 70 alleles; all variants are presented at the home page of the human CYP allele nomenclature committee (http://www.imm.ki.se/cypalleles/cyp2d6.htm)54.

The combination of these variants leads to four phenotypes; poor, intermediate, extensive, and ultrarapid metabolizers (PM, IM, EM, and UM, respectively). Subjects with a PM phenotype lack two functional alleles (FA); those with an IM have on reduced activity allele and one non-functional allele or two reduced activity alleles; whereas EM individuals have one or two FA; and the UM phenotype is associated with gene duplications of functional alleles, with an increased protein expression (Zanger et al., 2004, Bogni et al., 2005).

The polymorphism of CYP2D6 significantly affects the pharmacokinetics of about 30% of the drugs in clinical use, which are CYP2D6 substrates.

Subjects with multiple gene copies will metabolize drugs more rapidly and therapeutic plasma levels will not be achieved at ordinary drug dosages.

There are few data about the influence of CYP2D6 genotype on MDMA pharmacokinetics. Recently, a clinical trial examined the impact of genetics on the clinical pharmacology of MDMA, and reported that subjects' carriers of two FA for CYP2D6 presented higher mean values of HMMA plasma concentrations compared to volunteers with just one FA (Pardo-Lozano et al., 2012).

About 5-10% of Caucasian are PM, presenting a metabolic deficiency in CYP2D6 activity (Sachse et al., 1997). The genetic polymorphisms associated with individual differences in CYP2D6 activity created some expectations that subjects exposed to these types of drugs who were genotypically classified as PM would be more susceptible to acute toxic effects of these drugs or to a higher abuse liability (Henry and Hill, 1998). However, toxicological data do not seem to fully support these expectations because in a series of acute intoxications, no bias was observed toward an overrepresentation of genotypes leading to the PM phenotype (Gilhooly and Daly, 2002). In addition, CYP2D6 may also be the source of a number of drug–amphetamine interactions because it regulates the biotransformation of many therapeutic drugs. Antiretroviral drugs and MAO inhibitors have been reported to be the main cause of life threatening interactions with MDMA (Henry and Hill, 1998, Vuori et al., 2003). The phenomenon of CYP2D6 inhibition as well as the fact that other isoenzymes of cytochrome P450 may contribute to MDMA disposition may explain why the contribution of CYP2D6 genetic polymorphism to acute toxicity is probably less relevant than expected.

2.2.2. MDMA and COMT polymorphism

A single nucleotide polymorphism in the COMT gene encodes for high and low-activity forms of the enzyme. COMT gene displays a functional polymorphism at codon 158 producing a valine *(val)* to methionine *(met)* substitution (Val158Met, rs4680) resulting in three genotypes (*val/val*, *val/met*, and *met/met*). Individuals with the *met* allele have a lower enzyme activity (approximately 25% of the white population) (Zhu, 2002).
A recent study reported that subjects carriers of *met/** alleles showed a higher mean MDMA Ke compared to *val/val* individuals (Pardo-Lozano et al., 2012).

The COMT enzyme is responsible for the transformation of HHMA to HMMA. This enzyme is of interest because studies *in vitro* have shown that HMMA is even more potent than MDMA in releasing vasopressin (Forsling et al., 2002). The inappropriate secretion of antidiuretic hormone has been implicated in life-threatening cases of hyponatremia in acute MDMA poisoning (Hartung et al., 2002). Variability of COMT activity as a result of genetic polymorphisms may account for interindividual differences in vasopressin secretion after MDMA consumption.

Two of the main enzymes involved in MDMA disposition in humans, CYP2D6 and COMT, exhibit genetic polymorphisms which determine the amount of protein in the liver. The impact of CYP2D6 genetics on MDMA metabolism is still not clear and warrants further investigation.

2.2.3. CYP2D6 phenotype

Although cytochrome P450 isozyme CYP2D6 accounts for only a small percentage of total hepatic cytochrome P450 (1-2%), estimates reveal that around the 30% of all drugs in clinical use are metabolized at least in part by CYP2D6, including tricyclic antidepressants, selective serotonin reuptake inhibitor antidepressants (SSRI), opioids, and antipsychotic, antiemetic, antiarrhythmic, and amphetamine-like drugs (Ingelman-Sundberg et al., 1994).

The CYP2D6 phenotype is usually determined by administering a probe drug, such as DEX, or debrisoquine (Schmid et al., 1985, McElroy et al., 2000, Zanger et al., 2004, Bogni et al., 2005, Zhou, 2009). The three best known CYP2D6 drug probes are DEX (Schmid et al., 1985), debrisoquine (Mahgoub et al., 1977) and sparteine (Eichelbaum et al., 1975). 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.

In vitro, CYP2D6 contributes 80% to the formation DOR, and CYP3A4 contributes more than 90% to the formation of 3-methoxymorphinan (MM) from DEX as a substrate (Yu and Haining, 2001) and therefore is used to simultaneously assess CYP2D6 and CYP3A4 activities *in vivo*

based on the determination of urine MR of DEX/DOR and DEX/MM, respectively (Jacqz-Aigrain and Cresteil, 1992, Ducharme et al., 1996). Both DOR and MM are partially demethylated to hydroxymorphinan (HM; hydroxymorphinan-3-ol) and all are recovered in urine, as glucuronides (Chladek et al., 2000, Perfetti et al., 2009) (see figure 3).

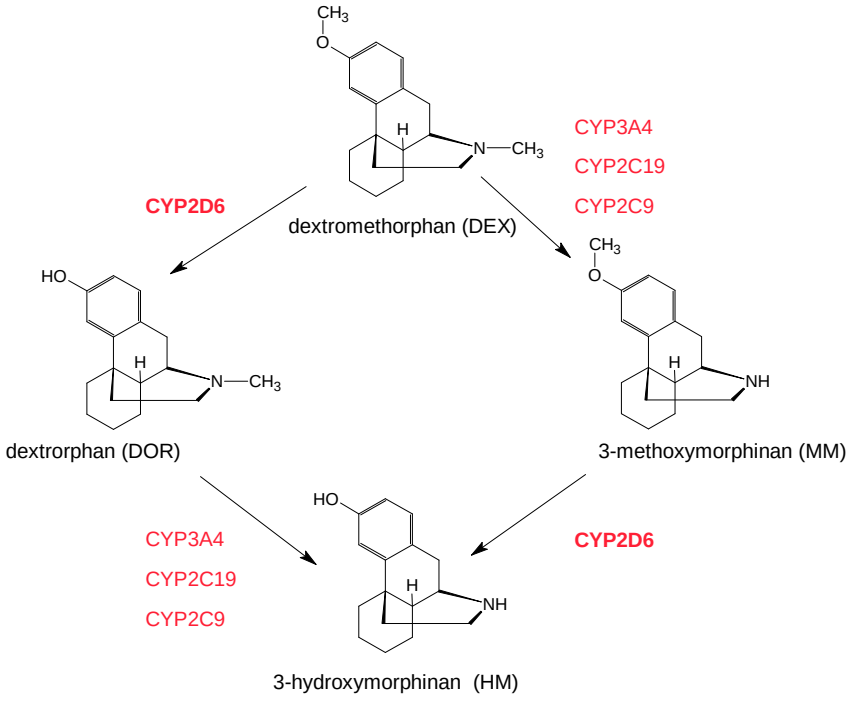

Figure 3. Pathways of dextromethorphan (DEX) metabolism in the human.

2.2.4. CYP1A2 phenotype

CYP1A2 is involved in the activation of environmental procarcinogens, such as arylamines, heterocyclic amines and aflatoxin B1. Its activity is considerably increased by xenobiotics and life style factors, including cigarette smoke and diet (Landi et al., 1999, Lampe et al., 2000). Caffeine has been extensively used as a probe drug to determine CYP1A2 phenotype (Rostami-Hodjegan et al., 1996) and to investigate gender and age differences in CYP1A2 activity (Simon et al., 2001). Although

CYP2E1, CYP2A6, CYP2C8/9, and CYP3A4 are involved in caffeine metabolism, 90% of its clearance is thought to be regulated by CYP1A2 (Valero et al., 1990, Miners and Birkett, 1996). 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 (Lelo et al., 1986). Hepatic metabolism is the dominant elimination mechanism, with less than 5% of an ingested dose being eliminated unchanged in urine.

Caffeine (1,3,7-trimethylxanthine; 137X) undergoes N-demethylation at three sites forming paraxanthine (1,7-dimethylxanthine; 17X: 84%), theophylline (13-dimethylxanthine: 4%), and theobromine (3,7-dimethylxanthine: 12%) (see Figure 4). Its plasma clearance, and in particular 17X/137X plasma concentration ratio is widely used for the measurement of CYP1A2 activity in humans (Rostami-Hodjegan et al., 1996).

Caffeine biotransformation is complex, and at least 17 urinary metabolites can be detected following its consumption. At least six different urinary metabolic ratios for caffeine have been proposed as *in vivo* probes for CYP1A2 activity and of these, the ratio 5-acetylamino-6-amino-3-methyluracil (AAMU) + 1-methylxanthine (1X) + 1-methyluric acid (1U)/ 1,7-dimethyluric acid (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 (Rostami-Hodjegan et al., 1996). The combination of both 137X and DEX to evaluate the contribution of several metabolic enzymes to the metabolic disposition of a third drug has already been applied successfully (Wenk et al., 2004).

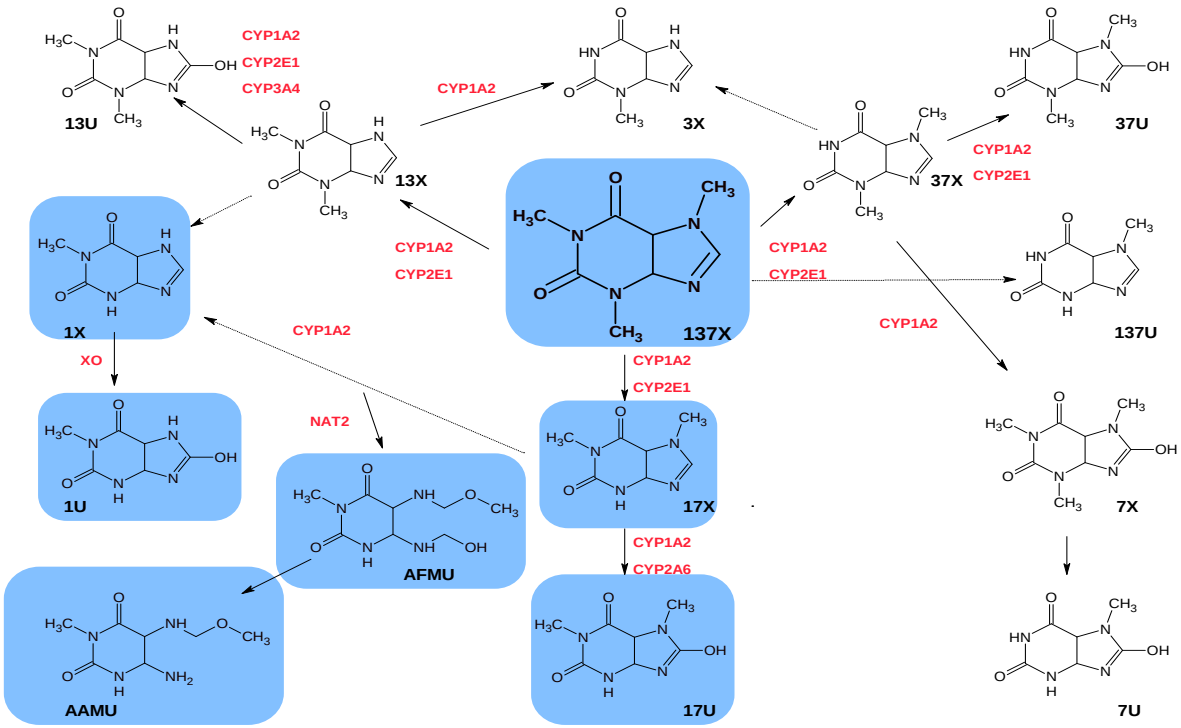

Figure 4. Principle pathways of caffeine metabolism in the human. Compounds frequently used in urinary metabolic ratios are highlighted in blue. 137X = 1,3,7-trimethylxanthine (caffeine); 17X = 1,7-dimethylxanthine (paraxanthine); 17U = 1,7-dimethyluric acid; 1X = 1-methylxanthine; 1U = 1-methyluric 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.

2.3. Gender differences in MDMA metabolism

Several studies suggest that women are more vulnerable to drug abuse than males (Becker and Hu, 2008). In some Western countries, female users present higher rates of consumption and dependence on ecstasy than men (UNODC, 2011) and MDMA has been shown to produce stronger effects in them (Liechti et al., 2001, Allott and Redman, 2007). Differences in drug disposition may contribute to this observation; nevertheless, few pharmacokinetic studies have been performed to assess this issue. One of the does not have sufficient sample size to address this issue (Kolbrich et al., 2008), whereas a more recent one done with 27 (12 women) recreational users of MDMA, showed no major gender differences for most of the pharmacokinetic parameters. The only significant difference observed between genders relied in a higher MDMA plasma clearance in men, and thus, a higher MDMA vs. HMMA ratio in women. Therefore, few data available show that no marked gender differences in MDMA disposition (Pardo-Lozano et al., 2012).

Recent data reported no gender differences in human MDMA pharmacokinetics. It would be interesting to assess CYP2D6 and CYP1A2 activities after MDMA intake and see if both enzymes behave similarly in men and women.

3. MDMA PHARMACODYNAMICS: THE SEROTONERGIC SYSTEM

3.1. Acute pharmacologic and adverse effects

Administration of MDMA has acute effects on brain monoaminergic systems, behaviour and physiology in rodents, non-human primates and humans (Greene et al., 2003). Following systemic MDMA administrations, DA increases its extracellular levels in the brain (Yamamoto et al., 1995, Rothman and Baumann, 2003), and 5-HT concentrations rise rapidly in the brain and in plasma (Colado et al., 1995, Zolkowska et al., 2006, Fonsart et al., 2008, Zolkowska et al., 2008)

The acute effects of ecstasy in humans have been extensively described in retrospective studies, surveys, and cases of intoxication, and more recently after the controlled administration of MDMA to recreational users in experimental conditions (Cami et al., 2000, Cole and Sumnall, 2003a, Kuypers et al., 2013, Peiro et al., 2013).

Physiological effects

Cardiovascular effects related to MDMA intake are blood pressure and HR increase, together with higher body temperature and mydriasis. MDMA also increases cortisol, prolactin, adrenocorticotropic hormone (ACTH), dehydroepiandrosterone (DHEA), and the antidiuretic hormone (ADH, vasopressin) secretion.

This drug has been shown to induce a transient immune dysfunction related to its plasma concentrations, with a decrease in circulating CD4 T-helper lymphocytes, an increase in NK cells, and impaired lymphocyte mitogen-induced proliferation (Pacifici et al., 1999, Pacifici et al., 2000,

Pacifici et al., 2001). The effects of repeated administration of MDMA seemed to potentiate the immune dysfunction and extend it over time.

Acute side effects most often reported are (in order of frequency) lack of appetite, jaw clenching, dry mouth, thirst, restlessness, palpitations, impaired balance, difficulty in concentration, dizziness, feeling and sensitivity to cold, drowsiness, nystagmus, hot flashes, muscular tension, weakness, insomnia, confusion, anxiety, and tremor. MDMA can also produce panic attacks, delirium, and brief psychotic episodes that usually resolve rapidly when the drug action wears off.

Short-term side effects (up to 24 hours after ecstasy consumption) most often reported are (in order of frequency) fatigue, heavy legs, dry mouth, loss of appetite, insomnia, drowsiness, weakness, muscular tension, lack of energy, difficulty concentrating, and headache.

Late short-term residual side effects (up to 7 days after ecstasy use) include fatigue, irritability, anxiety, lack of energy, fatigue, depressed mood, insomnia, drowsiness, and muscular tension.

Subjective effects

Some effects of MDMA such as closeness to others, facilitation of interpersonal relationships, and empathy have been referred to by some authors as "entactogen" properties.

The most frequent effects after MDMA administration are euphoria, well-being, happiness, stimulation, increased energy, extroversion, feeling close to others, increased empathy, increased sociability, enhanced mood, mild perceptual disturbances, changed perception of colors and sounds, somatic symptoms related to its cardiovascular and autonomic effects, and moderate derealisation but not hallucinations.

A recent study done with 27 (12 women) recreational users of MDMA, showed that women experienced heightened physiological effects, in particular, cardiovascular ones; more intense effects in systolic blood pressure (SBP), HR, and also oral temperature (OT) than men. They also reported greater negative subjective effects (e.g. dizziness, depression/sadness, and sedation) (Pardo-Lozano et al., 2012).

MDMA intake causes a boost in central and peripheral monoamine activity, which is responsible for the well-known physiological and subjective effects of the drug; increase in BP, HR, body temperature, and induced euphoria, stimulation and well-being. After its consumption, the user can suffer from adverse effects, including fatigue, dry mouth, loss of appetite, insomnia, lack of energy, difficulty concentrating, and headache.

3.2. Action on 5-HT

3.2.1. Serotonergic system: the serotonin transporter (5-HTT).

In the central nervous system (CNS), 5-HT acts as a neuromodulatory neurotransmitter involved in a wide variety of functions, including sleep, appetite, memory, sexual behaviour, neuroendocrine function and mood.

Serotonergic signalling is controlled by the serotonin transporter (5-HTT), which clears 5-HT from extracellular spaces, modulating the strength and the duration of the signalling.
5-HTT is a member of the sodium-dependent transporter group characterized by 12 transmembrane-spanning domains, with the NH2- and COOH-terminals predicted to lie in the cytoplasm (Blakely et al., 1991,

Hoffman et al., 1991). In the brain, 5-HTT is expressed in presynaptic serotonergic nerve terminals, on axons and 5-HT cell bodies in the raphe nuclei (Blakely et al., 1998). Its main function is to regulate serotonergic transmission, selectively up-taking 5-HT, as well as Na+ and Cl− ions from the synaptic cleft into the presynaptic terminal, and releasing K+ ions (Rudnick, 2006). In the periphery, it is expressed in several tissues, notably in membranes of lung endothelial cells (Maclean and Dempsie, 2010), in systemic arteries and veins (Linder et al., 2008), in placenta and blood platelets (Blakely et al., 1994), in the gastrointestinal epithelium (Wade et al., 1996), adrenal gland (Schroeter et al., 1997), as well as in lymphocytes (Faraj et al., 1994).

5-HTT is a carrier of 5-HT molecules across the biological membrane (Lesch, 2007). It resembles other biogenic amine transporters structurally and functionally, such as NA and DA transporters. Transporters undergo conformational changes and move one or more molecules per 'cycle', unlike channels, which stay open or closed, thus allowing floods of molecules to move across bilayer membranes (Mitchell et al., 2004).
Both polypeptide termini are located within the cytoplasm and six putative phosphorylation sites, which are potential targets for protein kinase A and protein kinase C, exist in the same compartment. The areas important for selective serotonin affinity are localised within helices 1 to 3 and helices 8 to 12.

Over the last years, 5-HTT has received particular attention because the molecular sites for 5-HT binding and re-uptake, and for 5-HTT auto-regulation have proved to be viable targets for pharmacological intervention in various disorders including acute and chronic pain (Hains et al., 2002) migraine (Liu et al., 2011), anxiety (Narasimhan et al., 2011), depression (Porcelli et al., 2012), schizophrenia (Lindholm Carlstrom et

al., 2012) and hypertension (Dempsie and MacLean, 2008). In addition, several drugs of abuse, including amphetamines and cocaine are substrates for 5-HTT and increase 5-HT levels both in neurons (Rothman and Baumann, 2003), and plasma (Zolkowska et al., 2006).

Interest in determining both the molecular characteristics and the regulation of 5-HTT has been enormous, but the difficulty in obtaining human tissues and the ethical limitations in human experiments have turned researchers to look for alternative models.

Among the different tissues expressing the 5-HTT protein, platelets show several similarities with neurons in terms of serotonergic function (Campbell, 1981), that will be explained below. Therefore, platelets have been proposed as a reliable peripheral surrogate model to measure central 5-HTT activity in neuropsychiatric research, although it is still under debate (Rausch et al., 2005, Uebelhack et al., 2006).

5-HTT clears 5-HT from the brain and plasma, and is a key modulator of the serotonergic system. It is expressed in the brain and in other peripheral tissues. The difficulty in studying 5-HTT mechanisms in human brain lead researchers to look for alternative models.

3.2.2. 5-HTTLPR genotype

The human 5-HTT gene (*SCL6A4*) has been cloned, and its expression characterized in human brain and blood platelets. The same single-copy gene encodes for 5-HTT protein in lymphocytes, platelets and neurons, and polymorphisms affecting its expression have been shown to influence function in human platelets and brain in a similar manner (Blakely et al.,

1991, Hoffman et al., 1991). The gene is located in chromosome 17 (17q11.2), which contains 13 exons (Ramamoorthy et al., 1993) and encodes a protein of 630 amino acids.

In humans, transcriptional activity is modulated by a repetitive element of varying length found in the 5' flanking region. This region is known as the serotonin-transporter-linked polymorphic region or 5-HTTLPR (Murphy et al., 2004). The functional polymorphism of an insertion/deletion of 43 base pairs in this promoter region gives rise to the long (*l*) or short (*s*) allelic variants (Heils et al., 1996), and alters the transcriptional activity of the *SCL6A4*. The short variant reduces the transcriptional efficiency of the *SCL6A4* promoter, resulting in decreased 5-HTT expression and uptake activity (Lesch et al., 1996). On the contrary, *SCL6A4* expression is 30% higher in homozygous *l/l* carriers when compared to heterozygous *s/l* or homozygous s*/s*. This higher expression leads to a higher density of 5-HTT (30–40%), and about two times greater 5-HT uptake capacity (Heils et al., 1997). In the European population the genotype distribution is 32% *l/l*, 49% *l/s*, and 19% *s/s* (Lesch et al., 1996). Importantly, some studies found that in platelets, as in neurons, the long promoter variant was also associated with more rapid initial 5-HT uptake when compared to the short allele (Greenberg et al., 1999).

More than twelve different human behavioral traits and whole-body medical disorders are reported to be associated with *SLC6A4* variation. Reduced transporter expression and function resulting from variation in the 5-HTTLPR is associated with anxiety- and depression-related personality traits (Lesch et al., 1996). Similarly, the 5‑HTTLPR and other regulatory and structural variations (in the non-coding and coding regions, respectively) seem to have a role in neuropsychiatric conditions such as eating disorders, substance-abuse, autism, attention-deficit/hyperactivity

disorder and neurodegenerative disorders (Greenberg et al., 2000, Murphy et al., 2004, Hu et al., 2006). Pre-clinical data have reported an obesity phenotype in *SLC6A4* $^{-/-}$ mice at approximately 3 months of age and becomes more exaggerated throughout life. Plasma triglycerides, insulin, leptin and cholesterol were also elevated in these animals (Murphy and Lesch, 2008).

The 5-HTTLPR polymorphism also plays an important role in deficits in verbal fluency associated to MDMA use (Fagundo et al., 2010), and have been associated with primary mood disorders (Martin-Santos et al., 2010). Moreover, data reported that users carrying the *s* allele may be at particular risk for emotional dysfunction (Roiser et al., 2005) after MDMA consumption.

5-HTTLPR genotype is also related to drug response. A recent study showed that after MDMA intake, *l*/* allele carriers presented higher values of BP and HR than s/*s* carriers (Pardo-Lozano et al., 2012).

5-HTT expression and uptake activity depends partially on 5-HTTLPR genotype. The short allele and low 5-HTT expression have been related to neuropsychiatric conditions such as bipolar disorder, depression, and anxiety. More recently, 5-HTTLPR genotype has also shown to play an important role in MDMA drug response, although few data are available and further clinical investigations are warranted.

3.2.3. Serotonergic function in platelets and 5-HT neurons

The precursor for 5-HT synthesis is the amino acid tryptophan (TRP) coming mainly from the diet. TRP is taken up from the blood into nerve terminals, where it is converted into 5-hydroxytryptophan (5-HTP) by the TH. The conversion to 5-HTP is a key regulatory step in 5-HT synthesis, and 5-HTP is quickly converted into 5-HT by the action of the aromatic L-amino acid decarboxylase. Outside the CNS, 5-HT is mainly synthesized by enterochromaffin cells (Bertrand and Bertrand, 2010) and 99% of the circulating 5-HT is stored in platelets (Zolkowska et al., 2006). Thus, one important difference between platelets and neurons is that platelets do not synthesize 5-HT, and their amine content is solely a result of transporter-mediated uptake from the surrounding plasma.

In platelets, 5-HT storage results from a two-step process. First, it is taken-up across the plasma membrane through 5-HTT, and then it is either sequestered into dense granules by vesicular monoamine transporters (VMAT) (Mercado and Kilic, 2010), or degraded by a mitochondrial enzyme; the monoamine oxidase (MAO). In neurons, the process is very similar; presynaptic terminals also contain small dense core storage vesicles that resemble in many features platelet-dense granules (Jedlitschky et al., 2012).

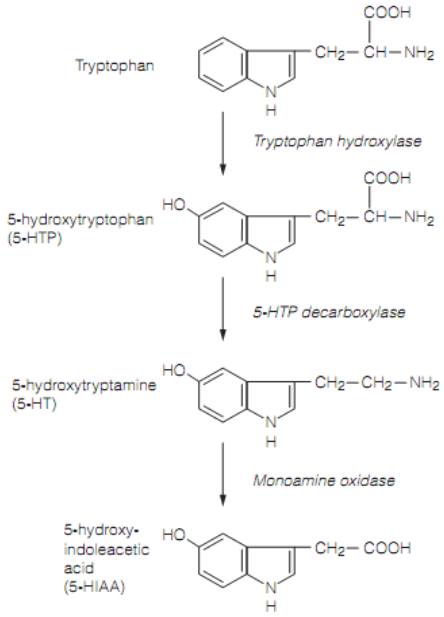

Figure 5. Serotonin biosynthesis pathway.

5-HT, once is inside the vesicles in the presynaptic area, is released into the synapse following an action potential. Once in the synapse, 5-HT can interact with both presynaptic and postsynaptic receptors. However, immediately after interaction it is critically important for 5-HT to be removed from the system. Reuptake, the process of removing neurotransmitters from the synaptic cleft after release, determines the extent, duration and spatial domain of receptor activation (Vialou et al., 2007). Any neurotransmitter not removed from the synaptic cleft prevents further signals from passing through. Active removal reduces the level of neurotransmitter in the cleft faster than diffusion, constrains the effects of released neurotransmitter to smaller areas and allows at least part of the released chemical to be recycled for further use. Reuptake is carried out by transporter proteins that bind to the released neurotransmitter and carry it across the plasma membrane and back into the presynaptic neurone. In

the case of 5-HT, reuptake is carried out by the serotonin transporter (5-HTT).

Neurons and platelets show several important similarities regarding 5-HT re-uptake. A peripheral model of serotonergic activity in the brain could be potentially useful for understanding the mechanisms involved in the regulation of serotonergic system and its role in pathologies related to its functionality.

3.2.4. MDMA and Serotonin transporter

MDMA causes the release of 5-HT, DA and NA from neurons by acting as substrates for monoamine transporter proteins (Crespi et al., 1997, Rothman and Baumann, 2003). *In vivo* microdialysis studies in rat brain demonstrate that MDMA evokes large dose-dependent increases in extracellular 5-HT, and to a lesser extent DA (Gudelsky and Nash, 1996, Baumann et al., 2008).

Like other transporter substrates, MDMA binds to plasma membrane transporters and are translocated into the cytoplasm where they promote non-exocytotic transmitter release (Rudnick and Wall, 1992, Verrico et al., 2007). The precise mechanism underlying transporter-mediated release is not completely understood, but probably involves drug-induced phosphorylation of cytoplasmic domains on the transporter, which triggers reversal of normal transporter flux (Robertson et al., 2009, Sitte and Freissmuth, 2010). MDMA-induced release of 5-HT is blocked by the uptake inhibitor fluoxetine, confirming the critical role of 5-HTT.

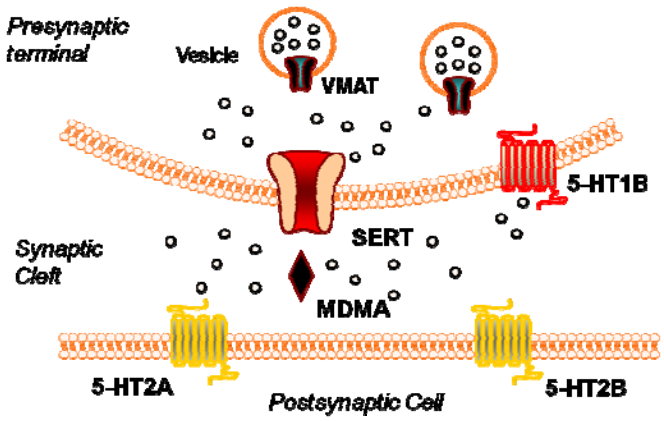

Figure 6. MDMA binds to 5-HTT and is translocated to the cytoplasm where it promotes non-exocytotic transmitter release.

The activity of 5-HTT controls the concentration of free 5-HT in the extracellular space and therefore the efficacy of serotonergic signalling likely depends not only on the activity of single 5-HTT molecules, but is also modulated by the density of cell surface-expressed transporter molecules.

MDMA affects 5-HTT at different levels; a) limiting its synthesis by decreasing TH activity; (b) altering 5-HT gene expression, which could lead to c) changes in 5-HTT protein levels.

b) There are few data available concerning 5-HTT gene expression before and after MDMA intake. All data reflect an alteration of the serotonergic system at the expression level but results are contradictory.

A recent work showed that rats treated with 4 subcutaneous injections of 10 mg/kg of MDMA with an interdose interval of 1 h, presented a striking decrease in 5-HTT gene expression in pooled dorsal and median raphe tissue punches (Biezonski and Meyer, 2010). Other data studied 5-HTT

gene expression 7 days after MDMA administration (15 mg/kg), and significantly elevated 5-HTT mRNA levels were found in the raphe pallidus and obscurus. This up-regulation of 5-HTT mRNA 1 week after MDMA injections might indicate the potential recovery of the serotonin system (Kovacs et al., 2007). 5-HTT mRNA expression increased 7 days after MDMA treatment followed by transient decreases at 21 days (Kirilly, 2010). Another recent work reported a significantly lower gene expression of the 5-HTT seven days after MDMA administration (Cuyas et al., 2013).

c) MDMA and other drugs of abuse such as amphetamine provoke a decrease 5-HTT protein expression (McCann et al., 2005, Urban et al., 2012). Several studies showed that MDMA does not affect the total amount of 5-HTT protein in the cells, but the transporter is rapidly downregulated from the cell surface and relocalized to intracellular vesicles (Greene et al., 2003, Capela et al., 2009). This data was confirmed afterwards by other studies which showed that MDMA-mediated 5-HT release activated 5-HT2B receptors, which mediated the phosphorylation of 5-HTT and subsequent internalization (Kittler et al., 2010). The transporter needs to be phosphorilated to be internalized but the mechanism is not completely understood yet. Some data revealed that this down-regulation of 5-HTT is independent of p38-mitogen protein protein kinase (p38 MAPK) phosphorylation (Kivell et al., 2010), a known signalling pathway that modulates 5-HTT cell surface expression (Samuvel et al., 2005).

MDMA administration alters 5-HTT gene expression and causes an internalization of the transporter from the cell membrane to intracellular vesicles. Only pre-clinical data is available concerning this regulatory mechanism; therefore there is a need to study MDMA induced 5-HTT alterations in human subjects.

3.2.5. Need to develop a method to measure 5-HT in blood

5-HTT sites are widely expressed in non-neuronal tissues such as blood and lung (Ramamoorthy et al., 1993, Ni and Watts, 2006), therefore it seems logical to assume that MDMA has direct actions in the periphery.
In the bloodstream, plasma 5-HT levels are kept very low (i.e., ~1 nM) by 5-HTT-mediated uptake of 5-HT into platelets and by metabolism to 5-hydroxyindolacetic acid (5-HIAA) (Mercado and Kilic, 2010).

Recent studies in rats demonstrate that administration of MDMA and other amphetamine analogs causes dose-dependent elevations in plasma 5-HT. The ability of amphetamine-like drugs to increase plasma 5-HT is related to their potency as 5-HTT substrates, suggesting that platelet 5-HTT proteins are involved in this process (Zolkowska et al., 2006).

Along decades, several procedures have been used to obtain platelet-poor plasma (PPP) and different methods such as high performance liquid chromatography (HPLC) coupled to electrochemical detectors (HPLC/ECD) or to tandem mass spectrometry (HPLC/MS/MS) or immunoassays, have been applied to measure 5-HT afterwards. However, all of them seem unsuccessful in reporting real 5-HT levels in PPP and highly discrepant concentrations have been reported in the literature over the years (Brand and Anderson). Therefore, there is an urge for

standardized pre-analytical and analytical protocols to determine this small amount of free 5-HT present in blood.

The discrepancies in the results are due to the fact that measuring plasma 5-HT is technically challenging. Given that 99% of blood 5-HT is stored in platelets, and platelets are fragile; minor damage to platelets during specimen handling will cause platelet activation or lysis, generating artificial increases in plasma 5-HT. Factors that can activate platelets are the anticoagulant used (Middelkoop et al., 1993, White and Escolar, 2000, Ohkawa et al., 2005), sheer forces (Beck et al., 1993, Montgomery et al., 2012) or the temperatures during centrifugation or storage (Middelkoop et al., 1993, Ohkawa et al., 2005, van der Meer and de Korte, 2011).

To be able to use platelets as a surrogate biomarker of the serotonergic neurotransmission system in the CNS, a method to measure 5-HT levels in the bloodstream is required. Unfortunately, highly discrepant results have been reported over the years. Therefore, there is an urge to develop a reliable method to quantify these low levels of 5-HT in blood.
In order to develop this methodology and considering the lack of reliability of previous reports, it would be necessary to study and evaluate how different pre-analytical conditions (like temperature, speed of centrifugation, etc.) modify 5-HT concentrations.

4. MEPHEDRONE PHARMACOLOGY

Mephedrone (4-methylmethcathinone; MEPH) is a synthetic derivative of cathinone, a psychostimulant drug chemically related to amphetamine, found in the plant *Catha edulis* (Wood et al., 2010b), that possesses powerful entactogenic and hallucinogenic effects (Schifano et al., 2011).

It is colloquially known as MCAT, meow-meow, or bubbles, and is a main constituent in substances marketed as "bath salts".
Recently, MEPH has gained growing popularity as a 'designer drug' or a 'research chemical' offered for sale under various names via Internet sites, head shops and recently, by street dealers (Winstock et al., 2010, Schifano et al., 2011, Winstock et al., 2011).

The users of MEPH have described its psychomimetic effects being comparable to amphetamine, cocaine and MDMA, causing euphoria, elevated mood, and stimulation, enhanced appreciation for music, decreased hostility, improved mental function and mild sexual stimulation. A recent web-based survey from 1006 responders revealed that MEPH users consider its effects best compared with those of MDMA (Carhart-Harris et al., 2011).
Owing to its recent emergence, there are few formal pharmacodynamic or pharmacokinetic studies of mephedrone performed in humans.

Based on the spectrum of psychostimulant effects described by the drug users and the chemical similarity of mephedrone to substituted methcathinone and methamphetamine it has been speculated that mephedrone may act via increased release and re-uptake inhibition of 5-HT and DA (Schifano et al., 2011).

Microdialysis experiments reported concurrent elevations in extracellular DA and 5-HT *in vivo*, with preferential effects on 5-HT (Baumann et al., 2012), supporting the hypothesis that this drug targets monoamine transporters in a manner similar to MDMA.

A recent study by Kehr et al (2011) showed that MEPH increases extracellular DA and 5-HT in rat nucleus accumbens, and these effects could be related to inhibition of monoamine uptake (Hadlock et al., 2011). Moreover, *in vitro* data showed that mephedrone displays nanomolar potency as substrate for NET, DAT, and 5-HTT. More specifically, the drug evokes transporter-mediated release of monoamines via reversal of normal transporter flux (Fleckenstein et al., 2007, Sitte and Freissmuth, 2010).

4.1. Mephedrone pharmacokinetics

Mephedrone is ingested in a number of ways including nasal insufflations, orally, and intravenously (Winstock et al., 2010, Wood et al., 2010b), being most frequently used inhaled, and to a lesser extend, orally.

This drug is more soluble than amphetamine, due to the cetonic group in the molecule. This makes more difficult to pass the brain barrier, and then, a higher dose is required to produce the same effects (Gibbons and Zloh, 2010).Doses consumed depend on the administration route; from 5 to 75 mg for inhalatory administration, from 150 to 250 mg orally, and from 75 to 167 mg for IV and IM administrations. When the drug is administered orally, its effects start from 15 minutes to 2 hours after the intake, lasting up to 4 hours. If inhaled, it takes less time to have effects and last until 3 hours. The low bioavailability of mephedrone found after oral ingestion justifies why abusers preferably snort it.

CYP2D6 is the main enzyme responsible for the metabolism of mephedrone (Pedersen et al., 2013), and several metabolites have been identified in the urine (see figure 7). Recent pre-clinical data reported that Mephedrone presents non-linear pharmacokinetic, which could cause a dramatic increase in plasma levels, leading to enhanced toxicity.

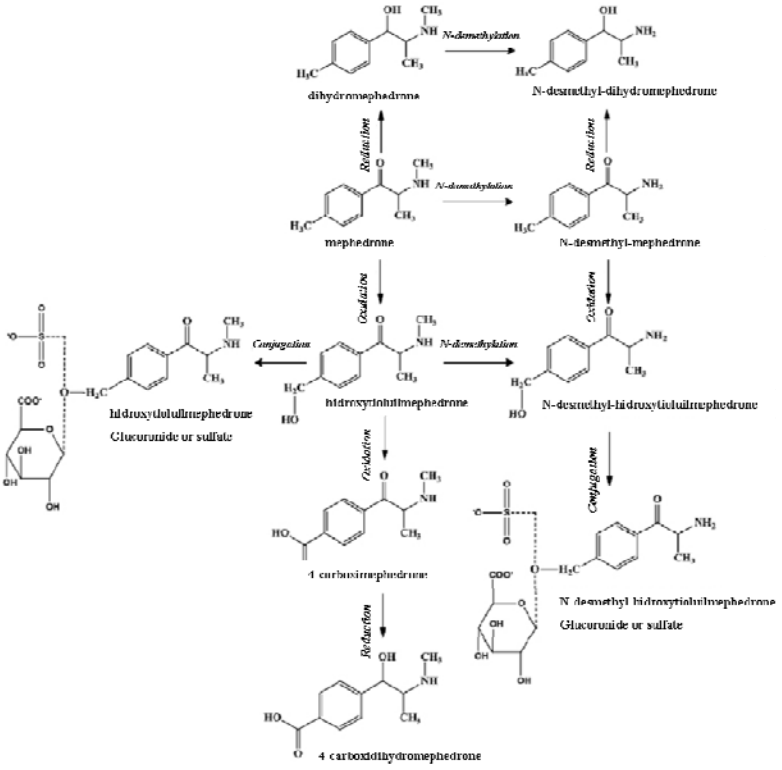

Figure 7. Mephedrone metabolism, with its main metabolites.

MEPH's half-life is shorter than that of MDMA which causes users to often redose, thus contributing to the appearance of addiction.

4.2. Mephedrone pharmacodynamics

Although it might be inferred from structural similarities with cathinone that designer analogs of methcathinone target monoamine transporters (Cozzi and Foley, 2003) or nicotinic acetylcholine receptors (Damaj et al., 2004, Carroll et al., 2010), only limited information is available regarding the pharmacodynamic mechanisms of mephedrone.

Some pre-clinical studies report that it causes locomotor activation (Motbey et al., 2012, Martinez-Clemente et al., 2013), and increases HR, blood pressure and cardiac contractility (Meng et al., 2012). Some case reports revealed several adverse effects on cardiovascular and neurological systems after MEPH consumption, with an acute sympathomimetic toxidrome (e.g. hypertension, tachycardia and agitation) (Wood et al., 2010a).

The nonmedical use of 'designer' cathinone analogs such as mephedrone is increasing worldwide, yet little information is available regarding the mechanism of action for these drugs.
Up to date, data demonstrate that mephedrone is a substrate for monoamine transporters, with a profile of transmitter-releasing activity comparable to MDMA.

On the basis of the background information provided, the main *research objectives* were defined as follows:

A) To investigate gender differences in the pharmacology of MDMA assessing MDMA-induced CYP2D6 autoinhibition.

To achieve this goal, the next series of experimental steps have been planned:

1) Evaluation of the duration of MDMA-induced CYP2D6 autoinhibition (MBI) in women by using the probe drug dextromethorphan (DEX) **(P-I)**.

2) Calculation of CYP2D6 enzyme activity recovery over time in women, and compare both genders **(P-I)**.

3) Investigation of the role of CYP1A2 in the metabolism of MDMA in humans by using the probe drug caffeine in both genders **(P-II)**.

4) Mini-review comprehending the aforementioned three points above **(P-III)**.

Hypothesis: *MDMA affects differently the activities of the cytochrome P450 enzymes responsible for its phase I metabolism, depending on gender.*

B) To investigate whether MDMA causes alterations in the central and peripheral serotonergic system, in a similar manner in animal models and its translation to human studies.

To achieve the aim we have planned the following tests to be performed:

1) Determine the effects of MDMA in 5-HT concentrations in brain and blood from rats **(P-IV)**.

2) Review published data regarding the assessment of platelet 5-HTT as a biomarker for 5-HTT activity in the brain **(P-V)**.

3) Gene expression assay of 5-HTT in lymphocytes from healthy control volunteers who have taken MDMA **(M-I)**.

4) Validate the analytical procedure developed in rats in humans for its further application in clinical studies.

    a. Do a standard 4-days validation method protocol, to study the recovery, the precision and accuracy of the method **(P-VI)**.

    b. Study the differences in blood and platelet-poor plasma (PPP) 5-HT in patients with a dysfunctional serotonergic system vs. healthy controls **(P-VI)**.

    c. Evaluate 5-HT concentrations in blood from healthy control volunteers after administration of mephedrone. **(M-II)**.

Hypothesis: *MDMA intake changes 5-HTT gene expression. Besides, MDMA causes the release of 5-HT to the extracellular space by reversing the flux of 5-HTT. This effect is not produced only in the brain, but also in platelets, showing a correlation between both compartments.*

C) To evaluate to which extend the induced serotonergic activity by MDMA and related drug mephedrone correlates with pharmacological effects measured in healthy recreational users exposed to these drugs.

To achieve this goal, the next series of experimental steps have been planed:

1) Measure MDMA, mephedrone plasmatic and 5-HT blood concentrations, and see if they are related **(M-II)**.

2) Study the relationship between 5-HTTLPR genotype and 5-HT blood levels **(M-II)**.

3) Assess the intensity of the pharmacological effects produced by the drug and 5-HT blood concentrations **(M-II)**.

***Hypothesis**: It is postulated that the more concentration of the drug in plasma the more increase in 5-HT concentrations occurs in a dose-dependent manner. It is also expected that 5-HT increase and intensity of the pharmacological effects will follow a concentration relationship, reflecting the mechanism of action of this drug.*

Yubero-Lahoz S, Pardo R, Farré M, O'Mahony B, Torrens M, Mustata C, Pérez-Mañá C, Carbó M, de la Torre R. Gender differences in 3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy) Induced CYP2D6 Inhibition in Humans

Clinical Pharmacokinetics. 2011;50(5):319-29.

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Yubero-Lahoz S, Robledo P, Farré M, de la Torre R.

Platelet 5-HTT as a Peripheral Biomarker of Serotonergic neurotransmisión in the central nervous system

Current medicinal Chemistry. 2013;20(11):1382-96.

Acute changes in serotonin tranporter gene expression after MDMA intake

Notes & Tips Analytical Biochemistry

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The entactogen 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") is a ring-substituted amphetamine derivative, the use of which is prevalent among adolescents and adults (Banken, 2004). It is widely reported that MDMA interacts with the serotonergic system in several ways; causing depletions in serotonin (5-hydroxytryptamine; 5-HT), decreasing in tryptophan hydroxylase (TH) activity, and reducing levels of the serotonin transporter (5-HTT) as measured either by membrane binding or autoradiography (Greene et al., 2003). Besides, MDMA has been reported to alter 5-HTT gene expression (Kovacs et al., 2007, Kirilly, 2010), which could lead to changes in protein levels. Evidence from biochemical studies has repeatedly demonstrated that MDMA-induced loss of 5-HT terminals includes a marked reduction of 5-HTT binding sites throughout these regions (Battaglia et al., 1991, Aguirre et al., 1995). Up to date, results are contradictory since some studies reveal a profound down-regulation of 5-HTT gene expression after MDMA administration (Biezonski and Meyer, 2010, 2011, Cuyas et al., 2013), whereas other see an increase (Kovacs et al., 2007, Kirilly, 2010), attributing it to a compensatory mechanism. These differences are likely due to different methodological approaches, rat strain models, and treatment regimens. Besides, clinical data is lacking to study this interaction.

Recently, 5-HT signaling in lymphocytes has been reported to be quite analogous to central nervous system serotonergic neurotransmission, and the idea that lymphocytes could offer a reliable surrogate for central 5-HT function is becoming relevant (Yubero-Lahoz et al., 2013).

In the present work, peripheral blood mononuclear cells (PBMCs) were extracted from healthy recreational MDMA users in order to study 5-HTT gene expression before and after drug administration. Our objective was to determine whether significant changes in PBMNCs 5-HTT gene expression after 3h of MDMA consumption could be observed

in recreational users. Subjects were split according to gender and 5-HTTLPR genotype. It is anticipated that these preliminary findings may serve as a baseline rationale to design future long-term experiments assessing gene expression together with protein analyses.

Five healthy male and eight female volunteers were recruited by word-of-mouth in the study. Eligibility required self-reported recreational use of MDMA on at least three occasions. Volunteers were considered to be in good health as assessed by medical history, physical examination, and routine blood and urine laboratory tests participated in the study. The protocol was approved by the local ethics committee and participants gave their written informed consent (Ref. NL34859.068.10). None had a history of adverse medical or psychiatric reactions after MDMA consumption. Subjects were interviewed by a psychiatrist to exclude those with major psychiatric disorders or with a history of such disorders (Torrens et al., 2004) and by a physician to exclude concomitant medical conditions. None met criteria for drug abuse or drug dependence (except for nicotine), and all had previous experience with other psychostimulants.

The study presented a double-blind randomised clinical trial design. Subjects participated in two experimental sessions; each session lasted 8 hours, with a minimum wash-out period of 7 days between sessions. Each session began at 09:00h following an overnight fast. 75 mg of MDMA or placebo was administered at 09:30h in both sessions. The dose of MDMA was chosen to be in the range reported for single recreational oral doses of MDMA (Parrott, 2004). A light meal was provided just before drug/placebo administration. Blood samples were obtained through an indwelling catheter inserted into a subcutaneous vein in the forearm of the non-dominant arm. Thereafter, the subjects remained seated in a quiet room. In both sessions, blood samples were taken at 9:00h and 3 hours later.

Sample genotyping was done by extracting DNA from peripheral blood leukocytes of participants using the Flexi Gene DNA kit (Qiagen Iberia, S.L., Spain) and applying a previously described protocol. (Fagundo et al., 2010). Each genotype was classified according to three existing types for serotonin-transporter-linked polymorphic region (5-HTTLPR); *l/l, l/s* and *s/s*. Table I depicts the subjects' anthropological data.

PBMNCs were isolated within 2 h after blood withdraws using Vacutainer CPT Tubes (Beckton Dickinson, Franklin Lakes, NJ) according to manufacturer's instructions. Harvested PBMNCs were preserved in 1 mL or RNA cell protect solution (Qiagen Iberia, S.L., Spain) and were stored at -80ºC prior to RNA extraction.

Variable	Age (in years)	Weight (in Kg)	Height (in cm)	BMI (in Kg/m^2)	Pills last year
Gender					
Men (n=5)	20.0±1.6 [18,21]	83.4±6.9 [74,91]	182.8±9.1 [173,193]	25.1±3.4 [24.0,30.2]	16.6±12.5 [4,30]
Women (n=8)	22.5±2.5 [21,26]	58.1±4.7 [50,65]	169.0±4.2 [162,176]	20.4±1.8 [19.2,23.3]	5.1±3.7 [1,12]
Genotype					
5-HTTLPR *l/l* (n=3)	23.3±2.9 [20,25]	60.3±4.0 [58,65]	167.6±4.3 [162,170]	21.6±1.4 [17.5,29.7]	4.7±2.3 [2,6]
5-HTTLPR *l/s* (n=9)	21.4±2.4 [18,26]	71.1±16.3 [50,91]	177.6±10.0 [167,193]	22.4±4.1 [17.5,29.7]	9.4±9.2 [1,30]
5-HTTLPR *s/s* (n=1)	19	74	175	24.2	30

Table 1. Anthropometric data of included volunteers according to gender and genotypes (mean ± SD [min, max]).

An automatized extraction method using Qiacube (Qiagen Iberia, S.L., Spain) was applied for total RNA extraction. All tRNA extractions

were performed following the manufacturers' instructions. The cDNAs were kept at -20 °C. All reagents, plastic ware, and supplies used in this study were sterile, nucleases free, and of molecular biology grade.

The total RNA (tRNA) was validated using quantitative (tRNA recovery) and qualitative (tRNA purity and integrity) criteria. tRNA recovery was calculated from the tRNA concentration measured by absorbance at 260nm (A_{260}) and expressed in micrograms of RNA isolated from 1ml of whole blood (µg/1ml whole blood). tRNA purity was determined spectrophotometrically as the A_{260}/A_{280} ratio. tRNA integrity was assessed by microcapillary gel electrophoresis with fluorescent detection (Bioanalyzer, RNA 6000 LabChip kit, Agilent Technologies, Wilmington, DE, USA).

tRNA integrity was expressed as the RNA integrity number (RIN value), calculated by Agilent 2100 Expert Software (http://www.chem.agilent.com/scripts). The purity of isolated individual total RNA samples was greater than 1.8 by A_{260}/A_{280} and 1.75 by $A_{260}/_{230}$, with integrity values not lower than 8.2 by RIN. Potential DNA contamination was assessed by an Agilent Bioanalyzer, and samples bearing DNA traces were omitted from evaluation.

The reverse transcription (RT) was performed using High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). The amount of tRNA was 100 ng per 20 µl of RT reaction.

The PCRs were performed in the real-time mode using TaqMan 5'-nuclease gene expression assays for SLC6A4 gene (Hs00984349), its expression being referred to that of human transferrin receptor (TFRC) (Hs00951083) as a housekeeping gene (Applied Biosystems). Gene expression measurements were performed in triplicate for each cDNA sample. Included in the assay there was a negative control without cDNA template.

For the evaluation of data from RNA isolation methods the Student *t* test (SPSS for Windows 18.0) was used. Basal condition was always numbered 1 and the second sample was compared to baseline for each subject. $p<0.05$ was considered statistically significant.

On the basis of the aforementioned parameters, 5-HTT gene expression in every subject was calculated before and after placebo or MDMA administration. Therefore, changes in MDMA were compared to changes in placebo from the same subject.

Figure 1 shows the amplification plot for 5-HTT and TFRC genes. Taken all the results globally, MDMA intake induced a 2.15 fold- increase in 5-HTT gene expression, whereas it was of 1.08 in the placebo condition. There were statistically significant gender differences in MDMA condition (see figure 2). Women reported a change in 5-HTT expression of 2.47 fold increase in the MDMA condition, and of 1.16 in the placebo condition. Men also showed a significant change although more modest, of 1.68-fold increase in the MDMA condition, and 0.98 in the placebo condition.

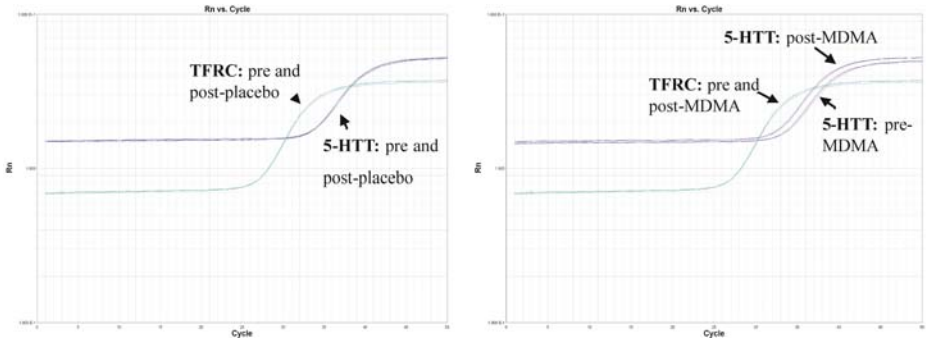

Figure 1. Quantitative RT-PCR analysis of 5-HTT transcript levels before and after 3 hours of placebo or MDMA administration. The plot shows representative amplification curves (n = 2 per group) for 5-HTT cDNA as a function of treatment, as well as curves for housekeeping gene loading control (transferring receptor; TFRC). MDMA caused a leftward shift in the cycle number at which 5-HTT cDNA was amplified beyond threshold foranalysis, indicating increased expression of the gene.

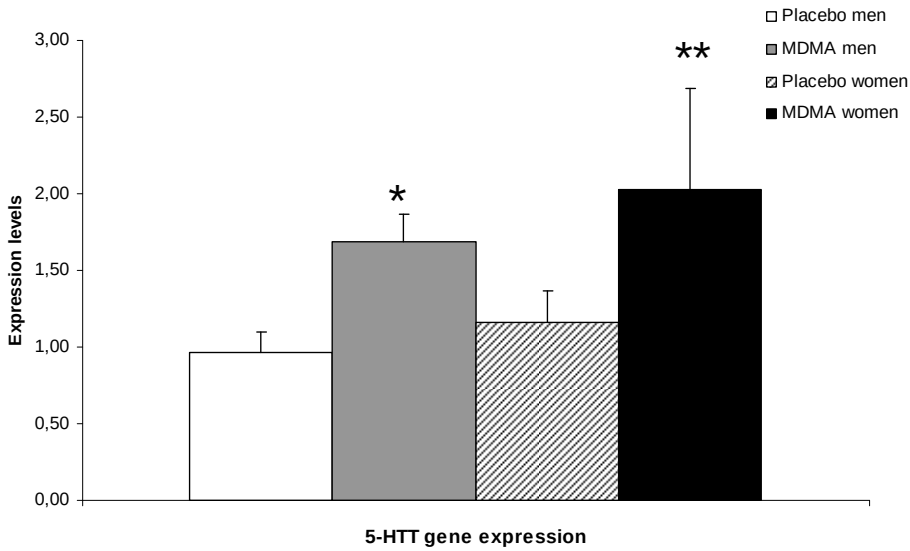

Figure 2. Quantitative comparison of 5-HTT gene expression in each treatment group, expressed as fold-difference in mean expression ± SEM compared with respective controls. Groups are denoted by treatment: gene as indicated in the graph legend. MDMA exposure increased 5-HTT expression 2,47- 1.68-fold increase in women and men, respectively. $p<0.05$ compared to placebo condition for each gender.

Moreover, there were differences depending on the genotype; subjects carriers of *l/l* showed a higher increased followed by *l/s* and by *s/s*, which show no differences to the placebo condition (see table 2 below).

l/l (n=3)		*l/s*(n=9)		*s/s* (n=1)	
Placebo	MDMA	Placebo	MDMA	Placebo	MDMA
0.96±0.63	3.98±1.68	1.10±0.40	1.59±0.80	1.37	1.14

Table 2. 5-HTT gene expression values in each treatment group depending on 5-HTTLPR genotype. Expressed as mean expression ± SD compared with respective pretreatment values.

This study was aimed to determine the effects of MDMA on the expression of peripheral serotonergic markers in humans. The main

experiment investigated MDMA-induced changes in levels of the 5-HTT gene three hours after administering 75 mg of the drug. Results show that subjects who received MDMA showed a striking increase in 5-HTT gene expression measured by quantitative RT-PCR in PBMNCs cells, when compared with the same subjects treated with placebo.

These results demonstrate that MDMA causes substantial regulatory changes in the expression of serotonergic markers, and it seems to be related to 5-HTTLPR genotype. A single administration of MDMA increases 5-HTT gene expression about 200% 3 hours later, this affectation being more pronounced in women compared to men. A larger sample size is required to confirm these results, and assess whether this change is translated to an alteration of 5-HTT protein expression in humans.

Title Page:

Determination of serotonin and its metabolite 5-HIAA in whole blood human samples with consideration to pre-analytical factors.

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Biomedical Chromatography

Abstract

Significant differences have been reported over the years in measuring physiological levels of serotonin (5-HT) in platelet poor plasma (PPP). This work shows that there are crucial pre-analytical factors in sample manipulation that can provoke an artifactual release of 5-HT from platelets, and that even when the sample is accurately processed to obtain PPP, 5-HT levels are approximately 2.8 times higher than those in whole blood. An alternative methodology consisting of *ex vivo* blood microdialysis coupled to high performance liquid chromatography–electrochemical detection (HPLC–ECD) is proposed and validated. It is considered the most accurate technique to measure physiological 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), due to its sensitivity (limits of quantification of 0.08 ng/mL) and reliability since there is no sample manipulation. 5-HT and 5-HIAA levels in whole blood and in PPP were studied in control subjects, hypertensive (HYP) and end-stage renal disease (ESRD) patients, who have deregulated serotonergic system. This work reveals that whole blood is the best matrix to determine 5-HT concentrations, and the clinical relevance of the accuracy of 5-HT determination is discussed.

Keywords: Serotonin, whole blood, platelet, platelet-poor plasma, microdialysis.

Introduction

Serotonin (5-HT) is an essential central nervous system (CNS) neurotransmitter that modulates neural activity and a wide range of neurophysiological processes such as digestion, energy balance, pain perception, and mood (Berger *et al.,* 2009). Dysregulation of the serotonergic system has been implicated in the pathogenesis of several psychiatric and neurological disorders such as depression, anxiety, and bipolar disorder. In addition, 5-HT is also involved in many important functions outside the CNS, including cardiovascular and pulmonary physiology. Indeed, about 95 % of the total body 5-HT is found in enterochromaffin cells of the gut, and almost all the blood circulating 5-HT is found sequestered within the dense granules of platelets. Peripheral 5-HT is metabolized mainly in the lung and the liver via enzymatic conversion by monoamine oxidase-A, resulting in urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) (Yubero-Lahoz *et al.,* 2013).

Peripheral 5-HT concentrations are biomarkers for central serotonergic activity (Yan *et al.,* 1993) and cardiovascular disease (Herve *et al.,* 1995). However, the typical approach of measuring the very small pool of 5-HT in platelet-poor plasma (PPP) has proven technically challenging as platelets are activated depending on several factors. Previous studies have tried to develop an optimal procedure to extract 5-HT from PPP assessing different parameters, like the anticoagulant used (Ohkawa *et al.,* 2005; Middelkoop *et al.,* 1993; White and Escolar., 2000), sheer forces (Beck *et al.,* 1993; Montgomery *et al.,* 2012) or the temperatures during centrifugation or storage (Ohkawa *et al.,* 2005; Middelkoop *et al.,* 1993; van der Meer and de Korte., 2011). A recent comprehensive review showed that along decades, several procedures have been used to obtain PPP and different methods (high-performance liquid chromatography; HPLC, immunoassay, tandem liquid chromatography-coupled to mass spectrometry) applied to measure 5-HT afterwards. All of them seem unsuccessful in obtaining real 5-HT levels in PPP and highly discrepant concentrations are reported in the literature (Brand and Anderson, 2011). Therefore, there is an urge for a standard protocol to determine this small amount of free 5-HT

present in blood.

We developed an analytical method that benefits from previous reports in animal and human studies using microdialysis for sample preparation and HPLC with electrochemical detection (ECD) as analytical approach (Yubero-Lahoz *et al.*, 2012; Zolkowska *et al.*, 2006 Middelkoop *et al.*, 1993; Cheng *et al.*, 1992; 1993, Paez and Hernandez, 1996; 1998). We believe that the combination of sample microdialysis and HPLC-ECD is the most appropriate for obtaining accurate results when determining 5-HT.

To demonstrate that measuring 5-HT directly from whole blood is the best assessment instead of PPP, we obtained PPP from the same blood samples and compared results. We evaluated pre-analytical conditions in order to minimize artifactual release of 5-HT from platelets in PPP, and assessed the effect of temperature, speed centrifugation and the addition of an antiaggregant on 5-HT levels. Taking into account data previously reported and all the factors stated earlier, we decided to obtain PPP following a reported procedure using double and slow centrifugations at room temperature (RT) (Javors *et al.*, 2005).

5-HT and 5-HIAA concentrations in blood and PPP were measured in two groups of patients suffering from hypertension (HYP) and end-stage renal disease (ESRD) which show a deregulated serotonin system (Mercado and Kilic, 2010; Kerr *et al.*, 1992), and in control subjects. 5-HT levels in PPP were compared to 5-HT levels in whole blood.

The objective of the present work was to develop a methodology that overcomes discrepancies in determining PPP 5-HT concentrations reported in the literature over decades. We report several essential pre-analytical factors that affect 5-HT determinations in human PPP samples, and shows that there are significant differences in determining 5-HT in whole blood or in PPP samples. We validate a sensitive and rapid microdialysis-HPLC-ECD, which was successfully applied to determine 5-HT levels in 3 groups of subjects.

Experimental

Reagents and chemicals

Standards of 5-HT and 5-HIAA were obtained from Sigma-Aldrich (Madrid, Spain). *In vitro* microdialysis probes (4 mm exchange surface) were purchased from SciPro Inc (Sanborn, NY, USA). Standards were prepared fresh daily in a Ringer's solution that contained 150 mM NaCl, 3 mM KCl, 1.4mM $CaCl_2$, and 0.8mM $MgCl_2$. Vacutainer tubes containing heparin citrate were acquired from BD (New Jersey, USA). The antiaggregant Prostaglandin I_2 (PGI_2) was supplied by Cayman Chemical Company (Michigan, USA), while all other reagents required for analytical assays were obtained from Sigma-Aldrich (Madrid, Spain).

Apparatus

The HPLC system consisted of an isocratic pump (Model 300), an autosampler (Model 200), and an ECD equipped with a glassy carbon electrode (Model 100) (Chromsystems, München, Germany). The chromatographic separation was performed using a 150 × 4.6 mm C18 column (Agilent Technologies). The mobile phase consisting of 75 mM sodium acetate, 240 µM Na_2EDTA, and 150 µM sodium octanesulfonic, with 22 % MeOH per liter of water (pH 5.00) was pumped at a flow rate of 1 ml/min. A mobile phase conditioning cell with a potential set at +0.60 V was connected between the pumping system and the injector.

Preparation of standards and quality control samples

A calibration curve was performed by first preparing a calibration stock solution (1 mg/ml) of 5-HT and 5-HIAA in perchloric acid (0.1 N) for its stability and storing it at 4 °C until analysis (Patel *et al.*, 2005). Then, we serially diluted the stock with Ringer's solution to obtain five working concentrations (0.1, 0.2, 0.5, 1 and 2 ng/ml). Calibration curves were fitted by least-squares linear regression (1/x weighting factor). Three inter-assay controls were prepared

(0.12, 0.80, and 1.70 ng/ml), and stored at −20°C until analysis. Controls were included in each analytical batch to check calibration, accuracy, precision, and stability of samples under storage conditions.

Validation procedures

Prior to application to real samples, the method was tested in a validation protocol following bioanalytical method validation standards (Dabase of Guidance for Industry). Selectivity, carryover, matrix effect, linearity, limits of detection and quantification, precision, accuracy, recovery, and stability were determined. The analytical methodology was validated following a 4-days protocol. Calibration curve linearity was tested over the range 0.1-2 ng/ml for 5-HT and 5-HIAA. Six and ten standard deviations (SD) of the calculated concentrations for the low control level were used to estimate the limits of detection and quantification, respectively. Intermediate precision was calculated as the relative SD of concentrations calculated for quality control samples. Inter-assay accuracy is expressed as the relative error with respect to the calculated concentrations.

Pre-analytical factors.

Before analyzing patients' samples, a series of experiments were carried out in control patients (n=15) to elucidate the best pre-analytical conditions to obtain reliable 5-HT levels in PPP. Four main factors were evaluated concerning sample manipulation: (i) anticoagulant agents, (ii) temperature, (iii) speed centrifugation, and (iv) the addition of the antiaggregant PGI_2.

(i) To determine the best anticoagulant agent, blood samples were centrifuged at RT at 2000g during 15 min in blood collection tubes containing different anticoagulants: K_3EDTA, lithium heparin and sodium citrate. The PPP samples obtained were dialyzed and 5-HT levels were measured.

(ii) The effect of temperature was assessed by centrifuging blood samples at 2000g, at RT and at 4°C . The PPP samples obtained were dialyzed and 5-HT levels were measured.

(iii) Concerning speed centrifugation, blood samples were centrifuged at several speeds: one single centrifugation at RT for 15 min at 2000g, and two consecutive centrifugations at low revolutions, the first one at 150g to obtain platelet-rich plasma (PRP) and the second at 550g.

(iv) Finally, and taking all these factors into account, 5-HT levels in PPP were assessed after the addition of PGI_2 and compared to 5-HT levels when it was not added.

5-HT levels obtained in PPP under the best pre-analytical conditions were compared to those concentrations obtained in whole blood of the same blood samples.

Biological samples

Blood samples used in this study were obtained from 12 patients with ESRD undergoing hemodialysis, 12 subjects with HYT, and 12 healthy controls who provided written informed consent before inclusion. Four ml of blood were withdrawn from the antecubital vein of each participant in the morning (8:00-9.00 a.m.) after fasting overnight. In hemodialysis patients, blood was obtained from the arteriovenous fistula immediately before hemodialysis. Blood (0.2 mL) was immediately dialyzed and an aliquot was centrifuged in parallel to obtain PPP. The study was conducted in accordance with the Declaration of Helsinki (2000) and approved by the institutional review board (CEIC-Parc de Salut Mar).

Sample collection and preparation

To avoid clotting, venous blood was collected in Vacutainer tubes containing heparin lithium. The tubes were gently inverted, and samples were transferred to 300 µl polypropylene tubes containing 50 µl of 1000 IU/ml heparin and kept at RT. Blood samples were immediately dialyzed and the efflux collected and injected to the HPLC system. For PPP preparation, specific conditions were applied in order to obtain the minimal artificial release of 5-HT from

platelets (Javors *et al.*, 2005) (i) blood was first centrifuged at low speed (150 g) at RT for 20 min, obtaining PRP, and (ii) PRP was centrifuged again at higher speed (550 g) at RT for 15 min adding 1 µl of the antiaggregant PGI_2 solution (300 ng/ml).

In vitro microdialysis probe recoveries were performed before and after microdialysis using a 100 pg/50 µl standard of 5-HT and 5-HIAA in Ringers' solution. Blood or PPP samples were perfused with Ringer's solution pumped at a flow rate of 2.0 µl/min. Dialysates were collected over a time period of 25 min, and 50 µl were injected into the HPLC column.

Statistical analyses

Statistically significant differences in pre-analytical factors data were assessed with a paired Student's t-test. Clinical samples data were analyzed using two-way repeated measures analysis of variance (ANOVA) with sample (blood and PPP), as a within subjects factor, and group (controls, HYT, ESRD), as a between subjects factor. Individual comparisons were carried out using the Fisher's least significant difference (LSD) and pair-wise comparisons *post-hoc* tests. A correlation analysis was performed between blood and PPP 5-HT and 5-HIAA values from all subjects. A value of $P < 0.05$ was considered the minimum criterion for statistical significance. Statistical analyses were carried out using the statistical software SPSS 12.0 for Windows (SPSS, Inc., 2003).

Results and Discussion

Method validation

Separation of 5-HT and 5-HIAA was completed in 9 min, with retention times of 4.9 and 6.0 min (Fig. 1). When the analyte concentration was higher than those of the upper calibrator, samples were re-processed once diluted 5 or 10 times.

Calibration curves were linear in the concentration range tested for each compound,

showing determination coefficients (r^2) from 0.992 to 0.999. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) values of each analyte were adequate (lower than the corresponding first concentration calibration level), as reported in Table 1. The calculated LLOQ tested for precision and accuracy presented coefficients of variation always lower than 20%. The intra and inter-assay imprecision and accuracy were lower than 13%.

Pre-analytical conditions

Results show that anticoagulant agent coated in the tub does not play an important role in avoiding artifactual release of 5-HT from platelets. The same samples centrifuged with three different anticoagulants did not differ in 5-HT PPP levels (mean values were 0.59, 0.60 and 0.55 ng/ml in tubes with EDTA, lithium heparin and sodium citrate, respectively).

On the contrary, centrifuging samples at RT was a key factor to avoid 5-HT release, as cold temperatures clearly activated platelets (see figure 2a). Moreover, slow speed centrifugation and the addition of PGI_2 improved the accuracy of 5-HT determinations. However, despite all these precautions, 5-HT levels in PPP under the best conditions assayed were significantly different from 5-HT concentrations obtained in whole blood (see figure 2b).

Analysis of clinical samples

The method of analysis was applied to samples from subjects diagnosed for HYT or ESRD. Preliminary studies have shown that *in vitro* probe recoveries determined in artificial salt solution do not necessarily reflect probe recovery characteristics in complex biological matrices. Thus, we did not "correct" 5-HT values for probe recovery.

Whole blood 5-HT concentrations were found to be lower in controls (0.22 ± 0.03 ng/ml), than in HYT (0.41 ± 0.12 ng/ml), and ESRD subjects (3.77 ± 1.44 ng/ml) (Fig 3a). 5-HT concentrations in PPP were also lower in controls (0.62 ± 0.12 ng/ml), than in HYT (1.52 ± 0.10 ng/ml) and ESRD (8.62 ± 3.06 ng/ml) subjects. Further analysis of the data revealed significant

main effects of group of subjects [$F(2,33) = 6.25$, $P < 0.01$], sample [$F(1,33) = 9.64$, $P < 0.01$] and interaction between factors [$F(2,33) = 7.09$, $P < 0.01$]. Blood 5-HIAA concentrations were lower for controls (0.80 ± 0.40 ng/ml) than for the HYT (1.93 ± 1.01 ng/ml) and the ESRD (14.21 ± 2.13 ng/ml) groups (Fig. 3b). 5-HIAA concentrations in PPP were also lower in controls (1.50 ± 0.25 ng/ml), than in HYT (3.98 ± 2.31 ng/ml) and ESRD (37.31 ± 5.66 ng/ml) subjects. The statistical analysis revealed significant effects of group [$F(2,33) = 46.41$, $P < 0.001$], sample [$F(1,33) = 34.81$, $P < 0.001$] and interaction between factors [$F(2,33) = 29.04$, $P < 0.001$] (see Table 2 for statistics). Analyzing all the volunteers together, significant positive correlation was observed between blood and PPP 5-HT and 5-HIAA concentrations ($r = 0.95$, $P < 0.001$; $r = 0.93$, $P < 0.001$, respectively) but concentrations are about 2.8 times higher in PPP.

In this study, we have developed a rapid and sensitive microdialysis-HPLC-ECD method for the simultaneous quantification of 5-HT and 5-HIAA in blood and PPP samples. Using this methodology, we show that PPP concentrations of these compounds are higher than in whole blood due to centrifugation. Previous studies where 5-HT was determined in PPP following centrifugation at cold temperatures (see Brand and Anderson, 2011 for a review) have overestimated the physiological concentrations of 5-HT present in blood. In this sense, the whole blood microdialysis method we report minimizes trauma to platelets since it does not require centrifugation. In addition, it provides a rapid and clean extraction of the sample through the microdialysis probe that can be directly injected into commonly used HPLC-ECD equipment.

The present work reports that exist pre-analytical factors which are of key importance in obtaining 5-HT reliable values from PPP samples, and shows that to determine 5-HT in whole blood instead of PPP is a much better approach to real 5-HT concentrations. At the same time, this methodology is easy to apply in any clinical trial; it overcomes some of the limitations of previous methods as it is less invasive (Paez and Hernandez, 1996; 1998).

All previous data reflect that there is no optimal procedure to obtain PPP from blood, but

slow speed centrifugation, RT procedures, and the use of an antiagreggant seem to play an important role in reducing platelet contamination.

Human 5-HT concentrations shown in this work fit in the lower range of 5-HT concentrations reported in PPP from healthy controls in several studies (Herve *et al.,* 1995; Ohkawa *et al.,* 2005; Matuchansky and Launay., 1995; Lederer *et al.,* 2008; Dupuis *et al.,* 2010) and agree with higher values of PPP 5-HT in HYT (Mercado and Kilic., 2010; Brenner *et al.,* 2007) and ESRD subjects (Kerr *et al.,* 1992; Sebekova *et al.,* 2001) which show a serotonergic dysfunction.

Conclusion

In summary, we have studied several key pre-analytical that affect 5-HT determinations in PPP samples. RT, slow speed centrifugation together with the addition of an antiaggregant are essential factors to avoid platelet contamination when manipulating the samples. Nevertheless, despite care being taken there is a significant artifactual release (5-HT values in PPP are 2.8 higher that those in whole blood). We highly recommend using use whole blood to determine 5-HT levels in human samples instead of PPP, followed by the microdialysis method as it provides the desired precision, accuracy and reproducibility.

Acknowledgements

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Table 1. Retention times, lower limits of quantification (LLOQs), limits of detection (LLODs), regression coefficients, precision and accuracy for 5-HT and 5-HIAA.

5-HT	Retention time (min)	LLOQ, (ng/ml)	LLOD (ng/ml)	Linearity (r^2)[a]	Slope[b]	*y* Intercept (ng/ml)	Conc (ng/ml)	No.obs.	Estimated conc ± SD (ng/ml)	Precision (CV)	Accuracy (error %)
							Intra-assay				
	6.0	0.082	0.027	0.995±0.002	0.139±0.001	-0.005±0.001	0.12	4	0.13±0.01	5.2	12.2
							0.80	4	0.86±0.03	3.3	7.2
							1.70	4	1.76±0.07	4.0	3.3
							Inter-assay				
							0.12	4	0.12±0.02	12.5	8.6
							0.80	4	0.77±0.06	7.4	6.8
							1.70	4	1.73±0.10	5.9	5.1
5-HIAA	**Retention time (min)**	**LLOQ, (ng/ml)**	**LLOD (ng/ml)**	**Linearity (r^2)[a]**	**Slope[b]**	***y* Intercept (ng/ml)**	**Conc (ng/ml)**	**No.obs.**	**Estimated conc ± SD (ng/mL)**	**Precision (RSD)**	**Accuracy (error %)**
							Intra-assay				
	4.9	0.073	0.024	0.998±0.001	0.091±0.001	-0.001±0.000	0.12	4	0.13±0.01	11.0	11.1
							0.80	4	0.81±0.07	8.9	7.1
							1.70	4	1.81±0.02	1.1	6.6
							Inter-assay				
							0.12	4	0.12±0.01	7.8	6.3
							0.80	4	0.77±0.06	7.4	6.8
							1.70	4	1.72±0.10	5.9	5.1

[a] Data are expressed as r^2 (SD).
[b] Data are expressed as mean (SD) (n=4).
5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid.

Table 2. ANOVA models with repeated measures of 5-HT and 5-HIAA concentrations in blood and PPP for the groups of subjects analysed.

Compound		Concentrations (ng/ml)			Group		Sample		Interaction	
		Controls	HYT	ESRD	F-value	p-value	F-value	p-value	F-value	p-value
5-HT	*Blood*	0.22±0.0.03††	0.41±0.12	3.77±1.44**§§†	$F_{(2,33)}$=6.249	<0.01	$F_{(1,33)}$=9.635	<0.01	$F_{(2,33)}$=7.092	<0.01
	PPP	0.62±0.12	0.62±0.10	8.62±3.06**§§						
5-HIAA	*Blood*	0.80±0.40†††	1.93±1.01	14.21±2.13***§§§†††	$F_{(2,33)}$=46.412	<0.001	$F_{(2,33)}$=34.806	<0.001	$F_{(2,33)}$=29.042	<0.001
	PPP	1.50±0.25	3.98±2.31	37.31±5.66***§§§						

5-HT and 5-HIAA mean concentrations in blood and PPP from controls (n=12), HYT (n=12), and ESRD subjects (n=12).

*P < 0.05, **P < 0.01, ***P < 0.001 (blood or PPP concentrations vs. controls). LSD *post hoc* test.

§P < 0.05, §§P < 0.01, §§§P < 0.001 (blood or PPP concentrations vs. HYT). LSD *post hoc* test.

†P < 0.05, ††P < 0.01, ††† P < 0.001 (PPP concentrations versus blood concentrations). Pairwise comparisons.

Figure Captions

Figure 1. A chromatogram of a dialyzed sample from an ESRD patient showing (1) 5-HIAA and (2) 5-HT peaks.

Figure 2. (a) 5-HT concentrations in PPP obtained from controls (n=15), being centrifuged at 2000g at RT or 4°C. (b) 5-HT concentrations in whole blood and PPP from controls (n=15), being centrifuged at different speeds and adding the antiaggregant PGI_2. The data represent the mean concentration of 5-HT + standard error of the mean (SEM). *P < 0.05, **P < 0.01, ***P < 0.001 (cold temperature vs. RT, and PPP concentrations vs. blood concentrations).

Figure 3. (a) 5-HT concentrations in blood and PPP from controls (n=12), HYT (n=12), and ESRD subjects (n=12). (b) 5-HIAA concentrations in blood and PPP from controls (n=12), HYT (n=12), and ESRD subjects (n=12). The data represent the mean concentration of 5-HT + standard error of the mean (SEM). *P < 0.05, **P < 0.01, ***P < 0.001 (PPP concentrations *vs.* blood concentrations).

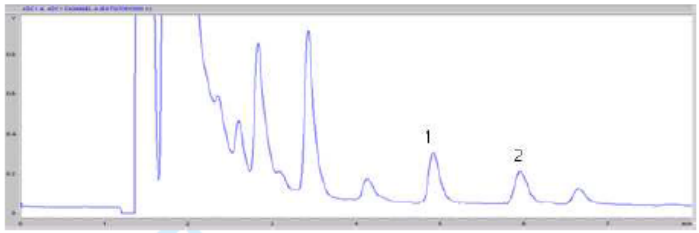


Figure 1. A chromatogram of a dialyzed sample from an ESRD patient showing (1) 5-HIAA and (2) 5-HT peaks.

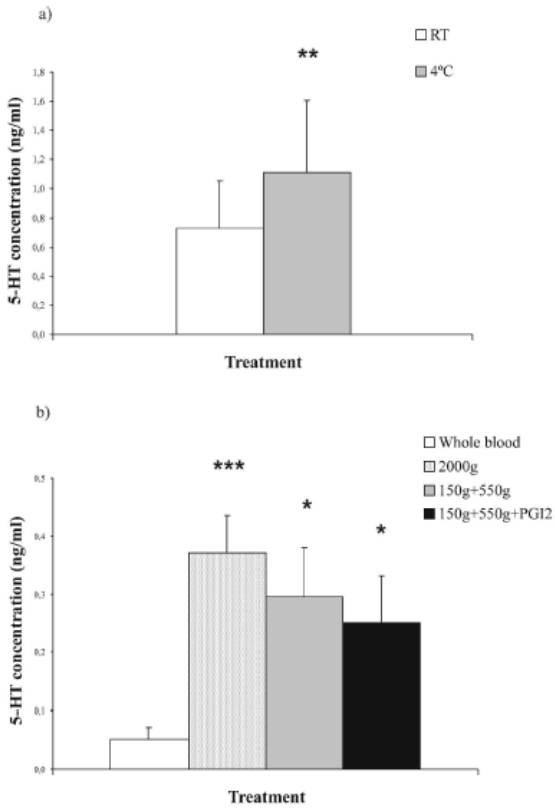


Figure 2. (a) 5-HT concentrations in PPP obtained from controls (n=15), all being centrifuged at 2000g, but at RT or 4ºC. (b) 5-HT concentrations in whole blood and PPP from controls (n=15), all being centrifuged at RT, but different speeds and adding the antiaggregant PGI_2. The data represent the mean concentration of 5-HT + standard error of the mean (SEM). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (cold temperature *vs.* RT, and PPP concentrations *vs.* blood concentrations).

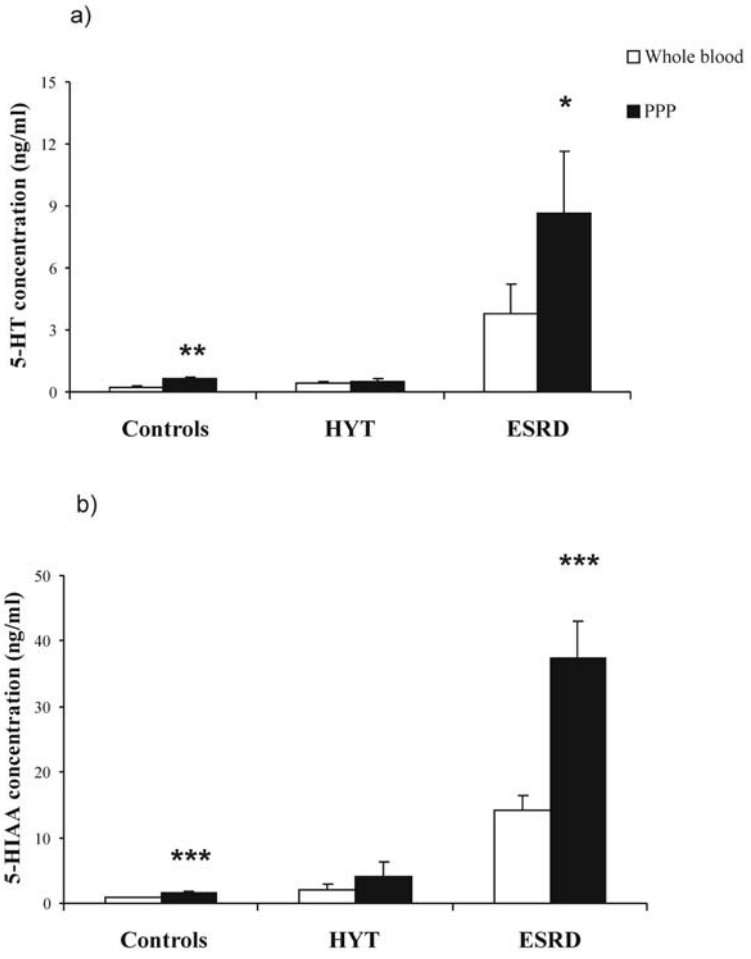
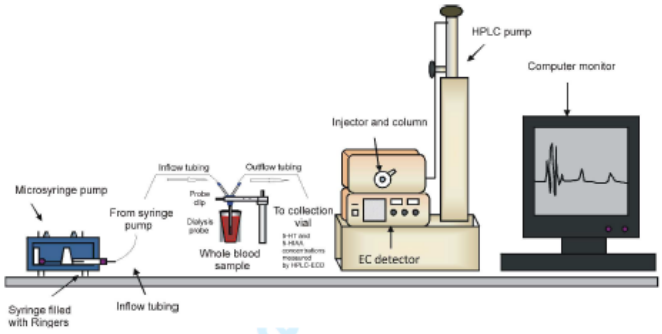


Figure 3. (a) 5-HT concentrations in whole blood and PPP from controls (n=12), HYT (n=12), and ESRD subjects (n=12). (b) 5-HIAA concentrations in blood and PPP from controls (n=12), HYT (n=12), and ESRD subjects (n=12). The data represent the mean concentration of 5-HT + standard error of the mean (SEM). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (PPP concentrations *vs.* blood concentrations).

Graphical Index



Dose finding clinical study of mephedrone with 3,4-Methylenedioxymethamphetamine (MDMA; Ecstasy) as reference drug

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Introduction

Mephedrone (4-methylmethcathinone; MEPH) is a new and popular psychostimulant chemically related to amphetamine, widely available on the Internet and still legal in some parts of the world (Schifano et al., 2011). MEPH is mainly sought for causing euphoria, social desinhibition, empathy, and increased libido (Ribeiro et al., 2012). Recent pre-clinical data revealed that MEPH is a nonselective substrate for plasma membrane monoamine transporters, similar to 3,4-methylenedioxymethamphetamine (MDMA) in potency and selectivity (Hadlock et al., 2011). *In vivo* microdialysis in rat nucleus accumbens showed that intravenous administration of 0.3 and 1.0 mg/kg of MEPH produced dose-related increases in extracellular dopamine and serotonin (5-HT) concentrations, with the magnitude of effect on 5-HT being greater (Kehr et al., 2011, Baumann et al., 2012). These results were confirmed in synaptosomes; MEPH inhibited 5-HT uptake with a lower IC_{50} value than that for the dopamine transporter (Martinez-Clemente et al., 2012). Other pre-clinical studies report that it causes locomotor activation (Motbey et al., 2012, Martinez-Clemente et al., 2013), and increases heart rate, blood pressure and cardiac contractility (Meng et al., 2012). Some case reports revealed several adverse effects on cardiovascular and neurological systems after MEPH consumption, with an acute sympathomimetic toxidrome (e.g. hypertension, tachycardia and agitation) (Wood et al., 2010a). Recent data reported that MEPH, as MDMA, presents non-linear pharmacokinetics, which could cause an increase in plasmatic MEPH concentrations, leading to an enhanced toxicity (Martinez-Clemente et al., 2013). However, the neurochemical actions of MEPH have scarcely been studied, and up to date, there is no data referring to its clinical pharmacology.

MDMA is known to cause the release of 5-HT, dopamine and norepinephrine from neurons by acting as substrate for monoamine

transporter proteins (Crespi et al., 1997, Rothman et al., 2001). The serotonin transporter (5-HTT) recycles back 5-HT from extracellular space to inside the cell. The same single-copy gene *SLC6A4* encodes for 5-HTT protein in platelets and neurons, and polymorphisms affecting its expression have been shown to influence function in human platelets and brain in a similar manner (Blakely et al., 1991, Hoffman et al., 1991). In humans, transcriptional activity is modulated by a repetitive element of varying length found in the 5' flanking region. This region is known as the serotonin-transporter-linked polymorphic region or 5-HTTLPR (Murphy et al., 2004). The functional polymorphism of an insertion/deletion of 43 base pairs in this promoter region gives rise to the long (l) or short (s) allelic variants (Heils et al., 1996), and alters the transcriptional activity of the *SCL6A4*. The short variant reduces the transcriptional efficiency of the *SCL6A4* promoter, resulting in decreased 5-HTT expression and uptake activity (Lesch et al., 1996). On the contrary, *SCL6A4* expression is 30% higher in homozygous *l/l* carriers when compared to heterozygous *s/l* or homozygous *s/s*.

Recent data have revealed platelet 5-HTT is involved with the elevation of plasma 5-HT produced by amphetamine-type drugs (Zolkowska et al., 2006, Zolkowska et al., 2008) and that MDMA causes an increase on circulating blood 5-HT (Yubero-Lahoz et al., 2012). Moreover, pre-clinical reports have shown that MDMA causes parallel changes in 5-HT levels in brain and blood in rats (Yubero-Lahoz et al., 2012, Collins et al., 2013). Therefore, platelet 5-HTT has been proposed as useful marker for brain 5-HTT functionality in the rat following acute administration of MDMA. However, no clinical data are available confirming these results.

The present study had three main objectives: firstly, to evaluate whether MDMA can provoke an increase on blood 5-HT in humans; secondly, to study the clinical pharmacology of MEPH, comparing to

MDMA, a widely studied drug of abuse, and lastly, to evaluate the relationship between 5-HTTLPR genotype and drug induced 5-HT release in blood.

Material and Methods

Study Participants

Eight healthy Caucasian males recruited by word of mouth were included in the study. Eligibility required the self-reported recreational use of MDMA on at least ten occasions and twice in the previous year. None had a history of adverse medical or psychiatric reactions after MDMA consumption. Subjects were interviewed by a psychiatrist to exclude those with a history of or actual major psychiatric disorders (Torrens et al., 2004) and by a physician to exclude concomitant medical conditions. Subjects underwent a general physical examination, routine laboratory tests, urinalysis, and 12-lead ECG. None met criteria of abuse or drug dependence (except for nicotine) and all had previous experience with other psychostimulants, cannabis or hallucinogens. Subjects were informed about the study and provided written informed consent before inclusion and were compensated for their participation. The study was conducted in accordance with the Declaration of Helsinki (2000), approved by the local Institutional Review Board (CEIC-IMAS). All participants were phenotyped for CYP2D6 activity using dextromethorphan; the extensive metabolizer (EM) phenotype was required for participation in the study (Schmid et al., 1985). Subjects were requested to refrain from consuming drugs of abuse two weeks before and throughout the duration of the study and asked to follow a tryptophan-free diet 48 hours prior to the beginning of each session. Regular ingestion of medication in the month preceding the study was an exclusion criterion although single doses of symptomatic medication

were accepted up to the week preceding the trial. At each session and before drug administration, urine samples were collected for drug testing (opiates, cocaine, amphetamines and cannabis) by a rapid test device (Instant-View®, Alpha Scientific Designs, Inc, Poway, CA, USA). A positive screen test was considered an exclusion criterion.

Study Design and drugs

The present study comprehends three pilots, each one including three experimental sessions.

The first pilot was first conducted in three individuals administering a low dose of MEPH (50 and 100 mg) to evaluate its safety, with a placebo condition (see table 1). The second pilot study was carried out in three individuals administering a higher dose of MEPH (150 and 200 mg), and placebo, to determine the optimal drug dose. The third pilot was performed with two subjects, administering MDMA instead of placebo, as a drug reference (MDMA, 150 and 200 mg of MEPH).

In each pilot study, subjects participated as outpatients in three experimental sessions. Each session lasted over 12 hours, with a wash-out period of 7 days between each session. The study design was a double-blind, randomised cross-over and controlled trial with placebo in the first two pilots, and MDMA in the third.

Pilot 1	Pilot 2	Pilot 3
Placebo	Placebo	100 mg MDMA
50 mg MEPH	150 mg MEPH	150 mg MEPH
100 mg MEPH	200 mg MEPH	200 mg MEPH

Table 1. Schedule of drug administration in the three pilot studies.

Dextromethorphan tablets (Romilar®; Roche Farma, SA, Madrid, Spain) were supplied by the Pharmacy of the Hospital del Mar, Barcelona, Spain. (R,S)-MDMA and MEPH were supplied by the Spanish Ministry of Health, and prepared in white, opaque soft gelatine capsules by the Hospital del Mar Pharmacy Department.

Each session began at 07:30 h following an overnight fast. Drug administration commenced at 08:30 h. The dose of MDMA was chosen to be in the range of reported among single recreational doses of MDMA (Parrott, 2004). All drugs were administered by the oral route. A light meal was provided 2 h after initial drug administration. Blood samples were obtained through an indwelling catheter inserted into a subcutaneous vein in the forearm of the non-dominant arm. Thereafter, the subjects remained seated in a quiet room. Samples (8 mL) were taken at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 25 h after placebo, MEPH or MDMA administration. After centrifugation at 4ºC, four 1 mL aliquots of plasma were stored at -20ºC until analysis. During sessions cardiovascular effects were recorded for safety reasons (continuous ECG, blood pressure, heart rate and temperature) but not presented in this manuscript.

Genotyping

Genomic DNA was extracted from the peripheral blood leukocytes of participants using the Flexi Gene DNA kit (Qiagen Iberia, S.L., Spain). 5-HTTLPR genotyping was performed using polymerase chain reaction (PCR) as previously described (Fagundo et al., 2010). Subjects were split according to genotype and associated functionality (5-HTTLPR, carrying the *l*/* or *s*/*s* alleles).

Pharmacokinetic Measurements

All chemical reagents were of the highest grade available. Dextromethorphan, DOR, MM and HM, its internal standard levallorphan, 5-HT and its metabolite 5-Hydroxyindoleacetic acid (5-HIAA), were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain). Aliquots of urine and plasma were assayed for dextromethorphan, DOR, MM and HM, using a previously reported method for phenotyping (de la Torre et al., 2005) based on solid-liquid extraction with Bond Elut Certify (Varian, Palo Alto, CA, USA) columns and analysis by high-performance liquid chromatography and fluorescence detection. Plasma MDMA, HMMA MDA (3,4-methylenedioxyamphetamine), and HMA (3-methoxy-4-hydroxyamphetamine) were analyzed following a previously reported method based on solid-liquid extraction and gas chromatography–mass spectrometry (GC/MS) (Pizarro et al., 2002). Mephedrone plasma concentrations were also determined by GC/MS (unpublished method).

Ex vivo microdialysis in blood

For whole blood 5-HT analyses, blood samples (4 mL) were collected at pre-dose, and at 1, 2, 4, 6, and 8 hours after drug administration and specimens were shortly after transferred gently to 300 µl polypropylene tubes containing 20 µl of 1000 IU/ml heparin and kept at room temperature. Microdialysis probes (4 mm exchange surface, MAB 6, SciPro, Inc., Sanborn, NY, USA) were immediately placed into blood specimens and perfused with Ringer's solution pumped at a flow rate of 2.0 µl/min. Dialysate efflux was collected at 25 min intervals and assayed for 5-HT and 5-HIAA using high performance liquid chromatography with electrochemical detection (HPLC-ECD). One baseline dialysate samples was collected before administration of test drugs. *In vitro* probe recoveries were performed before and after microdialysis using a 10 pg/5

µl standard of 5-HT in Ringers' solution. The concentration of 5-HT in dialysate samples was compared to known standards, and the lower limit of detection was 0.027 ng/ml and 0.024 ng/ml for 5-HT and 5-HIAA, respectively.

HPLC-ECD analysis of 5-HT and 5-HIAA

The HPLC system consisted of an isocratic pump (Model 300), an autosampler (Model 200), and an ECD equipped with a glassy carbon electrode (Model 100) (Chromsystems, München, Germany). The chromatographic separation was performed using a 150 × 4.6 mm C18 column (Agilent Technologies). The mobile phase consisting of 75 mM sodium acetate, 240 µM Na_2EDTA, and 150 µM sodium octanesulfonic, with 22 % MeOH (pH 5.00) was pumped at a flow rate of 1 ml/min. A mobile phase conditioning cell with a potential set at +0.60 V was connected between the pumping system and the injector.

Physiological Measures

Readings of non-invasive systolic blood pressure (SBP), diastolic blood pressure (DBP), HR, oral temperature (OT), pupil diameter (PD), and esophoria (ESO) were taken 15 minutes prior to drug administration, at baseline (time 0), and at 0.33, 0.66, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, and 24 hours after drug administration. SBP, DBP, HR, and OT were recorded using a CarescapeTM V100 monitor (GE Healthcare. Milwaukee, WI). Pupil diameter was calculated using a pupil gauge (Haab scale). The Maddox-wing device (AM, Clement Clark, London, U.K) was used to measure the balance of extraocular muscles and quantify exophoria and esophoria (drug effect) (Mas et al., 1999). For safety reasons, ECG was

continuously monitored during the first 12 h with a DashH 3000 patient monitor (GE Healthcare).

Rating Scales of Subjective Effects

Subjective effects were measured using a set of visual analogue scales (VAS), the Addiction Research Center Inventory (ARCI), and the Evaluation of the Subjective Effects of Substances with Abuse Potential (VESSPA) questionnaire at baseline and at 0.33*, 0.66*, 1, 1.5*, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h (* only VAS) after drug administration. VAS: Twenty one 100-mm VAS labelled with different adjectives marked at opposite ends with ‘‘not at all’’ and ‘‘extremely’’ were administered (Cami et al., 2000). ARCI: A Spanish validated version of a 49-item short form of ARCI was used (Cami et al., 2000, Farre et al., 2007). VESSPA is a validated questionnaire measuring MDMA induced changes in subjective variables (Farre et al., 2007).

Results

Baseline 5-HT levels in blood

For the eight subjects included in the present study, the mean basal concentration of dialysate 5-HT in blood was 0.078±0.025 ng/ml. It should be noted that microdialysis probes had *in vitro* recovery rates of approximately 25% and this value did not change before, during or after experiments where probes were immersed in sequential blood specimens. Preliminary studies have shown that *in vitro* probe recoveries determined in artificial salt solution do not necessarily reflect probe recovery characteristics in complex biological matrices. Thus, we did not “correct” 5-HT values for probe recovery.

Analysis of clinical samples

The MDMA single dose of 100 mg produced the typical effects described for this substance in an experimental laboratory setting (de la Torre et al., 2000) and no adverse effects were reported. The mean Cmax of MDMA was 107.6±16.2 ng/mL with a tmax of 1.0±1.0 hours and AUC from 0 to 25 hours of 1448.4±331.9 mg•h/mL.

The administration of 100 mg of MDMA caused an increase in extracellular blood 5-HT. These results have to be interpreted cautiously, since the number of subject is very small (n=2). Nevertheless, in these two subjects MDMA caused a 12-fold increase in 5-HT blood levels, from 0.060 to 1.01 ng/ml. The 5-HT concentrations in the first sample after drug intake were significantly elevated compared to preadministration baseline.

Figure 1 depicts 5-HT blood concentrations after MDMA administration and High scores. It shows a similar profile for both parameters; elevations in 5-HT levels occur along with changes in VAS scores.

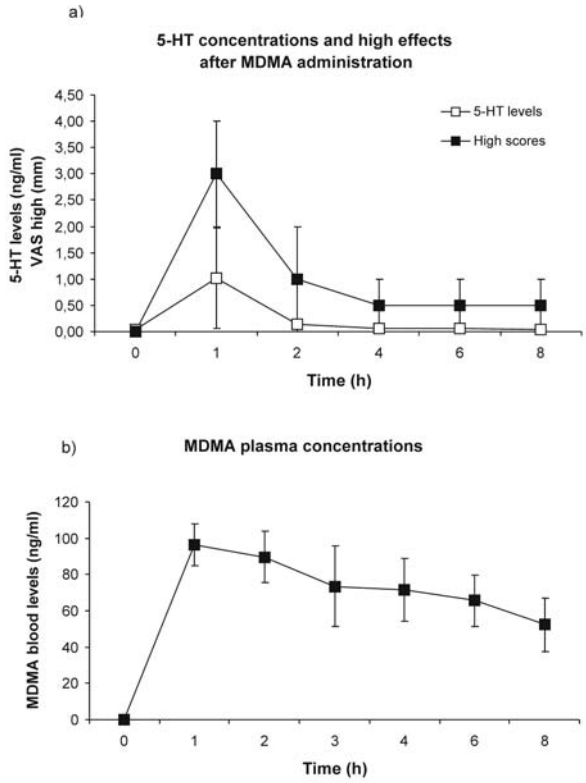


Figure 1. (a) Mean (± standard error) 5-HT blood concentrations of 5-HT and VAS scores; (b) MDMA plasma concentrations after administering 100 mg dose in men (n = 2).

5-HT blood levels values significantly correlated with some subjective and physiological effects, such as good effects, SBP and DBP (table 2).

Effects		**Whole blood 5-HT (ng/ml)**	
		r	***P-value***
Subjective	**High**	0.975	<0.01
	Good effects	0.815	<0.05
Physiological	**SBP**	0.946	<0.01
	DBP	0.900	<0.05

Table 2. Relationship between drug-induced increases in plasma 5-HT and values for subjective and physiological tests performed during the clinical trial. Mean effects of MDMA on plasma 5-HT were plotted against the results of several psychological and physiological tests. Pearson's correlation coefficient "r" and P value for significance are given.

The administration of MEPH 50 and 100 mg in the first pilot did not produce significant changes in physiological or subjective parameters compared to placebo (data not shown).

The administration of MEPH 150 and 200 mg in the second and third pilot produced a significant increase in physiological parameters such as HR, SBP, and DBP. Subjective effects measured by VAS scales also changed in "high", "good effects" and "stimulated" scores. The mean Cmax of 150 mg oral dose was 127.6±34.0 ng/mL with a tmax of 1.0±0.6 hours and AUC from 0 to 25 hours of 487.3±196.0 mg*h/mL. The mean Cmax of 200 mg oral dose was 190.9±93.6 ng/mL with a tmax of 1.0±0.3 hours and AUC from 0 to 25 hours of 705.5±281.3 mg*h/mL

Figure 2 shows the effects MEPH intake on dialysate concentrations of 5-HT in blood from the five volunteers participating in the second and third pilot studies. MEPH increased dialysate 5-HT in blood after 150 mg and 200 mg doses. This stimulatory effect was dose dependent, with 150 mg and 200 mg doses elevating 5-HT to 6- and 10-fold above pre-administration baseline, respectively. This figure also shows that 5-HT release depends on the dose of the drug given, but also on the subject genotype. Volunteers carrying *s/s* genotype (n=2) report higher 5-HT blood concentrations (7.44±4.28 ng/ml) than *l/s* (n=2) (6.18±3.03 ng/ml) and *l/l* (n=1) (1.39±1.59 ng/ml) subjects. The data reveal a highly significant positive correlation between MEPH and 5-HT blood concentrations ($P<0.01$), suggesting that MEPH induces 5-HT release in a dose dependent manner.

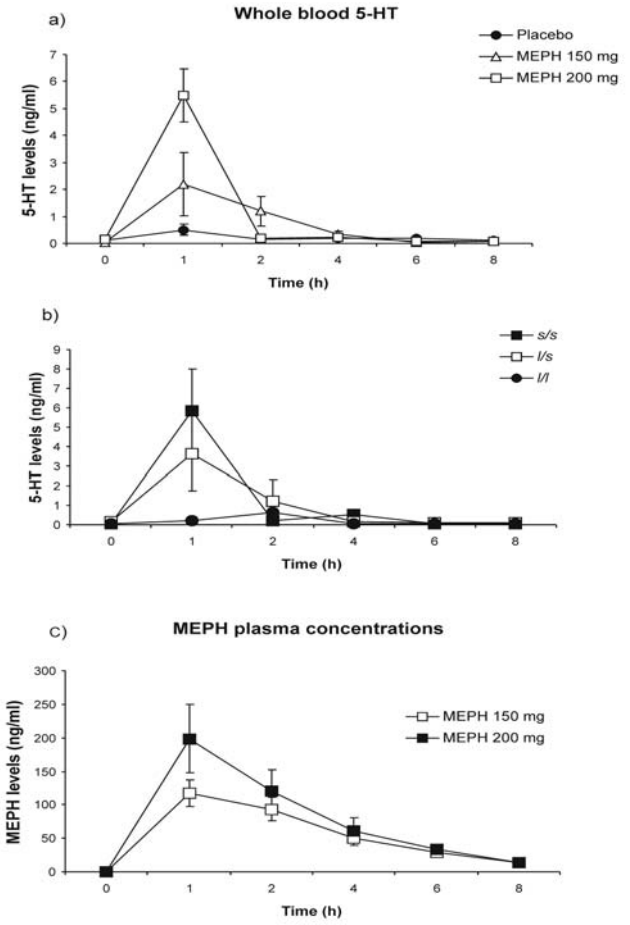


Figure 2. Mean (± standard error) blood concentrations of 5-HT depending on; (a) MEPH drug dose administered; (b) 5-HTTLPR genotype. (c) MEPH plasma concentrations after administering 150 mg or 200 mg doses in men (n = 5).

The findings in Table 3 indicate that there is a strong positive relationship between extracellular 5-HT levels in blood and the subjective and physiological effects observed. Importantly, inspection of the raw data revealed that there was a highly variable response to MEPH among the individuals. While all subjects received the same doses, the values of 5-HT and magnitude of responses differed between them. However, subjects who showed low effects also presented low 5-HT levels in blood.

Effects		Whole blood 5-HT (ng/ml)	
		r	***P-value***
Subjective	**High**	0.977	<0.001
	Stimulated	0.976	<0.001
	Good effects	0.977	<0.001
Physiological	**SBP**	0.957	<0.001
	DBP	0.779	<0.005
	HR	0.787	<0.005

Table 3. Relationship between drug-induced increases in plasma 5-HT and values for subjective and physiological tests performed during the clinical trial. Mean effects of MEPH on plasma 5-HT were plotted against the results of several psychological and physiological tests. Pearson's correlation coefficient "r" and P value for significance are given.

Subjects presented differences in some physiological parameters depending on their genotype (see figure 3). Subjects presenting the genotype *s/s* reported higher values of HR and papillary diameter (mydriasis), compared to *l/** subjects.

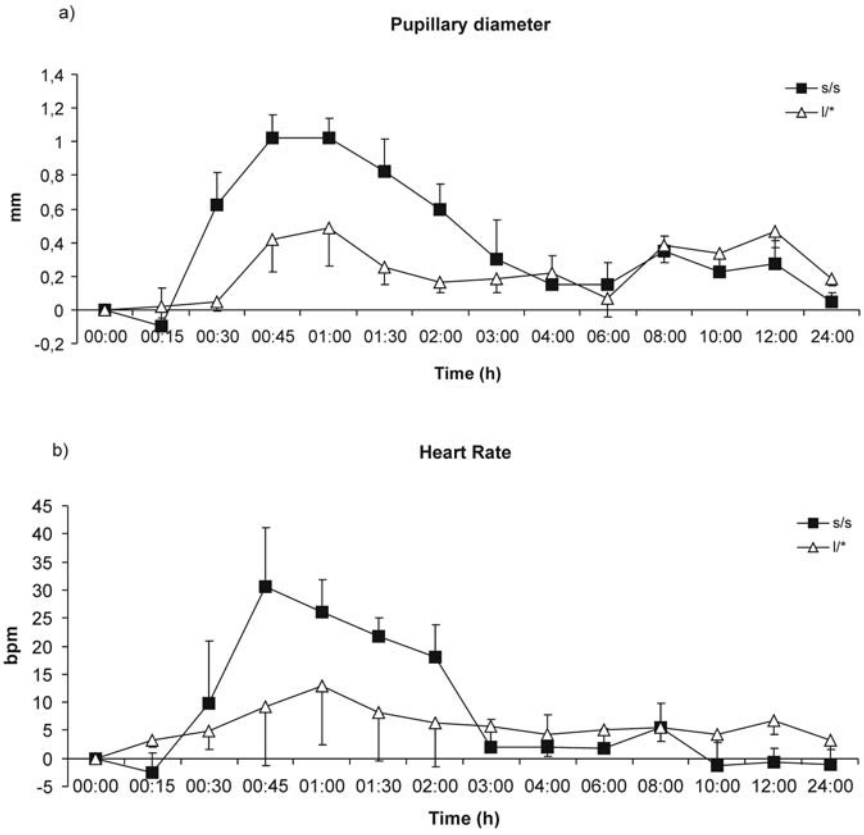


Figure 3. Affectation of 5-HTTLPR genotype on (a) mean (± standard error) change in pupillary diameter (b) change in heart rate (bites per minut), after administering 150 and 200 mg of MEPH.

Discussion

In the present study we report, for the first time, that MDMA increases blood 5-HT levels in humans. A single recreational dose of MDMA caused a 12-fold increase in 5-HT blood levels. Our data agrees with previous *in vitro* findings supporting the hypothesis that platelet 5-HTT is involved with the elevation of blood 5-HT produced by amphetamine-type drugs. The results presented here are preliminary from a pilot study and a small number of subjects were performed (n=2). Consequently, a larger sample is needed to confirm these findings, and

further evaluate some other factors (i.e. genetic factors such as 5-HTTLPR genotype) in this phenomenon.

MDMA was used in this study as a reference drug with two purposes; to determine whether, as it was previously reported in rats (Yubero-Lahoz et al., 2012), it was able to provoke a release on peripheral 5-HT (measured by using the method developed in the present thesis), and secondly, to compare its known clinical pharmacology to a newer abuse drug; MEPH.

This study is the first to report a controlled clinical trial evaluating the pharmacology of MEPH in healthy recreational users. The subjective and physiological effects observed after administering MEPH are similar to those of MDMA; showing an increase in HR, SBP, DBP, and also in subjective effects reported by the volunteers, such as "high", "good effects", and "stimulation". Nevertheless, the number of subjects is quite modest and a larger sample is required to confirm these results.

Regarding the volunteers who received 150 and 200 mg of MEPH (n=5), the increase in the aforementioned scores of subjective effects was parallel to an increase in 5-HT blood concentrations. MEPH plasma concentrations highly and significantly correlated with 5-HT blood concentrations. Although there was a variable response to MEPH among the individuals, these variations in drug effects were consistent with 5-HT concentrations. In other words, global values point out that MEPH provokes a release of 5-HT in a dose dependent manner. Nevertheless, analysing each subject individually, the same drug concentrations not always produced the same fold-increase in 5-HT blood levels, most likely due to differences in genetic factors (such as 5-HTTLPR genotype) between subjects. These results suggest that 5-HT blood levels play an important role in the physiological and psychological effects induced by MEPH administration, and therefore, the 5-HTTLPR genotype is also important in drug response. One hand, after drug administration and 5-HT

induced-release, subjects carriers of *s/s* genotype, which present low amounts of 5-HTT in platelets' membrane, have a limited capability to re-uptake 5-HT to platelets. On the other hand, carriers of *l/** genotype have a more dynamic serotonergic system, which allows to reuptake 5-HT more efficiently and return to a normal state more rapidly. Our thought is that *s/s* carriers will experience higher and longer drug effects compared to *l/** carriers, who will probably need higher drug doses to experience the desired pharmacological effects. This hypothesis was confirmed by grouping the subjects in 2 genotypes; *s/s* carriers (n=2) presented higher values of HR and papillary diameter when compared to baseline values, when compared to *l/** carriers (n=3).

Recent data reported that 5-HTT in platelets could be used as a peripheral biomarker for central serotonergic activity (Yubero-Lahoz et al., 2013). To have a reliable peripheral index of 5-HTT-mediated effects of drugs in the brain would be potentially useful as it is technically difficult to measure 5-HTT binding and function in living human brain (Huang et al., 2010).

The main goal of the present work was to measure the *in vivo* effects of MDMA and MEPH on blood 5-HT to gauge the effects of those drugs on platelet 5-HTT, and possibly relate this to brain 5-HTT function. In humans, interpreting this type of data is more complicated than in pre-clinical studies due to 5-HTT polymorphisms, life-time drug exposures, and other factors. Despite such limitations, our results suggest that 5-HT blood levels could be related to effects caused by the administered drug. The pharmacological effects observed in this pilot study correlate with 5-HT blood levels, and therefore, it is tempting to speculate that 5-HTT in platelets could be a reliable peripheral biomarker for central 5-HTT activity.

In summary, amphetamine type-drugs such as MDMA and MEPH caused an increase in 5-HT blood levels, which highly correlated to drug

physiological and psychological effects. These preliminary results put forward the hypothesis that 5-HTT present in platelets could be a surrogate biomarker of 5-HTT activity in the brain, implying an important role of the serotonergic system in the mechanism of action of these drugs. Further clinical investigations are warranted to confirm these observations.

GENERAL DISCUSSION

The studies presented in this thesis were aimed at investigating gender differences in MDMA pharmacology considering both pharmacokinetics, and the role of the serotonergic system in the pharmacodynamics of MDMA and related drug mephedrone.

MDMA is a potent mechanism based inhibitor of CYP2D6, an aspect that has a relevant impact in pharmacological effects seen in humans. The MDMA induced MBI phenomenon was first described in male subjects. **Our goal for the first part of the thesis was to study the pharmacokinetics of MDMA in females, focusing on CYP2D6 and CYP1A2 activities. Some preliminary reports suggest an enhanced sensitivity to MDMA effects by female subjects and we wanted to examine if pharmacokinetics and MDMA metabolic disposition may contribute to these observations.**

Several studies have provided evidence that MDMA is a substrate of CYP2D6 but at the same time it is also a potent MBI of the enzyme (de la Torre et al., 2000; Farré et al., 2004; Heydari et al., 2004; Yang et al., 2006). The MBI implies a rapid phenocopying of EM subjects to an apparent PM phenotype status after a single dose of MDMA. In practice, within 2 h after drug intake subjects display the PM phenotype, irrespective of their original genotype. In a previous clinical trial conducted in male subjects, results showed that the phenocopying phenomenon was observed in 67% of the participants. In the same study it was observed that MBI inactivated most hepatic CYP2D6 within 2 h, and it returned to baseline CYP2D6 activity after at least 10 days (O'Mathúna et al., 2008). This phenomenon is associated with a decrease in the availability of effective enzyme so that recovery of activity depends on its *de novo* synthesis (Liston et al., 2002).

Up to date, there was no available clinical data regarding MBI of the enzyme in female subjects, neither if gender differences existed.
The first publication **(P-I)** of the present thesis has provided, for the first time, the inhibition and recovery half-life of CYP2D6 in female subjects. These data were compared with the data obtained from a previous study in male subjects to assess gender differences.

Importantly, gender differences were already present at baseline, and persisted after MDMA administration with male displaying a stronger inhibition of the CYP2D6 enzyme by the drug than female subjects. Moreover, the phenocopying phenomenon was observed in 100% of the females, whereas in males was of about 67%.
Nevertheless, the time course of CYP2D6 recovery showed no significant between-gender differences in terms of time to reach the 50% of enzyme activity. In both genders, CYP2D6 activity recovered after 10 days to 90% of baseline activity, with a recovery half-life of 36.6±22.9 hours in females and a shorter recovery half-life (27.6±25.1 hours) in males.

Gender differences in *de novo* CYP2D6 protein synthesis after a MBI of the isoenzyme could be the cause of these gender differences observed in the steepness of the sigmoid curves, but further studies and different models are required to explain the higher pronounced shape of functional recovery of CYP2D6 in women.

These results are related with the publication **P-II** in which the activity of CYP1A2 of both genders was assessed in the same subjects and under the same conditions as in P-I. The evaluation of CYP1A2 activity is of relevance in order to conciliate pre-clinical observations suggesting that the activity of cytochrome P450 isozymes (other than CYP2D6) would be lowered by MDMA exposure, and the clinical observation showing that it

is metabolically cleared in less than 48 hours. Therefore, a relevant discrepancy exists between *in vitro* studies foreseeing a full inhibition of MDMA metabolic disposition in which the drug inhibits its own metabolism by inhibiting P450 CYPs, and the clinical observation that the drug is metabolically cleared from the body.

Besides, there were no data available in the literature reporting the *in vivo* activity of CYP1A2 after MDMA intake.

The publication **P-II** of the present thesis assesses activity of CYP1A2 *in vivo* after CYP2D6 had been inhibited by MDMA, and compares the activity of this isoenzyme in both genders.

Taken together P-I and P-II, results show that as it was hypothesized, MDMA affected differently the activities of the cytochrome P450 enzymes in both genders. In fact, there were already gender differences in CYP2D6 and CYP1A2 enzyme activities at baseline condition, females showing a lower CYP2D6 and CYP1A2 activities compared to males.

It is noteworthy to point out that all subjects included in this study were extensive metabolizers on the basis of a DEX phenotyping test. Behind this phenotype, certain heterogeneity on the genotype associated to it is expected. However, in the present studies, gender differences observed in DEX disposition are not attributable to a dissimilar distribution of CYP2D6 genotypes as their distribution among subjects was quite the same.

Regarding CYP1A2, P-II provided evidences of an apparent increase effect on CYP1A2 activity when CYP2D6 had been inhibited by the drug. Gender differences were also present in this case; women increased 40% its enzyme activity *vs.* 20% in men. In this study a pharmacokinetic model was also built to provide more data and predicted parameters. It was

shown that once CYP2D6 is inhibited, an increase in 17X formation is observed in both genders.

The present work shows an increase of one of the CYP450 isozymes *in vivo* when another is inhibited by MDMA. It seems, therefore, that a **compensatory mechanism** exists between the CYP450 isozymes in order to metabolically clear the drug.

Summary

Women display a slower CYP2D6 and CYP1A2 activity when compared to men.

After MDMA administration, the MBI occurs and CYP2D6 is inhibited by the drug. Women are less inhibited by the drug but it takes longer to recover. However, after 10 days, both genders have recovered the 90% of CYP2D6 activity.

CYP1A2 changes its phenotype after CYP2D6 has been inhibited. An increase of its activity takes place in both genders, being of about the 40% in women, and 20% in men.

The increase in CYP1A2 activity may further contribute to MDMA metabolic disposition once CYP2D6 has been irreversibly inhibited by the drug. A good correlation between CYP2D6 and CYP1A2 activities indicated that the more CYP2D6 is inhibited, the more CYP1A2 activity is enhanced.

The present work holds that CYP2D6 and CYP1A2 metabolic activities are not independent from each other, this helps to give a deeper insight into drug-drug interactions *in vivo*. This observation has some **clinical implications:** (i) after MDMA CYP2D6 is fully inhibited, the intake of drugs whose metabolism is regulated by this isozyme should be avoided. Subjects display the PM phenotype after MDMA intake irrespective of

their original genotype. Therefore, recreational MDMA users are exposed to a higher probability of relative overdose and, therefore, an increased risk of suffering adverse effects from CYP2D6 substrates including MDMA. (ii) After MDMA there is a temporary increase in CYP1A2 activity that compensates for the decreased CYP2D6 activity. This helps to explain why MDMA is still metabolically cleared even after repeated doses; (iii) an enhanced clearance of medications whose metabolism is regulated by CYP1A2 is expected. This is of importance since MDMA use has been associated with psychiatric symptoms and psychological problems (more frequently in women) that may require psychopharmacological treatment with antidepressant drugs, some of which are known CYP2D6 substrates (Martin-Santos et al., 2010).

Our goal for the second part of the thesis was to study the relationship between the peripheral serotonergic system and the pharmacological effects of MDMA and mephedrone. In other words, to see whether induced serotonergic activity by MDMA and related drug mephedrone correlates with pharmacological effects measured in healthy recreational users exposed to the drug.

MDMA is known to cause the release of 5-HT, DA and NA from neurons by acting as substrates for monoamine transporter proteins (Crespi et al., 1997, Rothman and Baumann, 2003).

It is generally accepted that 5-HTT protein expressed in platelets is identical to the one found in neurons, displaying similar structural and functional properties in both tissues. The interest in determining both the molecular characteristics and the regulation of 5-HTT has been enormous over the last decade, but the difficulty in obtaining human tissues and the

ethical limitations in human experiments have turned researchers to look for alternative models.

The concept of identifying peripheral biomarkers for central 5-HTT activity is of great interest.

Two approaches have been used in this thesis to study peripheral serotonergic activity after MDMA administration:

A) 5-HTT gene expression (human lymphocytes).

B) 5-HT concurrent concentrations in blood and brain (rats).

A) The best blood cell candidate to do so would be lymphocytes, as lymphocytes express the same 5-HTT protein, and 5-HT signalling is quite analogous to CNS serotonergic neurotransmission (Rivera-Baltanas et al., 2012).

MDMA has been reported to alter 5-HTT gene expression (Kovacs et al., 2007, Kirilly, 2010), which could lead to changes in protein concentrations. There is no agreement in the affectation of this gene caused by the MDMA; as some studies reveal a profound down-regulation of 5-HTT gene expression after drug administration (Biezonski and Meyer, 2010, 2011, Cuyas et al., 2013), whereas other see an increase (Kovacs et al., 2007, Kirilly, 2010), attributing it to a compensatory mechanism. Up to date, there are no data available regarding 5-HTT gene expression in human samples after MDMA intake, and which is the importance of 5-HTTPLR in this response.

Therefore, the aim of the present work **M-I**, was to study 5-HTT gene expression in peripheral blood mononuclear cells (PBMCs) extracted from healthy recreational MDMA users.

The administration of 75 mg of MDMA significantly changed 5-HTT gene expression 3 hours later. The drug seems to duplicate the expression

of this gene considering all subjects together. However, gender differences also take place in this study, and women present a higher mean increase (2.5 fold-increase compared to placebo) in its expression compared to men (1.7).

Peripheral serotonergic system is affected after the administration of drugs such as MDMA in terms of 5-HTT gene expression, duplicating its concentrations in lymphocytes. In this assessment, high inter-individual differences were shown between subjects.

Although results are quite consistent, they are just a part of the final study in which a total amount of 21 subjects will be analysed. We believe that with a larger sample size, a better representation of all genotypes will be obtained.

B) The second approach to study serotonergic system was to determine 5-HT concentrations in brain and blood after MDMA administration.
The publication **P-IV** of this thesis assesses the hypothesis that induced serotonergic activity by MDMA is not produced only in the brain, but also in blood platelets, showing a correlation between both tissues.
In order to measure the release of 5-HT from blood and brain simultaneously, pre-clinical studies were done in rats. The animals underwent catheter surgery; one catheter was implanted into the jugular vein, and the second one was implanted with a proximal cannula tip resided just above the nucleus accumbens.

A method consisting of a whole blood microdialysis coupled to HPLC with electrochemical detection (ECD) was developed in order to measure 5-HIAA and 5-HT concentrations in blood in these experiments.

Results showed that MDMA significantly increased dialysate 5-HT in blood and brain after 0.3 mg/kg and 1.0 mg/kg doses. This stimulatory effect was dose dependent in both compartments.

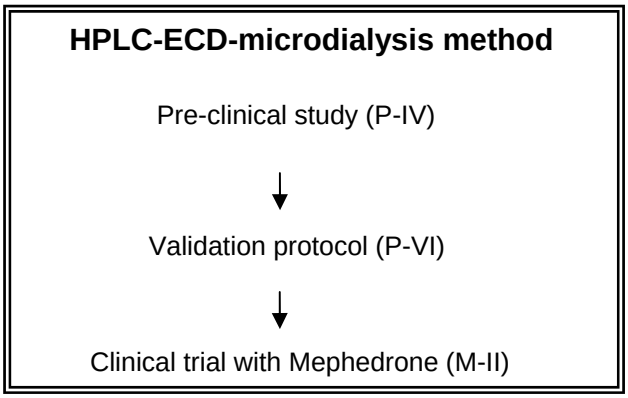
The data reveal a highly significant positive correlation between blood and brain dialysate 5-HT concentrations ($p<0.001$), suggesting that MDMA-induced 5-HT release in blood may reflect 5-HT release in the brain. However, inspection of the raw data revealed that there was a highly variable response to MDMA among the individual rats.

When examining the data from individual rats, we found that there is not always a significant correlation between 5-HT concentrations in blood and brain. While there is no simple explanation for the lack of consistent correlations between dialysate 5-HT in blood and brain, there are many possible sources of variability in 5-HT measures. First, there appears to be substantial individual differences in responsiveness to MDMA among the rats tested. For example, some rats exhibited robust behaviors (i.e., 5-HT syndrome) after MDMA administration while others did not (data not shown). The molecular underpinnings of individual differences in drug responsiveness are not known but could be related to differential expression of 5-HTT across subjects. Another factor giving rise to individual differences in plasma 5-HT could be the extent of non-specific 5-HT release from platelets across blood specimens.

There is a positive relationship between 5-HTT activity in brain and blood when analyzing the data of the whole group. Nevertheless, there is substantial variability between subjects in responsiveness to the drug, suggesting caution should be exercised when attempting to glean information about central 5-HTT function from assessment of transporter-mediated release of 5-HT in the bloodstream.

There are **potential clinical implications** of our findings. First, it seems that our method developed in rats could be refined and applied to clinical studies where repeated blood specimens are available for HPLC analysis. Clinical studies could measure the *in vivo* effects of therapeutic or abused drugs on plasma 5-HT to gauge the effects of those drugs on platelet 5-HTT, and possibly relate this to brain 5-HTT function.

After these experiments, the HPLC-ECD-microdialysis methodology was validated (**Publication-VI**) in human studies in order to apply it to clinical trials (**Manuscript -II**).



Until now, two different techniques have been used to determine whether MDMA administration affects serotonergic peripheral activity; measuring 5-HTT gene expression in lymphocytes and 5-HT concentrations in blood and brain in rats.

In order to apply the same methodology to clinical trials, a 4-day validation protocol following bioanalytical method validation was done (P-VI). The HPLC-ECD-microdialysis method showed optimal sensitivity, with low limits of detection and quantification, being precise and accurate.

It is essential to point out that significant differences have been reported over the years in measuring physiological concentrations of 5-HT in PPP, and nowadays there is no reference method to do so (Brand and Anderson).

This can be due to the fact that the **99% of blood 5-HT is stored in platelets**, and platelets are fragile, minor damage to platelets during specimen handling will cause platelet activation or lysis, generating artificial increases in plasma 5-HT.

To perform a complete validation with real samples, 5-HT and 5-HIAA concentrations in blood and PPP were measured in two groups of patients suffering from hypertension, and end-stage renal disease which show a deregulated serotonin system, and in control subjects.

The aim of the work P-VI was applying this method to measure the same samples comparing 5-HT and 5-HIAA concentrations in whole blood (without any sample manipulation) to their concentrations in PPP.
We realized that there was an enormous difference between both matrices, due to manipulating effect before microdialysis.
Therefore, a protocol to minimize the artifactual release of 5-HT from platelets in obtaining PPP was developed. Several factors regarding centrifugation speed, temperature and the addition of an antiaggregant were evaluated.

Once optimal conditions were established, the method was applied to humans to a population of 24 patients and 12 controls. Results showed that even when the sample is accurately processed to obtain PPP, 5-HT

concentrations are approximately 2.8 times higher than those in whole blood.

This work provides evidences that there are crucial pre-analytical factors in sample manipulation that can provoke an artifactual release of 5-HT from platelets. There is no optimal procedure to obtain PPP from blood, but slow speed centrifugation, RT procedures, and the use of an antiagreggant seem to play an important role in reducing platelet contamination.

Our method in whole blood provides reliable 5-HT determinations as **there is no sample manipulation**. Besides, a small amount of blood is needed to be withdrawn from the antecubital vein of the subject and 0.2 mL was immediately dialyzed. The eluted sample was injected in the HPLC system and the separation of the compounds was done in 9 minutes.

This analytical method had been already used in animal studies (Rothman and Baumann, 2003, Zolkowska et al., 2006, Zolkowska et al., 2008), and also in human 5-HT in PPP (Cheng et al., 1993).

Other authors also used this methodology to measure human 5-HT whole blood concentrations, but it was an invasive method, with an intravenous probe (Paez and Hernandez, 1998).

Therefore, the method developed in this thesis is applied, for the first time, to measure human *ex-vivo* 5-HT and 5-HIAA concentrations directly from whole blood concentrations, without invasive methods.

Once the method had been validated, it was applied to a pilot clinical trial consisting of 5 healthy volunteers' polydrug recreational users who

received 150 mg, 200 mg of Mephedrone and placebo, in three different sessions. MDMA was also administered and used as reference drug.

The goal of M-II was to apply the method developed to determine whether the oral administration of MDMA and mephedrone caused the release of 5-HT to the extracellular space in the peripheral serotonergic system.
Results reported that although there is a substantial variability between subjects in responsiveness to the drug, MDMA and mephedrone significantly increased whole blood 5-HT concentrations after its administration.

MDMA administration provoked a 12 fold-increase in 5-HT blood levels. Mephedrone was also able to stimulate 5-HT release from platelets, this stimulatory effect being dose-dependent, with 150 mg and 200 mg doses elevating 6- and 10-fold 5-HT blood levels, respectively.

It is the first time that 5-HT concentrations in blood are measured in a clinical trial administering MDMA or mephedrone. Due to the recent misuse of mephedrone, scarce data on its human pharmacology is available and 5-HT blood concentrations have not even been determined in pre-clinical studies.

The peripheral serotonergic system is altered after the administration of drugs such as MDMA (rats and humans) and mephedrone (humans) in terms of blood 5-HT concentrations; although in both cases there is a high inter-individual differences in drug response.

The subjective and physiological effects observed after administering MEPH are similar to those of MDMA; showing an increase in HR, SBP,

DBP, and also in subjective effects reported by the volunteers, such as "high", "good effects", and "stimulation". Nevertheless, a larger sample is required to confirm these results.

Regarding the volunteers who received 150 and 200 mg of MEPH (n=5), the increase in pharmacological effects was parallel to an increase in 5-HT blood concentrations. MEPH plasma concentrations highly and significantly correlated with 5-HT blood concentrations. Globally, the more concentration of the drug in plasma the more increase in 5-HT concentrations occurs in a dose-dependent manner.

Globally, higher concentrations of the drug in plasma were accompanied with more intense drug effects. The main hypothesis of this thesis seems to be confirmed, as 5-HT blood levels seem to play a relevant role in mediating drug effects. Results suggest that drug administration stimulate 5-HT release, which mediates drug effects. Besides, 5-HTTLPR genotype has also a key role, determining 5-HT reuptake velocitiy, and therefore, drug effects intensity and duration.

In this study, subjects carriers of *s/s* presented higher 5-HT blood levels and higher physiological scores compared to to those with *l/** genotype.

We realize that this pilot study has a small number size, and the comparison between MDMA and MEPH is quite limited (n=2). These preliminary results were worthy to include them in this thesis, as they demonstrate the successful application of the method developed and confirm our hypothesis. Nevertheless, a definitive clinical trial will be carried out shortly, and therefore, a better comparison will be possible.

Summary

Amphetamine type-drugs such as MDMA and MEPH caused an increase in 5-HT blood levels, which highly correlated to drug physiological and psychological effects. 5-HTTLPR genotype seems to regulate 5-HT blood levels, and therefore, drug response. These preliminary results put forward the hypothesis that serotonergic activity has an important role in in the mechanism of action of these drugs.

CONCLUDING REMARKS

Main achievements of the present research project, obtained according to the determined objectives of the study, are summarized below:

1. Women display a slower CYP2D6 and CYP1A2 activity when compared to men in baseline conditions.

2. After MDMA administration, the MBI occurs and CYP2D6 is inhibited by the drug in both genders although the phenomenon of phenocopying is observed in 100% of female and in 67% of male subjects. Nevertheless inhibition is more pronounced in men, although they recover faster their CYP2D6 activity than women. However, after 10 days, both genders have recovered the 90% of CYP2D6 activity.

3. CYP1A2 activity is enhanced after CYP2D6 has been inhibited. An increase of its activity takes place in both genders, being of about the 40% in women, and 20% in men.

4. The increase in CYP1A2 activity contributes to MDMA metabolic disposition once CYP2D6 has been irreversibly inhibited by the drug. A good correlation between CYP2D6 and CYP1A2 activities indicated that the more CYP2D6 is inhibited, the more CYP1A2 activity is enhanced.

General conclusion of the previous achievements: Although there are gender differences at baseline in some key activities involved MDMA metabolic disposition, compensatory mechanisms after MBI are quite similar and therefore metabolism most probably does not play a major role in gender differences seen in drug effects.

5. *Ex vivo* administration of MDMA and its metabolites show that test drugs evoke dose-related increases in whole blood 5-HT, with MDMA and its metabolite, MDA, producing the largest effects. HMMA and HMA were also able to increase extracellular 5-HT concentrations, but more modestly.

6. In pre-clinical studies, *in vivo* administration of MDMA significantly increased dialysate 5-HT in brain being this stimulatory effect dose dependent. Concurrent blood determinations reported that MDMA also significantly increased whole blood 5-HT dose-dependently.

7. The data reveal a highly significant positive correlation between blood and brain dialysate 5-HT concentrations, suggesting MDMA-induced 5-HT release in blood may reflect 5-HT release in the brain. However, inspection of the raw data revealed that there was a highly variable response to MDMA among the individual rats.

General conclusion of the previous achievements: MDMA induces the release of 5-HT in a dose dependent manner both in blood and brain. The good correlation between both tissues was the basis for looking at 5-HT in blood as a surrogate biomarker of MDMA mechanism of action. Other surrogate biomarkers tested in clinical studies where serotonin transporter (5-HTT) gene expression and a genetic polymorphism of this protein regulating gene expression (5-HTTLPR).

8. The methodology used in pre-clinical studies was validated in human blood samples in order to apply it to clinical trials. The

method showed optimal sensitivity, with low limits of detection and quantification, being precise and accurate.

9. The determination of 5-HT done in plasma is hampered by a number of pre-analytical factors (temperature, speed centrifugation, preservatives…) responsible for its artifactual release from platelets.

10. 5-HT and 5-HIAA concentrations in blood were measured in two groups of patients suffering from hypertension and end-stage renal disease which show a deregulated serotonin system, and in control subjects. Both groups showed higher concentrations of 5-HT and 5-HIAA when compared to controls, the end-stage renal disease patients reporting the highest differences in both compounds.

General conclusion of the previous achievements: Even controlling for factors responsible for artifactual results in 5-HT determination in plasma, whole blood 5-HT determinations are far more accurate and reliable than in plasma.

11. After the administration of 75 mg of MDMA significant changes in 5-HTT gene expression 3 hours post drug administration are observed. Nevertheless a high inter-individual variability exists between subjects.

12. Gender differences in gene expression are observed women displaying a larger increase (2.5 fold-increase compared to placebo) in its expression compared to men (1.7).

13. The administration of mephedrone in a clinical trial significantly increased whole blood 5-HT concentrations after 150 mg and 200

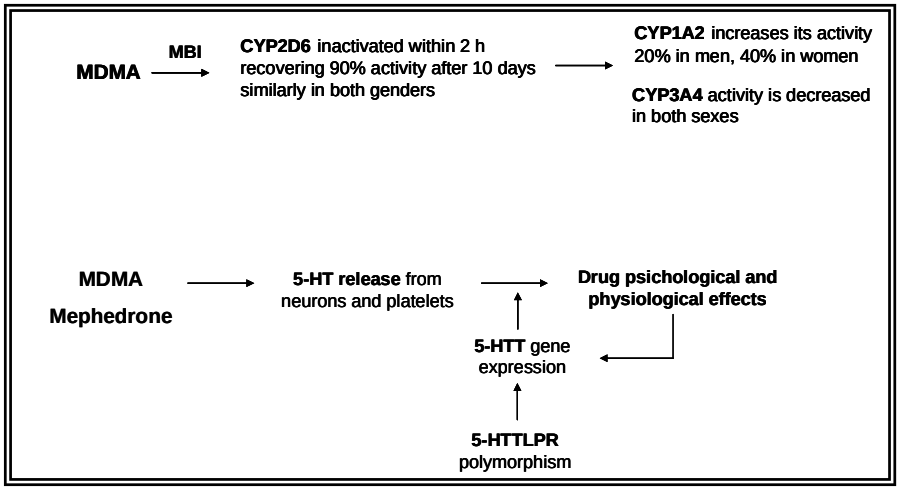
mg doses. This stimulatory effect was also dose-dependent, with 150 mg and 200mg doses elevating 5-HT blood concentrations 6- and 10-fold, respectively. A substantial individual variability in responsiveness to the drug, regarding drug effects and 5-HT and mephedrone blood concentrations is observed.

14. Blood 5-HT increases were different in subjects depending on its 5-HTTLPR genotype, the *s/s* genotype reported the highest 5-HT blood levels and acute physiological effects (HR and papillary diameter).

15. Blood 5-HT levels correlated with physiological and subjective effects observed after MDMA and mephedrone administration.

General conclusion of the previous achievements: MDMA and mephedrone provoke a significant increase in 5-HT blood concentration in humans. These levels correlated with drug pharmacological effects, meaning that, at least partly, the serotonergic system seems to play an important role in the mechanism of action of these two drugs.

General conclusion of the thesis:



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APPENDICES

ABBREVIATIONS

137X,	1,3,7-trimethylxathine
17U,	1,7-dimethyluric acid
17X,	1,7-dimethylxanthine
1U,	1-methyluric acid
1X,	1-mehtylxanthine
5-HIAA,	5-hydroxyindoleacetic acid
5-HT,	5-hydroxytryptamine
5-HTP,	5-hydroxytriptophan
5-HTT,	Serotonin Transporter
5-HTTLPR,	Serotonin-transporter-linked polymorphic region
AAMU,	5-acetylamino-6-amino-3-methyluracil
ACTH,	Adrenocorticotropic hormone
ADH,	Antidiuretic hormone
AUC,	Area Under the Curve
BP,	Blood pressure
$CaCl_2$,	Calcium Chloride
cDNA,	Complementary DNA
CL,	Clearance
C_{max},	Maximum Concentration
CNS,	Central Nervous System
COMT,	Catechol-O-methyltransferase
CYP,	Cytochrome P450 isozyme
DA,	Dopamine
DAT,	Dopamine Transporter
DEX,	Dextromethorphan

DOR,	Dextrorphan
DSM-IV,	Diagnostic and Statistical Manual of Mental Disorders
ECD,	Electrochemical detection
EM,	Extensive metabolizer
FA,	Functional allele
GSH,	Glutathione
HCl,	Hydrochloric acid
HHA,	3,4-dihydroxyamphetamine
HHMA,	3,4-dihydroxymethamphetamine
HM,	Hydroxymorphinan-3-ol
HMA,	4-methoxy-3-hydroxyamphetamine
HMMA,	4-methoxy-3-hydroxymethamphetamine
HPLC,	High Performance Liquid Chromatography
HR,	Heart Rate
IM,	Intermediate metabolizer
LC,	Liquid chromatography
LOD,	Limit of detection
LOQ,	Limit of quantification
KCl,	Potassium Chloride
K_e,	Elimination Rate constant
MAO,	Monoamine oxidase
MBI,	Mechanism-based inhibition
MDA,	3,4-mehtylenedioxyamphetamine
MDEA,	3,4-methylenedioxyethylamphetamine
MDMA,	3,4-methylenedioxymethamphetamine
Met,	Methionine
MEPH,	Mephedrone
$MgCl_2$,	Magnesium Chloride

MM,	Methoxymorphinan
MR,	Molar Metabolic Ratio
mRNA,	Messenger RNA
MS,	Mass spectometry
NA,	Noradrenaline
NaCl,	Sodium Chloride
NET,	Norepinephrine Transporter
OT,	Oral temperature
PBMCs,	Peripheral Blood Mononuclear Cells
PM,	Poor metabolizer
PRP,	Platelet-rich plasma
PPP,	Platelet-poor plasma
PRISM,	Psychiatric Research Interview for Substance and Mental Disorders
qPCR	Quantitative PCR
RIN,	RNA integrity number
RT,	Retention Time
SBP,	Systolic blood pressure
SSRI,	Selective Serotonin Inhibitor
$t_{½e}$,	Half-life of elimination
TH,	Triptophan hydroxylase
tRNA,	Total RNA
TRP,	Tryptophan
UM,	Utra-rapid metabolizer
UV,	Ultraviolet
Val,	Valine
Vd,	Volume of Distribution
VMAT,	Vesicular Monoamine Transporter

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