Phase II Metabolism of 3,4-Methylenedioxymethamphetamine

Synthesis, Analysis, and Enantioselective *in vitro* and *in vivo* Kinetics

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Für Euch!

It is our choices (…) that show

what we truly are,

far more than our abilities.

Harry Potter and the Chamber of Secrets

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1 GENERAL PART

1.1 INTRODUCTION

1.1.1 3,4-Methylenedioxymethamphetamine

3,4-Methylenedioxymethamphetamine (MDMA), commonly named as Ecstasy, is a ring-substituted amphetamine with structural similarities to methamphetamine and mescaline. As other amphetamines, MDMA is a chiral compound carrying an asymmetric carbon atom in the side chain. It was first synthesized in Germany by Merck in 1914[1,2] and, although patented as an appetite suppressant, never marketed as a therapeutic drug.[3] Since 1985, MDMA is scheduled in the Controlled Drugs and Substances Act as a restricted drug in the United States and since 1986, in Germany. It has become popular in the beginning of the 1990s as a drug of abuse among young people, especially in the dance scene.[4,5] After decreasing numbers of MDMA users in recent years, most likely due to its non-availability on the illicit drug market, the Substance Abuse and Mental Health Services Administration has reported on increasing MDMA consumption in the United States again since 2010.[6] Usually it is consumed recreationally on weekends (1 to 2 pills of 75 to 120 mg every 1 to 4 weeks) in form of tablets or pills.[7] Preparations available on the illicit drug market usually contain the 1:1 racemate of *R*- and *S*-enantiomers.

1.1.2 Pharmacology and Toxicology

Similar to amphetamine or methamphetamine, MDMA acts in the central nervous system (CNS) as a stimulant through indirect release of monoamine neurotransmitters from presynaptic nerve terminals into the synaptic cleft where postsynaptic receptors can be stimulated.[3,7] Mainly serotonergic (5-HT), noradrenergic (NA), and with a smaller effect dopaminergic (DA) neurotransmission is enhanced.

The distinctive effects are described as an altered state of consciousness, euphoria, energy and a desire to socialize.[3,8] However, MDMA also can induce severe acute

toxic symptoms, such as tachycardia, hypertension, hyperthermia, and hepatotoxicity. Severe and even fatal intoxications were described.[3]

Concerning chronic toxicity, preclinical animal data suggest that MDMA causes irreversible damage to serotonergic nerve terminals in the CNS.[3,9-11] In humans, chronic MDMA toxicity is still controversially discussed, as some recent publications suggest that animal doses may be too high compared to human pharmacokinetics.[12,13] Other studies with recreational MDMA users, found decreased levels of 5-hydroxyindoleacetic acid, the main metabolite of 5-HT in the cerebrospinal fluid[14] and a reduced density of serotonin transporters in the brain as determined by positron emission computed tomography with a ligand selective for these transporters.[15] Unfortunately, these studies were performed with recreational users, so it cannot be excluded that the indicated neurotoxicity might also be due to use of other recreational drugs especially since polydrug use is not uncommon. Admittedly, direct MDMA injection into rat brain failed to reproduce neurotoxic effects seen after systemic administration.[16] Furthermore, alteration of cytochrome P450 (CYP)-mediated MDMA metabolism influenced MDMA-induced neurotoxicity.[16,17] Therefore, MDMA metabolism may be an important contributor to neurotoxicity.[18-21] Metabolites such as 3,4-dihydroxymethamphetamine (DHMA) can easily be oxidized to their corresponding quinones which can form adducts with glutathione and other thiol-containing compounds.[18-20] Recently, such adducts have been implicated in MDMA neurotoxicity.[22,23]

For the two enantiomers, different pharmacological properties were observed.[3] While *S*-MDMA is generally more potent and responsible for the described psychostimulant and empathic effects, the *R*-isomer exhibits more hallucinogenic-type properties.[10] *R*- and *S*-MDMA also differ in their dose-response curves for changes in serotonergic function and neurotoxicity and their *in vivo* kinetics are known to be different.[3,8,24-27]

1.1.3 Metabolism

In vivo and *in vitro* MDMA studies revealed two main metabolic pathways as shown in Figure 1. The predominant pathway in humans involves multiple CYP enzyme-catalyzed *O*-demethylenation of MDMA to DHMA, followed by catechol-*O*-methyltransferase (COMT)-catalyzed *O*-methylation, primarily to 4-hydroxy-3-methoxymethamphetamine (HMMA). DHMA and HMMA also may be conjugated by

uridine diphosphate glucuronyltransferases (UGT) to DHMA 3-glucuronide, DHMA 4-glucuronide, and HMMA glucuronide, or by sulfotransferases (SULT) to DHMA 3-sulfate, DHMA 4-sulfate, and HMMA sulfate. A minor pathway includes demethylation to 3,4-methylendioxyamphetamine (MDA) followed by demethylenation to 3,4-dihydroxyamphetamine (DHA), *O*-methylation to 4-hydroxy-3-methoxyamphetamine (HMA), and respective conjugation.[10,28-30] The catechols DHMA and DHA, formed via metabolic demethylenation of MDMA, are suspected to be oxidized to their corresponding ortho-quinones which in turn can form adducts with glutathione and other thiol-containing compounds.[19,31]

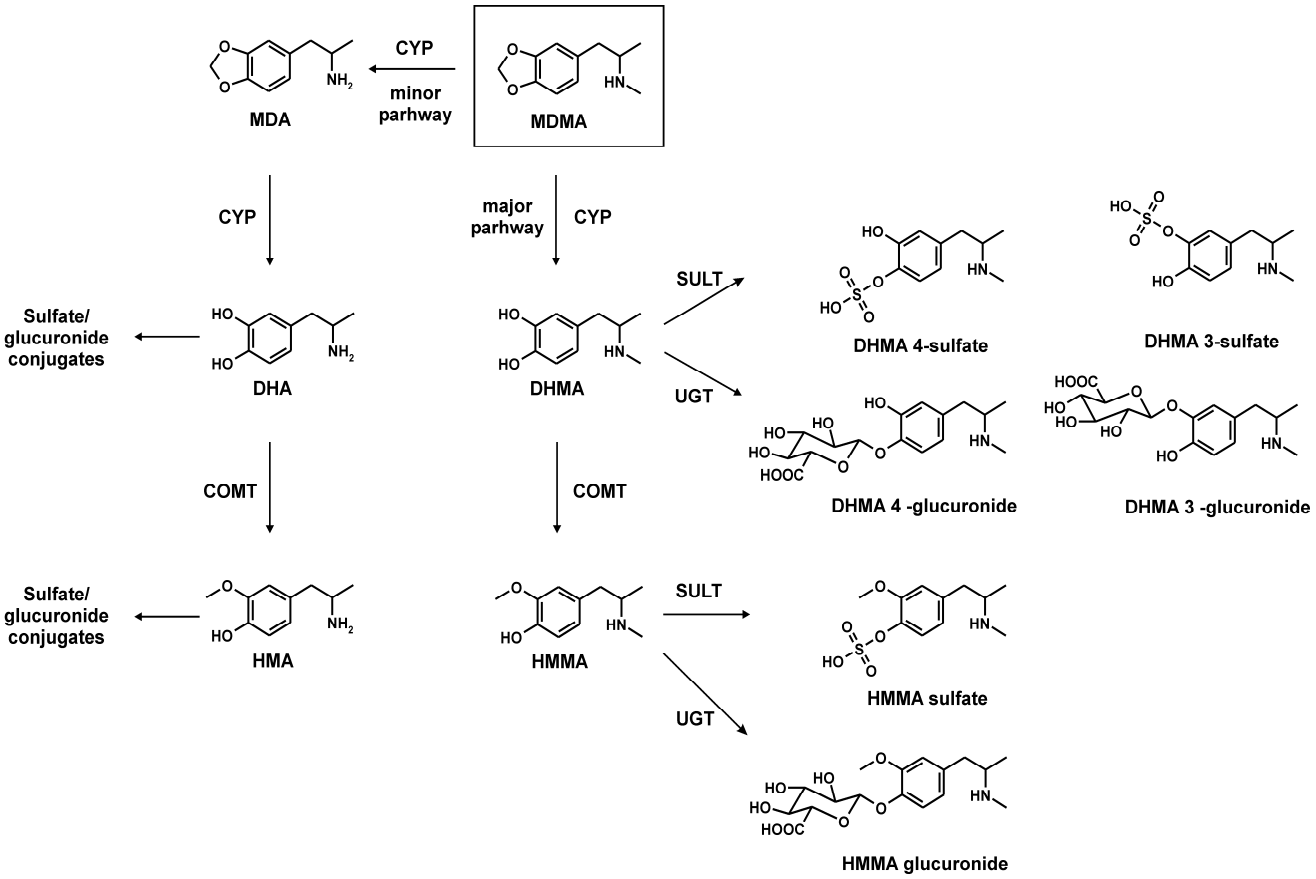

Fig. 1 Metabolic pathways of MDMA in humans

Different pharmacokinetic properties have been observed for the two MDMA enantiomers. The *S*-enantiomer is eliminated from plasma at a higher rate than the *R*-enantiomer[3,8,24-27] most likely explained by stereoselective metabolism. *In vitro* experiments concerning CYP-*N*-demethylation, CYP-*O*-demethylenation, and COMT-

methylation of DHMA to HMMA indeed revealed metabolic preferences for the *S*-enantiomers.[32,33]

1.1.4 Phase II Metabolizing Enzymes

Numerous enzymes are capable to metabolize xenobiotics, usually resulting in decreased toxicity and increased hydrophilicity compared to the parent compounds, which promotes their excretion. Generally, these biotransformations can be divided in two steps: phase I and phase II metabolism. Phase I metabolism is referred to as functionalization which mainly involves oxidation, reduction, or hydrolysis. Phase II type reactions are conjugative reactions, catalyzing among others, the transfer of hydrophilic residues such as glucuronic acid or activated sulfate. However, conjugation is not necessarily a secondary phase reaction as many endogenous compounds or xenobiotics can be directly glucuronidated or sulfated.

1.1.4.1 UDP-Glucuronyltransferase (UGT)

UGTs represent a superfamily of endoplasmic reticulum membrane-bound enzymes, postulated to reside on the luminal surface. Based on primary amino acid identity, they are divided into two families, UGT1 and UGT2. At present, 15 different isoenzymes are known in humans: UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 and UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, and UGT2B17,[34-36] whereas UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 are considered to be of greatest importance in hepatic drug elimination.[36] Although the liver is recognized as the major site of glucuronidation, numerous organs, e.g. small intestine, lung, kidney, brain, etc. significantly contribute to the overall glucuronidation capacity.

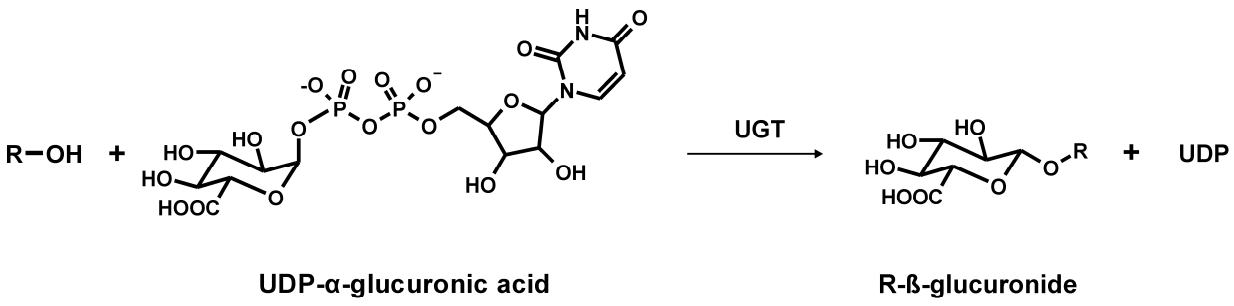

Fig. 2 Schematic of the glucuronidation reaction

UGTs catalyze the transfer of glucuronic acid from the co-substrate uridine 5'-diphosphoglucuronic acid (UDPGA) to a multitude of functional groups as shown schematically in Figure 2. The underlying mechanism is a S_N2 reaction where the configuration of the glucuronic acid changes from α- to β-anomer. Virtually all classes of drugs are substrates for UGTs, hence about 35% of phase II drug metabolism are estimated to underlie this pathway.[37] Although, glucuronidation generally results in the formation of water-soluble, inactive metabolites, it is known that also active and reactive glucuronides exist. For example, morphine 6-*O*-glucuronide shows greater pharmacologic activity than its parent compound morphine[35] and glucuronides of carboxylic acids exhibit electrophilic reactivity associated with cytotoxic, carcinogenic, and idiosyncratic hypersensitivity reactions.[35]

A number of polymorphisms have been described for different UGT isoenzymes and significant pharmacological impact have been demonstrated.[37] However, the clinical outcome of many polymorphisms is still controversial and additional studies are needed to promote the understanding of interindividual variations in the glucuronidation pathway.

1.1.4.2 Sulfotransferase (SULT)

In the mammalian organism, SULTs occur membrane-bound or soluble in cytosol. Membrane SULTs, localized in the Golgi apparatus, are responsible for the sulfation of endogenous structures, such as carbohydrates and proteins. Only cytosolic SULTs play a role in xenobiotic metabolism, as well as in the biotransformation of thyroid hormones, steroids, and neurotransmitters. Considerable numbers of cytosolic SULTs have been characterized and divided into several gene families based on similarity of their amino acid sequences.[38,39] Out of 13 human SULTs currently

known, the major isoforms responsible for human xenobiotic metabolism are SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1.[40] The widest tissue distribution was shown for the SULT1A subfamily, with SULT1A1 as the major isoform present in human liver, but also in the gastrointestinal tract, brain and placenta.[39,40] SULT1A3 is known to be only scarcely expressed in human liver, however highly expressed in the small intestine, brain and fetal liver.[39,40]

SULTs catalyze the transfer of a sulfonate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to nucleophilic sites of their substrates. Sulfation is a high affinity and low capacity phase II reaction, with overlapping substrates spectra for glucuronidation. Sulfation predominates at low substrate concentrations and glucuronidation at high substrate concentrations, when sulfation is saturated.[39] The limiting factor for sulfation is the availability of PAPS. Although it can be rapidly synthesized, it depends on the hepatic sulfate concentrations, which are largely dependent on equilibrium with circulating inorganic sulfate.[39]

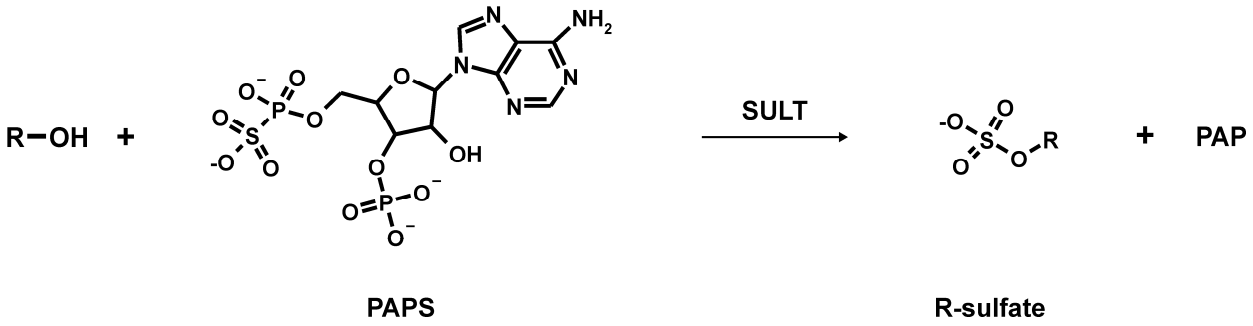

Fig. 3 Schematic of the sulfation reaction

Generally, sulfation is a detoxification process, however, labile and chemically reactive intermediates are sometimes formed, which can undergo DNA binding, leading to mutagenicity and carcinogenicity. Some sulfate esters including minoxidil, triamterene and morphine were reported to be more pharmacologically active than the corresponding parent drugs.[41] At least some endogenous sulfate conjugates seem to play a role in the CNS. For example, dopamine 4-sulfate demonstrated vasopressor activity in the peripheral and central nervous system, whereas dopamine 3-sulfate acted as a central depressor.[41] Several xenobiotics, among them dietary and environmental chemicals, therapeutic drugs, etc. were shown to inhibit one or more SULT isoenzymes and may cause adverse effects on human health.[41]

1.1.5 Synthesis of Phase II Metabolites

Reference standards of metabolites are needed for *in vitro* and *in vivo* kinetic studies. However, the number of commercially available glucuronide or sulfate standards is limited, hence it usually requires their synthesis prior to kinetic studies.

1.1.5.1 Glucuronides

Synthesis of glucuronides can be achieved either by chemical[42,43] or enzymatic methods.[44] Chemical synthesis requires multiple steps, most commonly via acyl-protected intermediates. Hydrolytic stability of the aglycones is therefore a prerequisite necessary for the removal of protecting groups. α-Anomers and other byproducts in addition to the desired β-anomer can occur leading to more complicated purifications and low yields. In the case of aglycones that contain several possible glucuronidation sites, without further protecting groups mono- and polyglucuronides can be formed.[42,43,45] Shima et al.[46] previously synthesized HMMA *O*-glucuronide by chemical synthesis achieving yields of 6%, which seems rather low. Enzyme-assisted synthesis represents a suitable alternative to chemical synthesis, especially when milligram scale yields are sufficient. Isolated purified UGT enzymes or liver microsomes might be applied as convenient catalysts for glucuronidation. However, liver microsomes of different species (rat, mouse, dog, monkey, human) seems most appropriate due to easy preparation and handling. Use of enzymes does not require multiple steps and results in the formation of the natural configuration. Mainly mono-glucuronides and even regio- and stereoselective glucuronides are obtained.[45] Yields with up to 100% depending on the aglycone and the microsomal source used could be reached.[44,45] Therefore, an enzyme-assisted synthesis was chosen to produce milligram amounts of the diastereomeric HMMA glucuronides as described in detail under 2.1.

1.1.5.2 Sulfates

Synthesis of sulfate conjugates is usually performed with chemical methods. Only few data using enzymatic synthesis are available.[47-49] Although enzymatic sulfate synthesis bears the advantages of regio- and stereoselective conjugation, there are some major drawbacks limiting its usefulness. The main issue is the need for the co-substrate. PAPS is rather expensive and unstable. The formed product 3'-

phosphoadenosine 5'-phosphate (PAP) leads to product inhibition.[49] Incubations with subcellular fractions and the addition of PAPS therefore provide only low sulfation capacities. Uutela et al used rat liver S9 fractions with the addition of PAPS for regioselective sulfation of 5-HT, 5-HIAA, DOPAC, and HVA. However, the yields were less than 3 mg (less than 10%) and hence too low for NMR confirmation of the sulfation side.[47] Chemical synthesis seems to be the method of choice for sulfate synthesis of xenobiotics.[47,48] Different strategies have been described, e.g. use of sulfuric acid[47,48] or sulfur trioxide-amine complexes.[46,48] As H_2SO_4 is not amenable to sulfation for many sensitive scaffolds considering the strong acidity of sulfuric acid, SO_3 adducts with amine containing molecules link pyridine, trimethylamine, triethylamine, or DMF provide the most straightforward method.[48] Usually, yields with up to 90% could be achieved. Sulfates of DHMA and HMMA were synthesized using a pyridine SO_3 complex as described in detail under 2.2.

1.1.6 (Enantioselective) *In vitro* Enzyme Kinetic Studies

The characterization of humane enzymes involved in the metabolism of specific drugs and the determination of their enzyme kinetic parameters, such as K_M and V_{max} is an important aspect in toxicological risk assessment. They can be used as potential determinants of interindividual variability in pharmacokinetics, e.g. drug-drug interactions or genetic polymorphisms. K_M and V_{max} values represent descriptors of the enzyme kinetic behavior of a respective biotransformation reaction. Assuming simple kinetic systems, V_{max} is the maximum enzyme velocity at an infinite substrate concentration and in general represents the capacity of an enzymatic reaction. The K_M value is defined as the substrate concentration that will yield a reaction velocity that is half of V_{max} and reflects the substrate affinity to a certain enzyme. The overall effectiveness of a respective reaction is usually described by the V_{max}/K_M ratio and should increase the higher this ratio is. This fact sounds reasonable, as the catalytic efficiency value is getting higher with increasing affinity (low K_M) and increasing velocity (high V_{max}). Concerning differences in metabolic clearance of *R*- or *S*-stereoisomers, enantioselectivity can also be evaluated via the V_{max}/K_M values and marked enantioselectivity was previously defined as $V_{max}/K_{M(S\text{-stereoisomer})}/V_{max}/K_{M(R\text{-stereomer})} > 1.5$ or < 0.67).[32]

1.1.6.1 Product formation approach

Conventional determinations of enzyme kinetic parameters are made by assessing the rate of product (metabolite) formation at several substrate concentrations. Therefore, methods are required for measurement of metabolite concentrations in *in vitro* matrices. Such analytical methods themselves require that metabolites have been definitely identified, suitable chromatographic separation has been established and authentic standards prepared.[50] The simplest model to describe enzymatic biotransformation and hence to calculate K_M and V_{max} is fitting the initial rate velocities at various substrate concentrations to the Michaelis Menten equation (eq. 1).

$$V = \frac{V_{\max} \times [S]}{K_m + [S]} \tag{1}$$

A prerequisite are “initial” rate conditions, meaning protein concentrations and incubation time should be within the linear range of metabolite formation, and in total less than 20% of substrate should be consumed.

1.1.6.2 Substrate depletion approach

An alternative to the measurement of product formation is the determination of substrate depletion, which was successfully used for CYP reactions in both, human liver microsomes (HLM) and recombinant enzymes.[50,51] Substrate consumption over time can be used to calculate initial substrate depletion rates (k_{dep}) at various substrate concentrations. In theory, when substrate concentrations are well below K_M, the depletion should follow first-order decay kinetics.[52] As the substrate concentration is elevated through the K_M value, the measured values for k_{dep} should decline and become more zero-order in character. The infliction point of this relationship represents the K_M value and should occur at a substrate concentration that yields a k_{dep} value that is half of the theoretical maximum k_{dep} at an infinitesimally low-substrate concentration ($k_{dep([S]=0)}$).[50] Plotting of k_{dep} values versus substrate concentrations allows calculation of K_M according to equation 2.[50]

$$K_{dep} = K_{dep}([S] = 0) \times (1 - \frac{[S]}{K_m + [S]}) \tag{2}$$

The theoretical validity of this approach has been confirmed by Nath and Atkins,[53] who showed on a simulated data set that equation 2 can be derived from the Michaelis-Menten equation (eq. 1) and, as such, the kinetic parameters obtained should be comparable with those obtained by the traditional product-formation approach. The major advantage of the substrate-depletion approach is that reference standards of metabolites are not required. For some analytes, when (enantioselective) chromatographic separation of metabolites could not be accomplished sufficiently, (chiral) measurement of substrate consumption might be a versatile alternative to the conventional product formation. However, the substrate depletion approach possesses some practical limitations.[50] Substrates exhibiting low-intrinsic clearance will be difficult to examine, since measurement of substrate depletion requires a substantial consumption of the initial substrate concentration during the incubation period. Furthermore, enzyme kinetics of formation of individual metabolites cannot be determined, as the K_M and V_{max} values would only represent the sum of kinetic parameters for all single metabolic pathways.

1.2 AIMS AND SCOPES

Phase II metabolism represents an important detoxification process.[34,35,38] Investigation of glucuronidation and sulfation as a secondary metabolic step is especially important concerning the detoxification of reactive phase I metabolites. Such metabolites are known to be formed in humans after ingestion of MDMA, mainly through demethylenation to the catecholic metabolite DHMA and are suspected to contribute to MDMA's neurotoxic effects.[18,19,19,20,54] The qualitative and quantitative phase I metabolism of MDMA was studied extensively *in vitro* and *in vivo.*[10,28-30,32,33,55] Several pharmacokinetic studies in blood and urine following controlled MDMA administration to humans were performed, but DHMA, HMMA, and/or HMA urinary pharmacokinetic data were only obtained after conjugate cleavage. Only Shima et al. determined intact HMMA conjugates in 25 random urine samples and found that more than 70% of HMMA was eliminated as glucuronide or sulfate.[30] However, neither systematic *in vivo* nor *in vitro* kinetic studies were available concerning glucuronidation and sulfation of MDMA's phase I metabolites.

Furthermore, different pharmacological and pharmacokinetic properties were observed for the two enantiomers of MDMA[3,8,24-26] and enantiomeric preferences in the phase I metabolism were observed *in vitro*[32,33] and *in vivo.*[27] Elucidation whether the phase II metabolism also contributes to this phenomenon is important from the toxicological and pharmacological point of view.

Besides this, MDMA is known to be a potent mechanism-based inhibitor of CYP2D6[56] which is also assumed to influence MDMA-induced neurotoxicity.[16,17] DHMA was also shown to inhibit its own metabolism as well as the methylation of dopamine.[33] The inhibition potential of MDMA and/or its metabolites on other metabolic enzymes, such as UGTs or SULTs, is still unknown.

Therefore, the aims of the presented studies were:

- (Bio)Synthesis of MDMA's main phase II metabolites as reference standards for quantitative *in vitro* and *in vivo* kinetic studies

- Investigation of stereoselective enzyme kinetic data *in vitro* for HMMA glucuronidation in HLM and recombinant UGTs, and DHMA and HMMA sulfation in human liver cytosol (HLC) and recombinant SULT

- Determination of the inhibition potential of MDMA, DHMA, and HMMA on SULT

- Development and full validation of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) methods allowing the stereoselective analysis of MDMA, its phase I and phase II metabolites in human urine

- Evaluation of MDMA's phase II metabolites elimination kinetics in human urine following controlled oral MDMA administration

- Determination of stereoselective elimination kinetics of MDMA and its phase I and II metabolites in human urine following controlled oral MDMA administration

2 PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

2.1 THE ROLE OF HUMAN UGT-GLUCURONYLTRANSFERASES ON THE FORMATION OF THE METHYLENEDIOXYMETHAMPHETAMINE (ECSTASY) PHASE II METABOLITES R- AND S-3-METHOXYMETHAMPHETAMINE 4-O-GLUCURONIDES[57] (DOI: 10.1124/DMD.109.029215)

2.2 Sulfation of the 3,4-Methylenedioxymethamphetamine (MDMA) Metabolites 3,4-Dihydroxymethamphetamine (DHMA) and 4-Hydroxy-3-Methoxymethamphetamine (HMMA) and their Capability to Inhibit Human Sulfotransferases[58] (DOI: 10.1016/JTOXLET.2011.01.026)

2.3 Investigation on the Enantioselectivity of the Sulfation of the Methylenedioxymethamphetamine (MDMA) Metabolites 3,4-Dihydroxymethamphetamine (DHMA) and 4-Hydroxy-3-Methoxymethamphetamine (HMMA) using the Substrate Depletion Approach[59] (DOI: 10.1124/dmd.111.041129)

2.4 Development and Validation of LC-HRMS and GC-NICI-MS Methods for Stereoselective Determination of MDMA and its Phase I and II Metabolites in Human Urine[60] (DOI: 10.1002/JMS.1929)

2.5 Human MDMA and Phase I and Phase II Metabolite Urinary Excretion Kinetics Following Controlled MDMA Administration[61]

(DOI: 10.1373/clinchem.2011.172254)

2.6 Stereoselective Urinary MDMA and Metabolites Excretion Kinetics following Controlled MDMA Administration to Humans[62]

(DOI: 10.1016/J.BCP.2011.09.023)

3 CONCLUSIONS

The studies presented here provided systematic data on the *in vitro* glucuronidation and sulfation kinetics of the designer drug 3,4-methylenedioxymethamphetamine, (MDMA, Ecstasy). These data suggested, that sulfation was the predominant conjugation step with regioselective sulfation of the catecholic metabolite DHMA in position 3.[57,58] Inhibition studies performed with MDMA, DHMA, and HMMA towards typical sulfation reactions clearly indicated a mixed-type or competitive inhibition of dopamine sulfation by DHMA and HMMA, respectively, with IC_{50} values likely to cause significant inhibition *in vivo* after recreational MDMA doses.[63] In the author's opinion, a part of the described neurotoxicity of MDMA[3,9-11] could be explained by inhibition of the dopamine sulfation in the CNS. As MDMA and related drugs are able to increase the concentration of dopamine and other neurotransmitters in the CNS[64] and as they additionally could inhibit the inactivation of these compounds,[33] the described dopamine induced neurotoxicity might be enhanced.[65]

Additionally, evaluation with respect to a possible enantioselective phase II metabolism was performed. It could be shown, that HMMA glucuronidation by UGT1A9 was markedly stereoselective with preferences for the formation of the *S*-diastereomer whereas its glucuronidation by UGT2B7 favored the *R*-isomer. UGT2B15 and UBT2B17 revealed only slight preferences for *S*-HMMA. In human liver microsomes, which contain a physiological mixture of all liver UGT isoenzymes, and should therefore reflect the *in vivo* situation, slight preferences for *S*-HMMA were observed. Sulfation of HMMA was mainly catalyzed by SULT1A3 and to a minor extent by SULT1E1. Neither for SULT1A3 nor in human liver cytosol enantiomeric preferences could be observed. On the other hand, the efficiency for *S*-DHMA 3-sulfate formation was twice as high as for its *R*-enantiomer, both in SULT1A3 and human liver cytosol. One reason for this difference in enantioselectivity might be the position for sulfation. DHMA was mainly sulfated in position 3, whereas HMMA could only be sulfated in position 4.

To further obtain systematic *in vivo* data on MDMA's phase II metabolism and its enantioselectivity, liquid chromatography-high resolution mass spectrometry (LC-HRMS) and gas chromatography-negative ion chemical ionization- mass spectrometry (GC-NICI-MS) methods were successfully developed and validated.[60] These methods were shown to be applicable for the analysis of urine samples of 10

human subjects collected for up to 7 days following controlled oral placebo, low, and high dose MDMA administration.[61,62] Human MDMA urinary metabolites are primarily sulfate and glucuronide conjugates, with sulfates present in higher concentrations than glucuronides. HMMA sulfate was shown to be the major urinary metabolite providing the longest detection time for MDMA consumption with up to 168 h. All metabolites exhibited changes in enantiomeric disposition over time. MDMA, DHMA, and HMMA sulfate revealed preferences for the *R*-stereoisomers, all other metabolites showed conversely more *S*-isomer within the first 24 h after ingestion. Generally, initial stereoisomer preferences mimicked those observed in previous *in vitro* experiments.[32,33,57,59] In the later excretion phase (after 24 h), *R/S* ratios were >1 for all compounds. This is quite remarkable, as the enantiomeric ratios of at least one metabolite should be reversed from that of MDMA. However, it must be considered that urinary analysis reflects not only metabolite formation, but also distribution and elimination processes. Metabolism is represented mainly within the first 12 to 24 h, whereas later on, elimination is more relevant. One explanation for the observed time-dependency could be substrate availability. With increasing time, the amount of *R*- relative to *S*-enantiomers could increase, leading to increased metabolism of *R*-enantiomers, although affinity for S-enantiomers is higher. However, this only applies for analytes with initial preferences for *S*-enantiomers. On the other hand, distribution processes, including transport protein availability, could play a major role in enantioselective disposition and metabolite excretion. Changes in the *R/S* ratios over time could be used for estimation of ingestion time and to distinguish between recent (within 24 h) or earlier ingestion MDMA consumption. *R/S* cut-offs $\geq$ 2 for MDMA, HMMA sulfate, and HMMA glucuronide, and $\geq$ 1 for MDA, HMMA, and DHMA sulfate correctly predicted time of ingestion in more than 87% of all samples. However, so far these calculations were only performed after administration of a single MDMA dose. Recreational users might ingest repeated MDMA doses which would require further studies to show the applicability of such an estimation model after multiple doses.

4 SUMMARY

In the presented studies, the phase II metabolism of MDMA was investigated *in vitro* and *in vivo*. Furthermore, evaluation with respect to a possible stereoselective phase I and II metabolism was performed. The *in vitro* data indicated that sulfation is the major conjugation step with regioselective preferences for position 3 of DHMA. Both MDMA phase I metabolites, DHMA and HMMA, showed inhibition potential towards dopamine sulfation with IC_{50} values likely to be reached after recreational MDMA doses. Inhibition of dopamine degradation occurring in the central nervous system could be another reason for the drug-induced irreversible damage to central nerve terminals associated with MDMA consumption. Enantioselectivity was observed for DHMA sulfation and HMMA glucuronidation, but not for HMMA sulfation. *In vivo* urinary data obtained from 10 participants following controlled placebo, low and high dose MDMA administration supported the results from the *in vitro* experiments. HMMA sulfate was shown to be the major urinary metabolite providing the longest detection time for MDMA consumption. Enantiomeric ratios of all metabolites showed steady increases of *R*-isomers as a function of ingestion time allowing distinguishing between recent or earlier MDMA ingestion.

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6 ABBREVIATIONS

MDMA	3,4-methylenedioxymethamphetamine
NA	noradrenaline
5-HT	serotonin
DA	dopamine
CNS	central nervous system
CYP	Cytochrome P450
DHMA	3,4-dihydroxymethamphetamine
COMT	catechol-O-methyltransferase
HMMA	4-hydroxy-3-methoxymethamphetamine
UGT	uridine diphosphate glucuronyltransferase
SULT	sulfotransferase
MDA	3,4-methylenedioxyamphetamine
DHA	3,4-dihydroxyamphetamine
HMA	4-hydroxy-3-methoxyamphetamine
UDPGA	uridine 5’-diphosphoglucuronic acid
PAPS	3’-phosphoadenosine-5’-phosphosulfate
PAP	3’-phosphoadenosine-5’-phosphate
HLM	human liver microsomes
HLC	human liver cytosol
GC	gas chromatography
MS	mass spectrometry
LC	liquid chromatography

7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurde der Phase II Metabolismus von MDMA *in vitro* und *in vivo* untersucht. Darüber hinaus wurden die Daten auf einen möglichen stereoselektiven Phase I und II Metabolismus hin ausgewertet. Die *in vitro* Experimente haben gezeigt, dass die Sulfatierung die Hauptkonjugationsreaktionen darstellt, wobei für DHMA eine Regioselektivität für die 3 Position beobachtet wurde. Es wurde ebenfalls gezeigt, dass DHMA und HMMA die Sulfatierung von Dopamin hemmen können, mit IC_{50}- Werten wie sie nach üblichem Gebrauch von MDMA erwartet werden. Diese Inhibition könnte, wenn sie im Zentralnervensystem auftritt, eine weitere Ursache für die MDMA-induzierte irreversible Schädigung von Neuronen sein. Die Sulfatierung von DHMA und die Glucuronidierung von HMMA, nicht aber die HMMA Sulfatierung waren enantioselektiv. Die Ergebnisse der *in vitro*-Experimente wurden bestätigt durch *in vivo* Daten von 10 Teilnehmern, die im Rahmen einer kontrollierten MDMA-Studie jeweils ein Placebo, eine Niedrig- oder eine Hochdosis erhalten haben. HMMA-Sulfat war *in vivo* der Hauptmetabolit, der die längste Nachweisbarkeit einer MDMA Einnahme ermöglicht. Die Enantiomerenverhältnisse aller untersuchter Verbindungen zeigten eine stetige Zunahme der *R*-Enantiomere über die Zeit, was es erlaubt zwischen einem rezenten und einem länger zurückliegenden MDMA Konsum zu unterscheiden.