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Phase II Metabolism of 3,4-Methylenedioxymethamphetamine: Synthesis, Analysis, and Enantioselective in vitro and in vivo Kinetics

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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA), commonly named as Ecstasy, is a chiral compound with structural similarities to methamphetamine and mescaline. It has become popular in the beginning of the 1990s as a drug of abuse among young people, especially in the dance scene [1; 2]. 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 again since 2010 [3]. 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 [4; 5]. The distinctive effects are described as an altered state of consciousness, euphoria, energy and a desire to socialize [4; 6]. However, MDMA can also induce severe acute toxic symptoms, such as tachycardia, hypertension, hyperthermia, rhabdomyolysis, and hepatotoxicity. Severe and even fatal intoxications were described [4]. Concerning chronic toxicity, preclinical animal data suggest that MDMA causes irreversible damage to serotonergic nerve terminals in the CNS [4; 7-9]. 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 [10; 11]. Admittedly, direct MDMA injection into rat brain failed to reproduce neurotoxic effects seen after systemic administration [12]. Furthermore, alteration of cytochrome P450 (CYP)-mediated MDMA metabolism influenced MDMA-induced neurotoxicity [12; 13]. Therefore, MDMA metabolism may be an important contributor to neurotoxicity [14-17]. *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 3,4-dihydroxy-methamphetamine (DHMA), followed by catechol-*O*-methyltransferase (COMT)-catalyzed *O*-methylation, primarily to 4-hydroxy-3-methoxymethamphetamine (HMMA). DHMA and HMMA may also 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 [8; 18-20]. 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 [15; 21]. Such adducts have been implicated in MDMA neurotoxicity [22; 23].

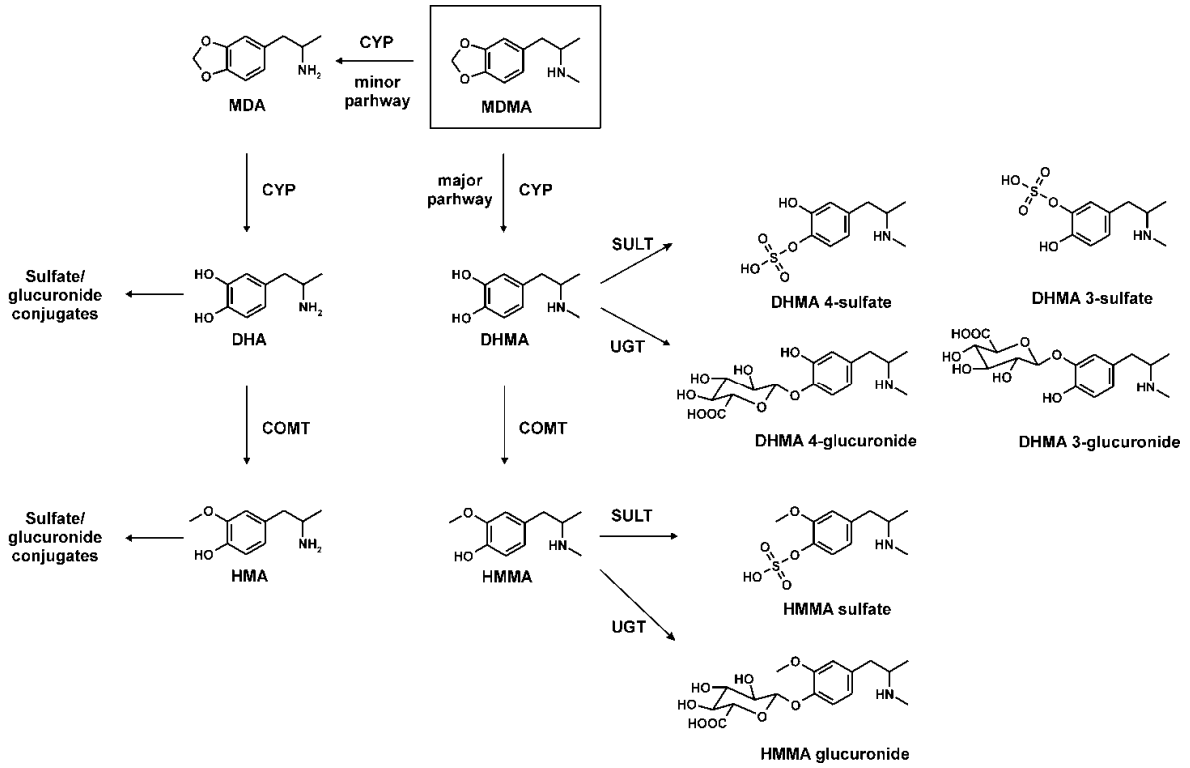

Fig. 1. Metabolic pathways of MDMA in humans.

2. Aims and Scopes

The qualitative and quantitative phase I metabolism of MDMA was studied extensively *in vitro* and *in vivo* [8; 18-20; 24-26]. 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. However, phase II metabolism represents an important detoxification process [27-29]. Investigation of glucuronidation and sulfation as a secondary metabolic step is especially important concerning the detoxification of reactive phase I metabolites.

Furthermore, for the two enantiomers of MDMA, different pharmacological and pharmacokinetic properties were observed [4]. While *S*-MDMA is generally more potent and responsible for the described psychostimulant and empathic effects, the *R*-isomer exhibits more hallucinogenic-type properties [8]. *R*- and *S*-MDMA also differ in their dose-response curves for changes in serotonergic function and neurotoxicity [4; 6; 30-33]. The *S*-enantiomer is eliminated from plasma at a higher rate than the *R*-enantiomer [4; 6; 30-33], most likely explained by stereoselective metabolism. Enantiomeric preferences in the phase I metabolism for CYP-*N*-demethylation, CYP-*O*-demethylenation, and COMT-methylation were observed *in vitro* [25; 26] and *in vivo* [33]. 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 [34] which is also assumed to influence MDMA-induced neurotoxicity [12; 13]. DHMA was also shown to inhibit its own metabolism as well as the methylation of dopamine [26]. 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, firstly to produce milligram quantities of MDMA's phase II metabolites as reference standards, secondly to investigate stereoselective glucuronidation and sulfation kinetics *in vitro* either in subcellular fractions (human liver microsomes, HLM or human liver cytosol, HLC) or in recombinant enzymes and assess a possible inhibition potential of MDMA and its phase I metabolites on sulfation. Thirdly, to further confirm the *in vitro* data, *in vivo* stereoselective elimination profiles should be performed in human urine after controlled MDMA administration to humans.

3. Results and Discussion

In order to investigate the phase II metabolism of MDMA's phase I metabolites, reference standards of respective glucuronides and sulfates were 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. An enzyme-assisted assay using rat liver microsomes and uridine-diphosphoglucuronic acid (UDPGA) was shown to be suitable to produce milligram amounts of the diastereomeric HMMA glucuronides with nearly 100% glucuronidation activity [35]. Highest yields of DHMA 3-sulfate (6 mg, 11%), DHMA 4-sulfate (6 mg, 11%) and HMMA sulfate (85 mg, 60 %) could be achieved by chemical synthesis with a pyridine SO_3 complex [36].

Adjacent, systematic analysis on the *in vitro* glucuronidation and sulfation kinetics of the MDMA phase I metabolites DHMA and HMMA was performed. Comparing the overall efficiency of the two conjugation reactions of HMMA, expressed by the V_{max}/K_m values from the enzyme kinetic determination, the one for the sulfation (270 µL/mg/min) [36] was approximately 25-fold higher than the one observed for the glucuronidation (10 µL/mg/min) [35]. To compare the activities of the conjugation reactions in a range of expected plasma concentrations (1-10 µM), ratios of interpolated pmol/mg/min values for both, sulfation and glucuronidation were calculated. The respective S/G ratios were 10 at lower plasma concentrations of HMMA decreasing to a ratio of 2 with increasing HMMA concentration. Sulfation of the catechol DHMA revealed regioselective preferences for sulfation in position 3 with approximately 10 times higher V_{max} values [35; 36]. DHMA glucuronidation in HLM was not observed in the concentration range used for HMMA, indicating that for DHMA conjugation, sulfation should also be the major pathway.

Additionally, evaluation with respect to a possible enantioselective phase II metabolism was performed [35]. 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 HLM, 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 [37]. Neither for SULT1A3 nor in HLC 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 HLC. One reason for this difference in enantioselectivity might be the position for sulfation. DHMA was mainly sulfated in position 3, whereas HMMA could, due to its chemical structure, only be sulfated in position 4.

Inhibition studies were performed with MDMA, DHMA, and HMMA towards typical sulfation reactions such as nitrophenol, dopamine, estradiol or dehydroepiandrostendione (DHEA) sulfation [36]. Only dopamine sulfation was inhibited by DHMA and HMMA, but not by MDMA. Further evaluation of the inhibition type clearly indicated a mixed-type or

competitive inhibition of dopamine sulfation by DHMA and HMMA, respectively, with K_i/IC_{50} values likely to cause significant inhibition *in vivo* after recreational MDMA doses [38]. In the author's opinion, a part of the described neurotoxicity of MDMA [4; 7-9] 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 [39] and as they additionally could inhibit the inactivation of these compounds [26], the described dopamine induced neurotoxicity might be enhanced [40].

To further obtain systematic *in vivo* data on MDMA's phase II metabolism and its stereoselectivity, liquid chromatography-high resolution mass spectrometry (LC-HRMS) and gas chromatography-negative ion chemical ionization- mass spectrometry (GC-NICI-MS) methods were developed and validated [41]. Unfortunately, as stereoselective chromatographic separation and analysis of MDMA and all its phase I and II metabolites was not possible in one method, three methods had to be developed. Method A allowed stereoselective determination of the HMMA glucuronides and only achiral determination of the intact sulfate conjugates of HMMA and DHMA after C18 solid-phase extraction by LC-HRMS with electrospray ionization. Method B allowed the determination of the enantiomer ratios of DHMA and HMMA sulfate conjugates after selective enzymatic cleavage and chiral analysis of the corresponding deconjugated metabolites after chiral derivatization with *S*-heptafluorobutyrylprolyl chloride using GC-NICI-MS. Method C allowed the chiral determination of MDMA and its unconjugated metabolites using method B without sulfate cleavage. The validation process including specificity, recovery, matrix effects, process efficiency, accuracy and precision, stabilities, and limits of quantification and detection showed that all methods were selective, sensitive, accurate and precise for all tested analytes and were therefore applicable for the analysis of urine samples of 10 human participants collected for up to 7 days following controlled oral placebo, low, and high dose MDMA administration [42].

Regarding urinary recovery, the major proportion of total urinary recovery occurred in the first 24 h [43]. To obtain comparable data for all participants, total urinary recovery was calculated over 5 days. After the low dose most MDMA was excreted in urine as HMMA sulfate (13%) while after the high dose, a greater percentage was excreted unchanged and the percentages of HMMA sulfate and glucuronide were significantly lower ($p<0.05$). The higher percentage of unchanged MDMA is best explained by MDMA's non-linear kinetics [42; 44; 45]. The significantly lower HMMA sulfate and glucuronide percentages, combined with no significant differences in DHMA and DHMA sulfate percentages, might indicate inhibition of COMT by DHMA, also demonstrated *in vitro* [26]. Unconjugated DHMA and HMMA accounted for less than 1% of the dose yielding 96 to 100% for DHMA conjugates and 88 to 98% for HMMA conjugates indicating that human MDMA urinary metabolites are primarily sulfate and glucuronide conjugates, with sulfates present in higher concentrations. Urinary ratios of sulfation/glucuronidation were in the same range as observed in the *in vitro* experiments [36; 43]. HMMA sulfate was shown to be the major urinary metabolite providing the longest detection time for MDMA consumption with up to 168 h [43].

All metabolites exhibited changes in enantiomeric disposition over time [46]. 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 [25; 26; 35; 37]. 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 MDMA consumption. *R/S* cut-offs ≥ 2 for MDMA, HMMA sulfate, and HMMA glucuronide, and ≥ 1 for MDA, HMMA, and DHMA sulfate correctly predicted time of ingestion in more than 87% of all samples [46]. However, so far, these calculations were only performed after administration of a single MDMA dose. Recreational users might ingest MDMA repeatedly which would require further studies to show the applicability of such an estimation model after multiple doses.

In conclusion, the presented work provided systematic data on the *in vitro* and *in vivo* kinetics of MDMA's phase II metabolism, also with respect to a possible stereoselectivity. These data might help to improve and optimize MDMA analysis and data interpretation and might contribute to further knowledge of the still controversially discussed neurotoxicity of MDMA in humans.

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