The Role of Human Hepatic Cytochrome P450 Isozymes in the Metabolism of Racemic MDMA and its Enantiomers

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Role of Human P450s in MDMA Enantiomers Metabolism

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Abbreviations used are: P450, cytochrome P450; ICM, insect cell microsomes; pHLM, pooled human liver microsomes; IS, internal standard; RAF, relative activity factor; TR, turnover rates; PS, probe substrate; SIM, selected-ion monitoring; MDMA, 3,4-methylenedioxymethamphetamine; DHMA, dihydroxymethamphetamine; MS, mass spectrometry; S-HFBPCl, S-heptafluorobutyrylprolyl chloride; GC, gas chromatography; MDA, 3,4-methylenedioxyamphetamine; DHBA, dihydroxybenzylamine; Na₂S₂O₅, Sodium metabisulfite; SPE, solid phase extraction; MAB3A4, monoclonal antibody inhibitory to 3A4; NICI, negative-ion chemical ionization; TEA, triethylamine.
Abstract

The entactogen 3,4-methylenedioxy-methamphetamine (MDMA), is a chiral drug which is mainly metabolized by N-demethylation and demethylenation. The involvement of cytochrome P450 (P450) isozymes in these metabolic steps has been studied by inhibition assays with human liver microsomes and, in part, with heterologously expressed human P450 isozymes. However, a comprehensive study on the involvement of all relevant human P450s has not been published yet. In addition, the chirality of this drug was not considered in these in-vitro studies. The aim of the present work was to study the contribution of human P450 isozymes in the N-demethylation and demethylenation of racemic MDMA and its single enantiomers. MDMA and its enantiomers were incubated using heterologously expressed human P450s and the metabolites were quantified by gas chromatography-mass spectrometry after derivatization with S-heptafluorobutyrylprolyl chloride (S-HFBPCl). The highest contribution for the N-demethylation as calculated from the enzyme kinetic data, were obtained for CYP2B6 (R,S-MDMA), CYP1A2 (R-MDMA), CYP2B6 (S-MDMA). In the case of the demethylenation, the isozyme with the highest contribution to net clearance for R,S-MDMA, R-MDMA, and S-MDMA was CYP2D6. For the first time, marked enantioselectivity was observed for N-demethylation and demethylenation by CYP2C19 with a preference for the S-enantiomers. In addition, CYP2D6 showed preference for S-MDMA in the case of demethylenation. None of the other isozymes showed major preferences for certain enantiomers. In conclusion the different pharmacokinetic properties of the MDMA enantiomers may therefore be caused by enantioselective metabolism by CYP2C19 and CYP2D6.
R,S-3,4-Methylenedioxy-methamphetamine (R,S-MDMA), also known as ‘Adam’ or ‘Ecstasy’, is a chiral compound and a very popular drug of abuse leading to feelings of euphoria and energy and a desire to socialize (Kalant, 2001). However, it can induce severe acute toxic symptoms such as tachycardia, hypertension, hyperthermia, and hepatotoxicity (Kalant, 2001). Many severe or even fatal intoxications have been described (Kalant, 2001).

Concerning chronic toxicity, data from animal experiments strongly suggest that these compound can cause irreversible damage to serotoninergic nerve terminals in the central nervous system (Kalant, 2001; Monks et al., 2004; de la Torre et al., 2004; Easton and Marsden, 2006). Decreased levels of the serotonin metabolite 5-hydroxyindoleacetic acid (McCann et al., 1994) and serotonin transporters (McCann et al., 1998) found in recreational MDMA users as compared to control subjects with no history of MDMA exposure point in the same direction. However, as the history of drug abuse of the studied MDMA users relied on self-report, it cannot be excluded that these findings were (in part) attributable to concomitant abuse of other drugs of abuse (Gouzoulis-Mayfrank and Daumann, 2006).

As shown in Fig. 1, in-vivo studies with MDMA revealed two main metabolic steps: N-demethylation and demethylenation (Maurer, 1996; Maurer et al., 2000; de la Torre et al., 2004). Systemic metabolism of MDMA may play a role in its neurotoxicity. This was concluded from the observation that direct injection of ecstasy into the brain fails to reproduce the neurotoxic effects seen after systemic administration (Esteban et al., 2001), and from the report that alteration of P450-mediated MDMA metabolism influences MDMA induced neurotoxicity (Gollamudi et al., 1989; Esteban et al., 2001). Metabolites such as 3,4-dihydroxymethamphetamine (DHMA) are easily oxidized to their corresponding quinones which can form adducts with glutathione and other thiol-containing compounds (Hiramatsu et al., 1990; Miller et al., 1997; Bai
et al., 1999). Recently, such adducts have been implicated in MDMA neurotoxicity (de la Torre and Farre, 2004; Capela et al., 2007).

The S-enantiomer of MDMA is known to be more potent than the R-enantiomer in producing the distinctive subjective effects that are characteristic of ecstasy. They also differ in their dose-response curves for changes in serotonergic function and neurotoxicity and their in-vivo kinetics are known to be different. It has been shown that after ingestion of racemic MDMA, the S-enantiomer is eliminated at a higher rate than the R-enantiomer (Fallon et al., 1999; Kalant, 2001; Kraemer and Maurer, 2002; Peters et al., 2003; Pizarro et al., 2004; Peters et al., 2005). Enantioselective metabolism is the most likely explanation for the enantioselective pharmacokinetics of MDMA. Data from inhibition studies (Maurer et al., 2000; Kreth et al., 2000) and experiments with recombinant CYP2D6 (Tucker et al., 1994; Lin et al., 1997; Kreth et al., 2000) indicated that CYP2D6, CYP3A4, CYP1A2, and CYP2B6 are involved in the N-demethylation and demethylenation of MDMA. Today, there is still only limited knowledge on the enantioselective metabolism of MDMA; data are only available for demethylenation of MDMA by recombinant CYP2D6 indicating a higher affinity for the S-enantiomer (Tucker et al., 1994; Lin et al., 1997). Therefore, the aim of the presented study was to obtain enantioselective enzyme kinetic data of N-demethylation and demethylenation of MDMA by the ten P450s most relevant in human drug metabolism.

**Materials and Methods**

Methanolic solution (100 mg/l) of racemic 3,4-methylenedioxyamphetamine (MDA)-d₅ was obtained from Promochem (Wesel, Germany), racemic hydrochlorides of MDMA and DHMA from Lipomed (Bad Saeckingen, Germany). Single MDMA enantiomers were obtained in the authors’ lab through enantioseparation of racemic MDMA as
described below. Sodium bicarbonate from Fluka (Steinheim, Germany); NADP⁺ from Biomol (Hamburg, Germany), isocitrate, isocitrate dehydrogenase, sodium metabisulfite (Na₂S₂O₅) from Carl Roth (Karlsruhe, Germany), and 3,4-dihydroxybenzylamine (DHBA) from Sigma (Taufkirchen, Germany). Water was purified in the authors' lab using a Millipore filtration unit. All other chemicals and reagents were obtained from Merck (Darmstadt, Germany). The following microsomes were from BD Gentest (Woburn, MA, USA) and delivered by NatuTec (Frankfurt/Main, Germany): baculovirus-infected insect cell microsomes (Supersomes; ICM) containing 1 nmol/ml human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 or 2 nmol/ml CYP2E1 and CYP3A5; wild-type baculovirus-infected ICM (control Supersomes), and pooled human liver microsomes (pHLM 20 mg microsomal protein/ml, 400 pmol total P450/mg protein). After delivery, the microsomes were thawed at 37°C, aliquoted, shock-frozen in liquid nitrogen and stored at –80°C until use. All chemicals were of analytical grade or the highest purity available. The monoclonal antibody inhibitory to CYP 3A4 (MAB3A4) (10 mg/mL MAB3A4 protein) was delivered by NatuTec (Frankfurt/Main, Germany). MAB3A4 is an ascites-based antibody preparation from mice and was tested to be selective for inhibition towards human CYP3A4. The derivatization reagent S-heptafluorobutyrylprolyl chloride (S-HFBPCl) was synthesized in the authors' laboratory according to (Peters et al., 2002).

**Separation of racemic MDMA and HPLC Conditions**

MDMA was separated using a Hewlett Packard Series 1050 semi-preparative HPLC system consisting of a pump and a variable wavelength detector (λ = 263 nm) which was coupled to an Advantec SF 2120 Super Fraction collector. The stationary phase
was a Merck Hibar HPLC ChiraDex column (250 x 10 mm, 5 µm). The mobile phase consisted of a mixture of 0.1 M ammonium acetate buffer adjusted to pH 6.5 with acetic acid 85% and acetonitrile 15%, flow rate 3 ml/min.

MDMA was separated in aliquots (100 µl) of an aqueous stock solution (5 mg/ml, 40 mg in total). The fractions were collected and checked for optical purity by gas chromatography-mass spectrometry (GC-MS) as described in detail under sample preparation.

The acetonitrile part of the HPLC solvent was evaporated. The remaining part was acidified with 1 ml 0.01 mol/l HCl and the enantiomers were isolated from the aqueous part by solid phase extraction (SPE) using Varian Bond Elut SCX HF cartridges (5 g, 20 ml), previously conditioned with 10 ml of methanol and 10 ml of water. After passage of the fractions, the cartridges were washed with 10 ml of 0.01 M hydrochloric acid and 10 ml of methanol. The compounds were eluted twice with 10 ml freshly prepared mixture of methanol/aqueous ammonia (96:4 v/v). The eluates were evaporated to dryness under reduced pressure and reconstituted in 1.0 ml 0.01M HCl and quantified according to (Peters et al., 2005).

Microsomal Incubations

Incubation mixtures (final volume: 50 µl) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM MgCl₂, 5 mM isocitrate, 1.2 mM NADP⁺, 0.5 U/ml isocitrate dehydrogenase, 200 U/ml superoxide dismutase and substrate at 37°C. The substrate was added after dilution of a 25 mM aqueous stock solution in the above-mentioned phosphate buffer. Reactions were started by addition of ice-cold microsomes and terminated with 5 µl of 60% (w/w) perchloric acid.

Initial Screening Studies
Incubations were performed with 50 µM R,S-MDMA, R-MDMA, or S-MDMA and 50 pmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5 for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM or 90 mM Tris buffer, respectively, according to the Gentest manual.

**Kinetic Studies**

Kinetic constants of N-demethylation (expressed as MDA formation) or demethylenation (expressed as DHMA formation) were derived from incubations with an incubation time of 20 min and a P450 concentration of 40 pmol/ml (N-demethylation) and 30 pmol/ml (demethylenation). The substrate concentrations as provided in Table 1 were used.

Enzyme kinetic constants were estimated by non-linear curve-fitting using GraphPad Prism 3.02 software (San Diego, CA). The Michaelis-Menten equation (equation 1) was used to calculate apparent $K_m$ and $V_{max}$ values for single-enzyme systems.

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

Eadie-Hofstee plots were used to check for biphasic kinetics (Clarke, 1998). If the Eadie-Hofstee plot indicated biphasic kinetics, equation 1 and the alternative equation 2 for a two site binding model (Clarke, 1998) were applied to the respective data. If equation 2 was found to fit the data significantly better (F-test, $P < 0.05$), biphasic kinetics were assumed.

$$V = \frac{V_{max,1} \times [S]}{K_{m,1} + [S]} + \frac{V_{max,2} \times [S]}{K_{m,2} + [S]} \quad (2)$$
Calculation of Relative Activity Factors, Contributions, and Percentages of Net Clearance

The relative activity factor (RAF) approach (Crespi and Miller, 1999; Venkatakrishnan et al., 2000; Grime and Riley, 2006) was used to account for differences in functional levels of redox partners between the two enzyme sources. The turnover rates (TR) of CYP2C19 [probe substrate (PS) S-mephenytoin], CYP2D6 (PS bufuralol), CYP2B6 (PS 7-ethoxy-4-trifluoromethylcoumarin) and CYP1A2 (PS phenacetin) in ICM and HLM were taken from the supplier's data sheets. The RAFs were calculated according to equation 3.

\[
RAF_{\text{enzyme}} = \frac{TR_{\text{ps}} \text{ in HLM} \left[ \text{pmol/min/mg protein} \right]}{TR_{\text{ps}} \text{ in ICM} \left[ \text{pmol/min/mg protein} \right]}
\]  

The enzyme velocities \( V_{\text{enzyme}} \) (see equation 1) for the respective metabolic reactions were calculated at different substrate concentrations and were then multiplied with the corresponding RAF leading to a value, which is defined as 'contribution'. The \( V_{\text{max}} \) and the \( K_m \) values (equation 1) were obtained from the incubations with cDNA-expressed P450s.

\[
\text{contribution}_{\text{enzyme}} = RAF_{\text{enzyme}} \times V_{\text{enzyme}} \]  

From these corrected activities (contributions) the percentages of net clearance by a particular P450 at a certain substrate concentration can be calculated according to equation 5:
Chemical Inhibition Studies

The effect of 3 µM quinidine (CYP2D6 inhibitor), 6 µM omeprazole (CYP2C19 inhibitor), or 30 µM α-naphthoflavone (CYP1A2 inhibitor) on DHMA formation was assessed in incubations containing 0.5 mg HLM protein/ml and 1 µM or 5 µM R,S-MDMA, R-MDMA, or S-MDMA (n=6 each). Control incubations (n=6) contained none of these chemical inhibitors. Significance of inhibition was tested by one-tailed unpaired t-tests using GraphPad Prism 3.02 software.

Inhibition Studies with MAB3A4

The effect of MAB3A4 to CYP3A4 racemic DHMA formation was assessed in incubations containing 0.4 mg HLM protein/mL and 0.02 mg MAB3A4 protein/mL for 20 min at a concentration of 1 µM or 5 µM of the drug (n=6 each). Control incubations contained none of this MAB3A4 (n=6 each). The MAB3A4 was added to the HLM incubated for 15 min on ice according to the NatuTec manual. As control, HLM were incubated for 15 min on ice without MAB3A4. After this pre-incubation HLM with and without MAB3A4 were added to the incubation mixture. Significance of inhibition was tested by one-tailed unpaired t-tests using GraphPad Prism 3.02 software.

Sample Preparation

After termination, the incubation mixtures were centrifuged and 45 µl of the supernatants were transferred to 1.5 ml reaction caps and diluted with 200 µl aqueous carbonate buffer (35 g/l sodium bicarbonate and 15 g/l sodium carbonate, pH 9) containing the respective internal standard (IS) racemic MDA-d₅, and DHBA.
(0.02 ng/ml, each) and 3% Na₂S₂O₅ and 3% EDTA-Na (250 mM each) for preservation of the dihydroxy compounds. Derivatization was performed according to (Peters et al., 2005), with slight modifications: after adding 20 µl derivatization reagent (0.1 mol/l S-HFBPCl in dichloromethane), the reaction vials were sealed and left on a rotary shaker at ambient temperature for 30 min. After addition of 100 µl cyclohexane to the reaction vials, they were resealed, and placed on a rotary shaker for 5 min. After phase separation by centrifugation (10000 g for 1 min), the cyclohexane phase was transferred to autosampler vials. Aliquots of 3 µl were injected into the GC-MS.

Enantioselective GC-NICI-MS Quantification

The samples were analyzed by an Agilent Technologies (AT) 6890 Series GC system combined with an AT 5973 network mass selective detector, an AT 7683 series injector, and an AT enhanced Chem Station G1701CA, version C.00.00 21-Dec-1999. For detection of R,S-MDA and R,S-MDA-d₅, the GC conditions were as follows: splitless injection mode; column, 5% phenyl methyl siloxane (HP-5MS; 30 m x 0.25 mm (i.d.); 250 nm film thickness); injection port temperature, 280°C; carrier gas, helium; flow rate, 1 ml/min; column temperature, 100°C increased to 200°C at 30°C/min, to 260°C at 5°C/min, and to 310°C at 30°C/min. The negative-ion chemical ionization (NICI)-MS conditions were as follows: transfer line heater, 280°C; NICI, methane (2 ml/min); source temperature, 150°C; solvent delay, 11 min; selected-ion monitoring (SIM) mode with the following ions: m/z 432 for MDA and 437 for MDA-d₅.

For detection of DHMA and DHBA, the GC and NICI-MS conditions were modified as follows: column temperature, 150°C increased to 280°C at 40°C/min and to 310°C at
2°C/min. Solvent delay, 13.5 min; SIM mode with the following ions: m/z 472 for DHMA and 430 for DHBA.

MDA and DHMA enantiomers were quantified by comparison of their peak-area ratios (enantiomers of analyte vs corresponding enantiomer of the IS) to calibration curves in which the peak-area ratios of enriched calibrators had been plotted vs their concentrations (0.1, 0.5, 1.0, 1.5, 5.0 µM) using a weighted (1/x) least-squares linear regression model.

**Results**

**Semi-preparative separation of R,S-MDMA**

Forty mg of racemic MDMA-HCl were separated under the described conditions. The chiral HPLC method provided almost base-line separation of the MDMA enantiomers (Fig. 2A). As illustrated in Figs. 2B and 2C, purity check of the isolated enantiomers by GC-MS indicated high optical purities of S-MDMA-HCl (97%) and R-MDMA-HCl (98%), respectively. The elution order was confirmed according to (Peters et al., 2005). The yield was approximately 60% or 12 mg per enantiomer.

**Enantioselective GC-NICI-MS Quantification**

The separation of the MDA enantiomers was comparable to that described by (Peters et al., 2005). The mass fragmentograms shown in Fig. 3 demonstrate that the modified GC-MS conditions applied for analysis of DHMA enantiomers provided sufficient separation. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and IS) and zero samples (control microsomes without substrate, but with IS). The method showed good linearity in a range of 0.01 to 5.0 µM DHMA or MDA (r² > 0.99).
Initial activity screening

N-demethylation of racemic MDMA and its enantiomers was catalyzed by CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4/3A5. Demethylenation of MDMA and its enantiomers was catalyzed by CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4/3A5.

Kinetic Studies

The kinetic parameters could not be determined for all P450 capable of catalyzing the monitored metabolic reactions, because the activity of CYP3A4/A5 with respect to the N-demethylation and CYP2B6 and CYP3A5 with respect to demethylenation of MDMA was too low. The same was true for CYP2C19 catalyzed demethylenation of R-MDMA in racemic MDMA. The Km and Vmax values for the other P450s are listed in Table 2. The data for CYP1A2, CYP2B6, and CYP3A4 (N-demethylation) and CYP1A2, CYP2D6, CYP2C19 (demethylenation) followed the expected classical hyperbolic Michaelis-Menten kinetics as exemplified for CYP2C19 in Fig. 4A. In contrast, the Eadie-Hofstee plots (data not shown) of the CYP2D6 (N-demethylation) and CYP3A4 (demethylenation) clearly indicated biphasic kinetics, which was statistically confirmed (F-test, p<0.05). Hence the kinetic parameters were estimated by fitting the data into equation 2 for a two-site binding model. The resulting Km1 and Vmax1 data are also reported in Table 2. Figure 5 shows plots of the percentages of net clearance as calculated from the RAF-corrected kinetic data versus substrate concentrations up to 10 µM. The data for N-demethylation and demethylenation of racemic MDMA, R-MDMA, and S-MDMA are shown in panels A-C and D-F, respectively. At low substrate concentrations (1 µM), N-demethylation and demethylenation were predominantly catalyzed by CYP2B6 (42-48%) and CYP2D6 (55-76%), respectively. At high substrate concentrations (10 µM), CYP2B6 (43-50%)
and CYP1A2 (36-42%) showed a comparable net clearance with respect to \( N \)-demethylation. The same was true for CYP2D6 (33-34%) and CYP1A2 (38-41%) with respect to demethylenation.

Marked enantioselectivity \( (k_{m}(R\text{-MDMA})/k_{m}(S\text{-MDMA}) > 1.5) \) was observed for both metabolic steps by CYP2C19 and for demethylenation additionally by CYP2D6. In addition, CYP1A2 showed a minor preference for \( S\)-MDMA with respect to demethylenation.

**Chemical Inhibition Studies**

The results of the experiments with the chemical inhibitors \( \alpha \)-naphthoflavone (CYP1A2), omeprazole (CYP2C19), quinidine (CYP2D6), or the MAB3A4 are presented in Fig. 6.

**Discussion**

Enantioselective metabolism is the most likely explanation for the enantioselective pharmacokinetics of MDMA. Data from inhibition studies (Maurer et al., 2000; Kreth et al., 2000) and experiments with recombinant CYP2D6 (Tucker et al., 1994; Lin et al., 1997; Kreth et al., 2000) indicated that CYP2D6, CYP3A4, CYP1A2, and CYP2B6 are involved in the demethylenation and \( N \)-demethylation of MDMA. Recombinant CYP2D6 was reported to have higher affinity for the \( S \)-enantiomer with respect to MDMA demethylenation (Tucker et al., 1994; Lin et al., 1997). The study presented here is the first providing enantioselective enzyme kinetic data of \( N \)-demethylation and demethylation of MDMA by the ten recombinant P450s, which are the most relevant in human drug metabolism. Among these enzymes was CYP2C19 which is known to be one of the five most abundant P450s involved in xenobiotic
metabolism (Guengerich, 2005), which had so far not been considered in studies on MDMA metabolism.

The experiments were performed with racemic MDMA as present in ecstasy tablets as well as single MDMA enantiomers to check whether certain P450s specifically metabolize certain enantiomers and whether the enantiomers influence each other's metabolism. Various buffers and buffer concentrations as well as organic modifiers were tested for semi-preparative isolation of the single enantiomers for these experiments. The best result in enantiomer separation was achieved with KH₂PO₄ 0.1M/acetonitrile 95/5 with triethylamine (TEA) 0.1% but the TEA content as well as the phosphate buffer turned out to be problematic in the following isolation of the enantiomers by SPE. The conditions described in the materials and methods section were finally preferred because they yielded sufficient separation combined with favorable properties for further workup. The final products were obtained as yellowish powders of high optical purities. Despite rather low recoveries (~ 60% per enantiomer), the isolated amounts were sufficient for further kinetic studies.

The described quantification method according to (Peters et al., 2005) was modified to be applicable for the presented kinetic studies. It is based on the derivatization of analyte enantiomers to diastereomers using an optically pure derivatization reagent. The diastereomeric derivatives can then be separated on an achiral GC column. In the present study, the amount of derivatization reagent had to be increased to achieve a reliable and reproducible (triple) derivatization of the DHMA enantiomers. In addition, the oven temperature program, the run-time and the ions monitored in SIM had to be modified to allow separation and detection of the triple derivatives of the dihydroxy compounds, DHMA and DHBA (Fig. 2).

According to the supplier's advice, the incubation conditions chosen in the initial screening experiment are applicable for checking the general involvement of
particular P450 enzymes. Because of the very low activity of CYP3A4/3A5 with respect to \( N\)-demethylation and the very low activity of CYP2B6 and CYP3A5 to demethylation, only the other P450s which had shown activity in the initial screening could be characterized with respect to their kinetic profiles. Duration and protein content of all incubations in these studies were within the linear range of metabolite formation (data not shown). Less than 20% of substrate was metabolized in all incubations with exception of the lowest substrate concentrations.

There are inherent differences in the \( K_m \) and \( V_{\text{max}} \) values of the single enantiomer kinetics vs. the values of racemic MDMA in incubations with racemic MDMA which are discussed in the following. \( R\)- and \( S\)-MDMA are competitors for the limited number of active sites in the incubation mixture. Each binding site can only transform one molecule at a time, either \( R\)-MDMA or \( S\)-MDMA. Hence, at saturation, approximately half of the active sites are busy transforming \( R\)-MDMA while the other half is busy transforming \( S\)-MDMA. In incubations of single enantiomers, however, all active sites are available for one enantiomer. Hence, at saturation, approximately twice as many molecules of the respective enantiomer can be transformed at the same time as in incubations of racemate. This clearly explains the higher \( V_{\text{max}} \) values of the single enantiomer kinetics.

The differences of the \( K_m \) values can be explained in the same way. Saturation is reached when all available active sites are busy transforming MDMA molecules. In incubations of the racemate, half of the active sites are occupied with \( R\)-MDMA and half with \( S\)-MDMA. In incubations of single enantiomers, this enantiomer must occupy all active sites and hence its concentrations must be much higher as in the respective incubations of the racemate to reach saturation. Considering that the \( K_m \) is defined as the substrate concentration where transformation is half maximal, it is
obvious that $K_m$ values obtained from single enantiomer incubations must inherently be higher than those of the respective enantiomer in incubations of the racemate. CYP2D6 turned out to have the highest affinity towards both main metabolic steps of racemic MDMA as well as for its enantiomers (Table 2). The obvious difference in the $K_m$ values of racemic MDMA and the respective enantiomers might be caused by interactions of $R$- and $S$-MDMA in incubations of the racemate. For the difference in the $V_{\text{max}}$ values observed in the case of CYP1A2, there seems to be no straightforward explanation. It might be attributable to a decreased turnover and complex interactions, e.g. cobinding at the active site of the enzyme, during incubation of racemic MDMA. However, for the time being, interpretation of this obvious difference remains speculative. CYP2D6 also had the highest capacity for demethylenation of $R,S$-MDMA and $R$-MDMA, whereas the highest $V_{\text{max}}$ value for $S$-MDMA was observed for CYP2C19.

The enzyme kinetic data reported here are considerably different to those reported by Kreth et al. (Kreth et al., 2000). This can be explained by the fact that the $K_m$ and $V_{\text{max}}$ data of Kreth et al. were derived from inhibition experiments with human liver microsomes rather than cDNA expressed single enzymes as described in our study. More precisely, Kreth et al. had observed biphasic enzyme kinetics in human liver microsomes and assumed the high affinity component to represent CYP2D6 and the low affinity component to represent CYP1A2. Based on this assumption, they derived their reported $K_m$ and $V_{\text{max}}$ from the two kinetic components. Considering that not only these two enzymes, but also CYP2B6, CYP3A4, and, as shown in our study, also CYP2C19 are involved in MDMA metabolism, the approach by Kreth et al. is certainly an oversimplification. In addition, Kreth et al. assessed the contribution of the various isoenzymes involved in MDMA metabolism solely by inhibition experiments with supposedly specific chemical inhibitors. However, orphenadrine
that was used as “specific” inhibitor of CYP2B6 also decreases the marker activity of CYP2D6 and CYP2C9 (Guo et al., 1997) which may have affected the contribution data reported by Kreth et al.

Common methods of calculating the net clearance are based on the assumption that substrate concentrations are lower than 10% of $K_m$ of the respective isozymes (Crespi, 1995; Venkatakrishnan et al., 2000). This is critical if the isozymes involved in the metabolic reaction have a very low $K_m$ value which is considerably below expected plasma concentrations. In the present study, $K_m$ values of 0.2-0.3 µM were found for MDMA demethylenation by CYP2D6. These were well below expected plasma concentrations of MDMA after recreational doses, which are usually in the range of 1 µM (194 µg/l) (Fallon et al., 1999; de la Torre et al., 2000; Logan and Couper, 2001; Peters et al., 2003; Pizarro et al., 2004; de la Torre et al., 2004; Peters et al., 2005), but can reach 10 µM and higher in severe intoxications (Peters et al., 2003; Schifano, 2004). In the present study, we chose to calculate the percentages of net clearance for substrate concentrations ranging from 1 to 10 µM (Fig. 5) to model the involvement of the studied P450s over the relevant concentration range. At 1 µM and 5 µM, CYP2D6 accounts for 55-78% and 42-46% of net clearance with respect to demethylenation, respectively. This percentage continuously decreases with increasing substrate concentrations reaching 33-34% at 10 µM. In contrast, CYP1A2 becomes increasingly important with rising substrate concentrations reaching 38-41% of net clearance at 10 µM. A similar situation is observed for $N$-demethylation, where the net clearance of CYP2B6 remains rather constant from 38-48% (1 µM) over 41-49% (5 µM) to 43-50% (10 µM), while that of CYP1A2 moderately increases from 33-34% over 35-39 (5 µM) to 36-41%, respectively. This shows that in intoxication cases, the activity of CYP1A2 may play an important role in MDMA metabolism. The difference in the percentage of net clearance observed for
CYP2D6 in the case of racemic MDMA and its enantiomers is caused by differences in the $K_m$ values for the respective compounds, as the $K_m$ value takes part in calculation of the net clearance (see equation 1 and 5). To confirm the role of CYP1A2, CYP2D6, CYP2C19, and CYP3A4 in the most important step in MDMA metabolism which leads to the toxic metabolite DHMA, inhibition experiments were performed using pHLM, with the chemical inhibitors α-naphthoflavone, quinidine, and omeprazole at concentrations according to the literature (Newton et al., 1995; Bourrie et al., 1996; Ko et al., 1997; Clarke, 1998; Venkatakrishnan et al., 2001), and the MAB3A4. These experiments were performed at two substrate concentrations representing concentrations expected in recreational users, namely 1 µM and 5 µM to account for the above-mentioned concentration dependence of the involvement of individual P450s in MDMA demethylation. Generally, the demethylation was inhibited significantly at both substrate concentrations. The only exceptions were CYP1A2 at 1 µM racemic MDMA and CYP3A4 at 5 µM S-MDMA and 1 µM R-MDMA (Fig. 6). Quinidine had the strongest inhibition effects at both substrate concentrations (Fig. 6). This is in line with the calculated percentages of net clearance as shown in Fig. 5, although the extent of inhibition by quinidine is somewhat larger as expected based on the calculations. The importance of CYP2D6 is critical from the toxicological point of view, because MDMA is known to be a mechanism-based inhibitor of this isozyme (Heydari et al., 2004). This means that in case of repeated doses, the metabolism of MDMA should be considerably reduced. Indeed, the area under the curve and the maximum plasma concentration values increased by 77% and 29%, respectively, after repeated doses of MDMA (de la Torre et al., 2004). The stronger inhibition effects of the CYP1A2 inhibitor α-naphthoflavone at higher substrate concentrations is also in good agreement with the calculated percentages of net clearance increasing with substrate
concentrations. As expected from the calculations of net clearance, inhibition with the CYP2C19 inhibitor omeprazole and the MAB3A4 did not show clear trends from the lower to the higher substrate concentrations. However, the observed inhibition effects were again somewhat larger than the respective percentages of net clearance. CYP2C19 showed the largest extent of enantioselectivity in both metabolic reactions with a marked preference for the S-enantiomer. The ratios of the $K_m$ values of the MDMA enantiomers ($R$ vs. $S$) with respect to demethylenation and N-demethylation were 2.1 and 2.0, respectively. CYP2D6 also showed marked enantioselectivity, but here the respective ratios were 1.6 and 1.7. The other isozymes showed little or no enantioselectivity. Considering these findings along with the fact that demethylenation is the major metabolic step of MDMA metabolism in-vivo, the different pharmacokinetic properties of the MDMA enantiomers are therefore most likely attributable to enantioselective demethylenation by CYP2C19 and CYP2D6. CYP2D6 should be most important in this context, because it accounts for more 33% of net clearance even at high substrate concentrations as compared to a maximum of 17% for CYP2C19. This must be considered when trying to estimate the time of ingestion from enantiomer ratios in plasma as proposed by (Fallon et al., 1999; Peters et al., 2003), because the time course of such ratios may be considerably different in CYP2D6 poor metabolizers or in case of inhibition of CYP2D6 by MDMA or other co-ingested drugs. In addition, it must be considered that correlation of the presented in-vitro data with the in-vivo situation is not straightforward, because in-vivo DHMA is further metabolized by $O$-methylation and/or glucuronidation/sulfation. Enantioselectivity of these phase II reactions might of course also influence the enantiomer ratios in plasma samples, especially those of DHMA.
Acknowledgements

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References


Legends to the Figures

**FIG. 1.** The two main metabolic steps of *R*- and *S*-MDMA leading to the formation of the corresponding enantiomers of dihydroxymethamphetamine (DHMA) and methylenedioxyamphetamine (MDA).

**FIG. 2.** A) Separation of MDMA using a Chiradex column under the described conditions (UV $\lambda$=263 nm). First peak is *R*-MDMA, second peak *S*-MDMA. B+C) GC-MS of aliquots of the collected *R*-MDMA (upper mass fragmentogram) and *S*-MDMA (lower mass fragmentogram) HPLC fractions after derivatization.

**FIG. 3.** Typical mass fragmentograms of ions $m/z$ 430 for DHBA and $m/z$ 472 for DHMA of an incubation mixture after derivatization.

**FIG. 4.** Michaelis-Menten plots for MDA (left) and DHMA (right) formation catalyzed by CYP2C19 (4A), CYP2D6 (4B), CYP1A2 (4C), and Michaelis-Menten plots for MDA formation catalyzed by CYP2B6 (4D left) and DHMA formation catalyzed by CYP3A4 (4D right). Data points represent means and ranges (error bars) of duplicate measurements. The solid curves represent formation of *R*-MDA/*R*-DHMA from racemic MDMA (upper part) and *R*-MDMA (lower part). The broken curves represent formation of *S*-MDA/*S*-DHMA from racemic MDMA (upper part) and *S*-MDMA (lower part).

**FIG. 5.** Plots of calculated net clearance vs. substrate concentration for the *N*-demethylation (upper part) and demethylation (lower part).
Fig. 6. Effect of the chemical inhibitors alpha-naphthoflavone, 30 µM (CYP1A2), omeprazole, 10 µM (CYP2C19), quinidine, 3 µM (CYP2D6), and the MAB3A4 on demethylenation in incubation mixtures containing 1 µM or 5 µM of R,S-, R- or S-MDMA. Metabolite formation of the control incubations was set to 100%. Each bar represents the mean of six incubations ± standard error of the mean. (*p<0.5, **p<0.05, ***p<0.005)
Table 1. Substrate concentration in µM used for microsomal incubations

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### Table 2. Kinetic Data for the Two Main Metabolic Steps of (R,S)-MDMA

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Units are: \( V_{\text{max}} \), pmol/min/pmol; \( K_m \), µM

* \( V_{\text{max}} \) and \( K_m \)
Figure 2

(A) Graph showing the mAU (molar absorbance units) over time in minutes.

(B) Graph showing the abundance over time in minutes for another compound.

(C) Graph showing the abundance over time in minutes for a third compound.
Figure 5

A. Clearance, %

B. Clearance, %

C. Clearance, %

S-MDMA, μM

R, S-MDMA, μM

R-MDMA, μM

CYP2D6
CYP1A2
CYP2B6
CYP2C19
CYP3A4