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

Markus R. Meyer, Frank T. Peters, and Hans H. Maurer

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Saarland University, D-66421 Homburg (Saar), Germany
Role of Human P450s in MDEA Enantiomers Metabolism

Corresponding author:

Prof. Dr. Dr. h.c. Hans H. Maurer
Department of Experimental and Clinical Toxicology
Institute of Experimental and Clinical Pharmacology and Toxicology
Saarland University, Building 46, D-66421 Homburg (Saar), Germany
Tel.: +49-6841-1626050, Fax: +49-6841-1626051
Email: hans.maurer@uks.eu

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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; MDMA, 3,4-methylenedioxymethamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; DHEA, dihydroxyethylamphetamine; 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 3,4-methylenedioxy-methamphetamine (MDMA)-related designer drug 3,4-methylenedioxyethylamphetamine (MDEA, Eve) is a chiral compound which is mainly metabolized by N-deethylation and demethylenation during phase I metabolism. The involvement of several cytochrome P450 (P450) isozymes in these metabolic steps has been demonstrated by inhibition assays using human liver microsomes. 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 first to elucidate the contribution of the relevant human P450 isozymes in the demethylenation as well as in the N-dealkylation of racemic MDEA and its single enantiomers and secondly to compare these findings with recently published data concerning the enantioselective metabolism of MDMA. Racemic MDEA and its single enantiomers were incubated using heterologously expressed human P450s and the corresponding metabolites dihydroxyethylamphetamine and methylenedioxyamphetamine were determined by gas chromatography-mass spectrometry after chiral derivatization with S-heptafluorobutyrylprolyl chloride. The highest contributions to both metabolic steps as calculated from the enzyme kinetic data were obtained for CYP3A4 and CYP2D6 at substrate concentrations corresponding to plasma concentrations of recreational users after intake of racemic MDEA. Both metabolic reactions were found to be enantioselective with a general preference for the S-enantiomers, which was particularly pronounced in the case of CYP2C19. In conclusion, different pharmacokinetic properties of MDEA enantiomers observed in vivo are therefore partially caused by P450-dependent enantioselective metabolism.
Introduction

3,4-Methylenedioxyethylamphetamine (MDEA, Eve) is chemically and pharmacologically related to 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy, Adam) (Freudenmann and Spitzer, 2004). The MDEA enantiomers have different pharmacokinetic properties, \(S\)-MDEA producing elevated mood and impairment in conceptually driven cognition and \(R\)-MDEA producing increased depression and enhanced visual feature processing. Concerning chronic toxicity, data from animal experiments strongly suggest that both MDMA and MDEA 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; Freudenmann and Spitzer, 2004; Easton and Marsden, 2006). However, most in vitro and animal studies indicate that in comparison to MDMA the neurotoxic potential of MDEA is lower (Kalant, 2001; Monks et al., 2004; Freudenmann and Spitzer, 2004).

Recently, we have published the involvement of human P450 in the metabolism of racemic MDMA as well as of its enantiomers (Meyer et al., 2008). In this MDMA study, we were able to show, that CYP2C19 (besides CYP2D6) is the most enantioselective P450 isozyme enzyme towards the two main metabolic steps, namely \(N\)-demethylation and demethylenation with a preference for the \(S\)-enantiomer. These results could in part explain the different pharmacokinetics of \(R\)- and \(S\)-MDMA in vivo. As shown in Figure 1, in vivo and in vitro studies showed that the main metabolic steps of MDEA are the same as of MDMA, namely \(N\)-deethylation and demethylenation (Maurer et al., 2000). Later, MDEA was investigated concerning enantioselective pharmacokinetics in vivo (Brunnenberg and Kovar, 2001; Buechler et al., 2003) and the plasma half life of \(R\)-MDEA was found to be longer than that of \(S\)-MDEA. Accordingly, the plasma concentrations of the \(S\)-enantiomers of the main metabolites \(N\)-ethyl-4-hydroxy-3-methoxyamphetamine and 3,4-
methylenedioxyamphetamine (MDA) were much higher than those of the R-enantiomers. Enantioselective pharmacokinetics of MDEA resulting in higher plasma concentrations of R-MDEA were also confirmed by other authors (Spitzer et al., 2001; Meyer et al., 2002; Peters et al., 2003). Enantioselective metabolism is the most likely explanation for the enantioselective pharmacokinetics of MDEA. Previously published data from inhibition studies and with recombinant P450 (Maurer et al., 2000; Kreth et al., 2000) indicated that CYP1A2, 2B6, 2D6, and 3A4 are involved in the N-deethylation and/or demethylenation of MDEA but did not provide any information about the possible enantioselectivity of these metabolic steps. Hence, no systematic enzyme kinetic data are available for (enantioselective) MDEA metabolism by recombinant isozymes. However, kinetic data for each involved isozyme could help to explain the differences in MDMA/MDEA metabolism as well as the different pharmacokinetics of the enantiomers in vivo. Therefore, the aim of the present study was to obtain enantioselective enzyme kinetic data of N-deethylation and demethylenation of MDEA by the ten P450s most relevant in human drug metabolism and to compare these data with kinetics of MDMA to reveal and explain the differences in enantioselective metabolism.

**Materials and Methods**

In principle, materials and methods were the same as described previously (Meyer et al., 2008). Therefore, only the differences are mentioned in the following section. Racemic hydrochlorides of MDEA (>98.5% purity) and N-ethyl-3,4-dihydroxyamphetamine were obtained from Lipomed (Bad Saeckingen, Germany) and ReseaChem (Burgdorf, Switzerland), respectively. The MDEA enantiomers (98% purity, each) were prepared in the author’s lab by enantioseparation as described below.
Separation of racemic MDEA and HPLC Conditions. Racemic MDEA was separated in aliquots (100 µl) of an aqueous stock solution (5 mg/ml, 30 mg in total) using a mobile phase composition of 0.1 M ammonium acetate buffer (pH 6.5):ACN 85:15 (v/v), a flow rate of 3 ml/min, and ultraviolet (UV) detection at λ = 263 nm. The cyclodextrin column was mounted into a freezer at 8°C. The fractions containing the separated enantiomers were collected and the enantiomers were isolated from the aqueous part by liquid/liquid extraction at pH 9 using ethyl acetate (three times using 150 ml each). The extracts were evaporated to dryness using a Rotavapor under reduced pressure and reconstituted in 1.0 ml of 0.01 M HCl. Thereafter, the concentrations of the MDEA enantiomers in the resulting solution was determined. The recovery was approximately 75 % per enantiomer.

Initial Screening Studies and Kinetic Studies. Incubations were performed with 50 µM R,S-MDEA, R-MDEA, and S-MDEA. Kinetic constants of N-deethylation (expressed as MDA formation) or demethylenation (expressed as DHEA formation) were derived from incubations with an incubation time of 20 min and a P450 concentration of 40 pmol/ml (N-deethylation) and 30 pmol/ml (demethylenation). The substrate concentrations shown in Table S1 were used. Calculation of enzyme kinetic constants was similar to the previously published method (Meyer et al., 2008). Briefly, the Michaelis-Menten equation (equation 1) was used to calculate apparent K_m and V_max values for single-enzyme systems. Eadie-Hofstee plots were used to check for biphasic kinetics. If the Eadie-Hofstee plot indicated biphasic kinetics, eq 1 and the alternative equation 2 for a two-enzyme model (Clarke, 1998) were applied to the respective data. For equation 2, CL_{int,2} represents the intrinsic clearance or V_{max}/K_m of the low affinity component (Clarke,
1998). If equation 2 was found to fit the data significantly better (F test, P < 0.05), biphasic kinetics were assumed.

\[
V = \frac{V_{\text{max}} \times [S]}{K_m + [S]}
\]

\[
V = \frac{V_{\text{max,1}} \times [S]}{K_{m,1} + [S]} + CL_{\text{int,2}} \times [S]
\]

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 corrected activities (contributions), the percentages of net clearance by a particular P450 at a certain substrate concentration, can be calculated according to equation 3:

\[
clearance_{\text{enzyme}} \% = \frac{\sum \text{contribution}_{\text{enzyme}}}{\sum \text{contribution}_{\text{enzyme}}} \times 100
\]

Inhibition Studies with Chemical Inhibitors or the Monoclonal Antibody (MAB) 3A4. The effect of 3 µM quinidine and 6 µM omeprazole on (R,S)-DHEA formation was assessed in incubations containing 0.5 mg HLM protein/ml and 1 µM or 5 µM R,S-MDEA, R-MDEA, or S-MDEA (n=6 each). Control incubations contained none of these chemical inhibitors. Significance of inhibition was tested by one-tailed unpaired t-test using GraphPad Prism 3.02 software. The effect of MAB inhibitory to CYP3A4 DHEA formation was assessed in the same way as described for MDMA (Meyer et al., 2008).

Sample Preparation. Sample preparation and metabolite quantification was also performed according to (Meyer et al., 2008) with the following modifications: For detection of (R,S)-DHEA and DHBA, 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, 150 °C increased to 250 °C at 40 °C/min, to 290 °C at 2 °C/min and finally to 310°C. For quantification, the following target ions (m/z) were used in the selected-ion monitoring mode: m/z 437 for MDA-d₅, 432 for MDA, 430 for DHBA, and 780 for DHEA.

**Results and Discussion**

The applied GC-MS conditions provided separation of DHEA enantiomers (supplemental data, Fig. S1) and the chosen target ions were selective for the analytes under these conditions as proven with blank samples (control microsomes without substrate and standard) and zero samples (control microsomes without substrate, but with standard). The method showed good linearity in a range of 0.05-5.0 µM DHEA or MDA.

In the initial activity screening, CYP1A2, CYP2B6, CYP2C19, CYP2D6, and CYP3A4/3A5 were found to be capable of catalyzing the N-deethylation and CYP1A2, CYP2C19, CYP2D6, and CYP3A4 were found to be capable of catalyzing the demethylenation of racemic MDEA and its single enantiomers. The kinetic parameters could not be determined for N-deethylation by CYP3A5 and for demethylenation by CYP1A2 because of insufficient activity of these isozymes with respect to these reactions. The resulting $K_m$ and $V_{max}$ values of the other isozymes are listed in Table 1. Figure 2 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 total metabolite formation are shown in panels A-C, respectively. Most kinetic data followed the expected classical hyperbolic Michaelis-Menten kinetics as shown in supplemental Fig. S2a-d. Visual inspection of the data in these figures gave evidence of biphasic kinetics in some cases, which led to significantly better results for equation 2 (F-test, p<0.05). The corresponding Eadie-Hofstee plot
clearly confirmed this (data not shown). Hence, the respective kinetic parameters were estimated by fitting the data into equation 2 for a biphasic kinetic. The second enzyme component is represented by $\text{CL}_{\text{int},2} \times [S]$. The corresponding $V_{\text{max}1}/K_{m1}$ values are presented in Table 1.

Enantioselective phase I metabolism might be the most likely explanation for the enantioselective pharmacokinetics of racemic MDEA. Data from inhibition studies and with recombinant P450 (Maurer et al., 2000; Kreth et al., 2000) indicated that 1A2, 2B6, 2D6, and 3A4 are involved in the $N$-deethylation and/or demethylenation of MDEA. CYP2D6 expressed in E. coli was investigated towards racemic MDEA by Keizers et al. (Keizers et al., 2005) and the $K_m$ determined for DHEA formation was in the same range but lower than in our study (1.1 µM vs 2.8 µM) which might be explained by different expression systems and/or different incubation conditions, particularly incubation time which was rather long in the aforementioned study (10 min). In the present study, we have observed that DHEA formation by CYP2D6 was only linear in a time range up to 2.5 min. In addition, Keizer et al. did not observe formation of MDA after incubation of CYP2D6. The reason for this could be insufficient sensitivity of the used analytical procedure.

The study presented here is the first providing enantioselective enzyme kinetic data of $N$-deethylation and demethylenation of MDEA by ten recombinant P450s, which are the most relevant in human drug metabolism. CYP2C19 was among these enzymes which is known to be one of the five most abundant P450s involved in xenobiotic metabolism (Guengerich, 2005), but had so far not been considered in studies on MDEA metabolism.

The experiments were performed with racemic MDEA as consumed by recreational users as well as single MDEA enantiomers to check whether certain P450s specifically metabolize certain enantiomers and whether the enantiomers influence...
each others’ metabolism. Duration and protein content of all incubations in the experiments on enzyme kinetics 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.

As already discussed for MDMA in our previous paper (Meyer et al., 2008), there are inherent differences in the $K_m$ and $V_{max}$ values of the single enantiomer kinetics vs. the values of racemic MDEA in incubations with racemic MDEA. Briefly, in incubations of racemic MDEA, $R$- and $S$-MDEA are competitors for the limited number of active sites in the incubation mixture. Hence, at saturation, approximately half of the active sites are busy transforming $R$-MDEA while the other half is busy transforming $S$-MDEA. In incubations of single enantiomers, however, these enantiomers must occupy all active sites and hence their concentrations must be much higher as in the respective incubations of the racemate to reach saturation.

CYP2D6 turned out to be the isozyme with the highest affinity towards racemic MDEA and its enantiomers (Table 1). In addition, there is a generally higher affinity towards $S$-MDEA than $R$-MDEA concerning all involved isozymes. The obvious difference in the $K_m$ values of racemic MDEA and the respective enantiomers might be caused by interactions of $R$- and $S$-MDEA in incubations of the racemate. CYP2D6 also had the highest capacity for demethylenation of racemic MDEA and its enantiomers. The enzyme kinetic data reported here are in part different to those reported by Kreth et al. (Kreth et al., 2000). This can firstly be explained by the substrate concentrations used by Kreth et al. which were 200 µM and therefore far away from the concentrations expected as plasma concentration of recreational users as used in our study and secondly by the fact that some data were derived from inhibition experiments with supposedly specific chemical inhibitors in human liver microsomes rather than cDNA expressed single enzymes as described here.
The latter is the most probable reason, why the *in vivo* contribution of some isozymes (e.g. CYP1A2) was considerably overestimated by Kreth et al.

In the present study, we calculated the percentages of net clearance for substrate concentrations ranging from 1 to 10 µM according to (Meyer et al., 2008) to model the involvement of the studied P450s over the relevant concentration range (Fig. 2). At low substrate concentrations (1 µM), MDEA biotransformation was predominantly catalyzed by CYP2D6 (40-50%) and CYP3A4 (30-40%). Whereas, monitoring only the *N*-deethylation, relevance of CYP2D6 decreased to 10-20% and contribution of CYP3A4 increased to 60%. Also, at high substrate concentrations (up to 10 µM), the relevance of CYP2D6 in total biotransformation decreased and the other isozymes became increasingly important.

To confirm the role of CYP2D6, CYP2C19, and CYP3A4 in the most important step in MDEA metabolism which leads to the potentially toxic metabolite DHEA, inhibition experiments were performed using pHLM according to the described procedure (Meyer et al., 2008). Quinones might be formed *in vivo* via oxidation of DHEA and two major mechanisms of quinone induced cytotoxicity have been identified ##. Quinones are reduced to radicals by cellular reductases and these radicals undergo rapid autoxidation with formation of superoxide and H$_2$O$_2$. Additionally, quinones can react with cellular nucleophiles.

The inhibition 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 MDEA demethylation. Generally, the demethylation was inhibited significantly at both substrate concentrations. Quinidine had the strongest inhibition effects at low substrate concentrations whereas inhibition by MAB3A4 had the strongest effect at higher concentrations (supplemental data, Fig. S3). This is in
line with the calculated percentages of net clearance as shown in Fig. 2. The importance of CYP2D6 and CYP3A4 is critical from the toxicological point of view, because both isozymes can be inhibited by quite a number of different xenobiotics. This means that in case of co-ingestion, the metabolism of MDEA might be considerably reduced.

Marked enantioselectivity for the S-enantiomer \( \left( \frac{k_{m(R-MDMA)}}{k_{m(S-MDMA)}} > 1.5 \right) \) was observed for both metabolic steps by CYP2C19, for demethylation by CYP2D6 and for \( N \)-deethylation by CYP3A4. In addition, CYP2D6 showed a preference for \( R \)-MDMA with respect to deethylation.

Considering these findings along with the fact that demethylation is the major metabolic step of MDEA metabolism \( \textit{in vivo} \), the different pharmacokinetic properties of the MDEA enantiomers are therefore most likely attributable to enantioselective metabolism by various P450s. CYP2D6 and CYP3A4 should be most important in this context, because together they account for approximately 80% of net clearance especially at low substrate concentrations. This must be considered when trying to estimate the time of ingestion from enantiomer ratios in plasma as proposed for e.g. MDMA 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/CYP3A4 by co-ingested drugs. In addition, it must be considered that correlation of the presented \( \textit{in vitro} \) data with the \( \textit{in vivo} \) situation is not straightforward, because \( \textit{in vivo} \) DHEA 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 DHEA.
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References


Legends to the Figures

Fig. 1. The two main metabolic steps of R- and S-MDEA leading to the formation of the corresponding enantiomers of dihydroxyethylamphetamine (DHEA) and methylenedioxyamphetamine (MDA).

Fig. 2. Plots of calculated total net clearance vs. substrate concentration for the N-deethylation and demethylenation.
TABLE 1

Kinetic data for the two main metabolic steps of (R,S-)MDEA

Units used are: $V_{\text{max}}$, picomoles per minute per picomole; and $K_m$, micromolar.

<table>
<thead>
<tr>
<th>MDA formation</th>
<th>DHEA formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td><strong>R,S-MDEA</strong></td>
<td></td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>19 ± 0.6</td>
</tr>
<tr>
<td>CYP 2B6</td>
<td>23 ± 0.8</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>22 ± 0.9</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>3.3 ± 0.4 *</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>2.4 ± 0.4 *</td>
</tr>
<tr>
<td><strong>R-MDEA</strong> (racemic MDEA)</td>
<td></td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>10 ± 0.4</td>
</tr>
<tr>
<td>CYP 2B6</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>3.2 ± 0.2 *</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>2.1 ± 0.2 *</td>
</tr>
<tr>
<td><strong>S-MDEA</strong> (racemic MDEA)</td>
<td></td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>CYP 2B6</td>
<td>11 ± 0.7</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>12 ± 0.4</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>2.2 ± 0.2 *</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>3.8 ± 0.3 *</td>
</tr>
<tr>
<td><strong>R-MDEA</strong> (single enantiomers)</td>
<td></td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>13 ± 0.5</td>
</tr>
<tr>
<td>CYP 2B6</td>
<td>18 ± 1.2</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>15 ± 0.4</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>2.5 ± 0.5*</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>13 ± 2.0*</td>
</tr>
<tr>
<td><strong>S-MDEA</strong> (single enantiomers)</td>
<td></td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>16 ± 0.8</td>
</tr>
<tr>
<td>CYP 2B6</td>
<td>19 ± 0.8</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>8.7 ± 1.5*</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>3.2 ± 0.4*</td>
</tr>
</tbody>
</table>

* kinetic data estimated according to eq. 2

+Not determined (ND)
Figure 1

S-MDEA

R-MDEA

S-DHEA

S-MDA

R-DHEA

R-MDA