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Identification of the Human Cytochromes P450 Involved in the Oxidative Metabolism of "Ecstasy"-Related Designer Drugs

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ABSTRACT. The human cytochrome P450 (CYP) isozymes catalyzing the oxidative metabolism of the widely abused amphetamine derivatives MDMA (*N*-methyl-3,4-methylenedioxyamphetamine, "Ecstasy"), MDE (*N*-ethyl-3,4-methylenedioxyamphetamine, "Eve"), and MDA (3,4-methylenedioxyamphetamine) were identified. Using a simplified non-extractive reversed-phase HPLC assay with fluorescence detection, biphasic Michaelis–Menten kinetics were obtained for formation of all three dihydroxyamphetamines in liver microsomes from a *CYP2D6* extensive metabolizer subject. In contrast, no low K_m component was detectable in microsomes from a poor metabolizer subject. Additional specific probes for CYP2D6 further confirmed this isozyme as the exclusive low K_m component for demethylenation. P450-selective inhibitors applied to CYP2D6-inhibited microsomes and activity measurements in a series of recombinant P450s suggested CYP1A2 as the major high K_m component with contributions by CYP2B6 and CYP3A4. Moreover, the relative CYP1A2 content of a panel of 12 human livers was weakly but significantly correlated to the high K_m demethylenase activity (Spearman rank correlation coefficient [r_s] = 0.58; $P < 0.05$). Microsomal maximal velocities for N-dealkylation were at least 7-fold lower than for demethylenation and were characterized by apparently monophasic kinetics. The most important isozyme for this reaction appeared to be CYP2B6, the microsomal content of which was found to be strongly correlated to N-deethylation of MDE (r_s = 0.90; $P < 0.001$). We conclude that, in addition to CP2D6 as the sole high-affinity demethylenase, several other P450 isozymes have the capacity to contribute to microsomal oxidative metabolism of methylenedioxyamphetamines. This may be of particular importance in individuals genetically lacking functional CYP2D6. BIOCHEM PHARMACOL **59**;12:1563–1571, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. CYP2D6; cytochrome P450; designer drugs; ecstasy; MDE; MDMA

MDMA§ (street name "Ecstasy", "Adam"), MDE ("Eve"), and MDA ("love pills") are methylenedioxyamphetamine-based designer drugs which are among the most commonly abused illicit drugs in the Western world. Their specific psychotropic effects, described as increased communicativeness, empathy, and self-knowledge, distinguish them from typical stimulants and hallucinogens and lead to their designation as "entactogens", a new entity of psychotropic agents [1]. Pharmacologically, they affect both the dopamine and serotonin systems primarily through indirect monoaminergic mechanisms, although serotoninergic effects appear to be more prominent [2]. For example, continued use of MDMA leads to chronic serotonin depletion and ultimately to irreversible damage of serotoninergic neurons in laboratory animals [3–5] as well as in human long-term users, as recently demonstrated by positron emission tomography studies [6] and further *in vivo* investigations [7, 8]. Although abusers commonly believe in the innocuousness of these drugs, acute somatic adverse effects such as hyperthermia, cardiovascular complications, renal and hepatic failure, occasionally even leading to death [9, 10], as well as unwanted neuropsychiatric reactions [11, 12] are increasingly being reported. As unexpected effects of drugs are often related to their metabolism, the question arises as to what impact individual drug metabolism capacity may have on the adverse and neurotoxic effects of these drugs.

The principal metabolic pathways of methylenedioxyamphetamine drugs (Fig. 1) have been elucidated and over a dozen metabolites identified in animals and in humans. The major oxidative degradation pathway of methylenedioxyamphetamines involves demethylenation, leading to reactive catechol metabolites which are further converted by methylation, glucuronidation, and sulfation be-

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§ *Abbreviations:* anti-LKM1, anti-liver/kidney microsome antibody type 1; CYP, cytochrome P450; EM, *CYP2D6* extensive metabolizer; FMO-3, flavin-containing monooxygenase 3; MDA, 3,4-methylenedioxyamphetamine; MDE, *N*-ethyl-3,4-methylenedioxyamphetamine; MDMA, *N*-methyl-3,4-methylenedioxyamphetamine; PM, *CYP2D6* poor metabolizer; and r_s, Spearman rank correlation coefficient.

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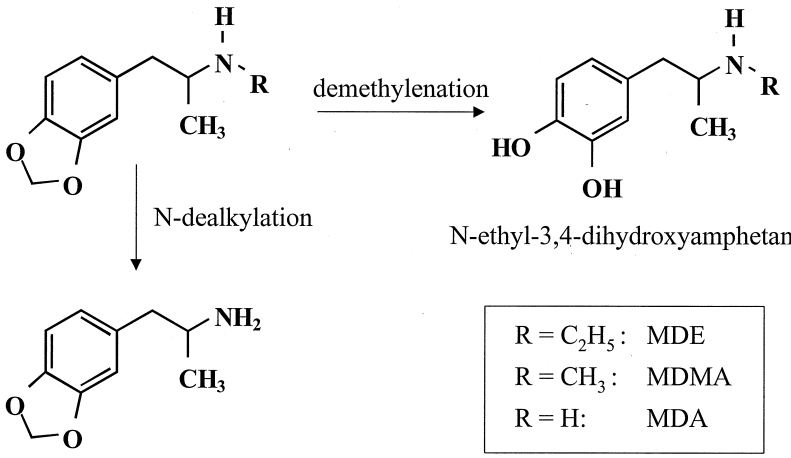

FIG. 1. Major pathways of oxidative microsomal metabolism of N-substituted methylenedioxyamphetamines.

fore they are excreted [13–15]. A parallel side-chain degradation pathway is initiated by N-dealkylation, first leading to the corresponding primary amines, which are then oxidatively deaminated to the final benzoic acid derivatives [15]. A third route via aromatic hydroxylation has been proposed to result in the production of neurotoxic trihydroxyamphetamines based on *in vitro* studies [16]. Kumagai *et al.* [17] investigated the demethylenation of MDMA kinetically in rat liver microsomes and provided evidence for the involvement of multiple enzymes, including one or more phenobarbital-inducible cytochrome P450 forms with high K_m and, in particular, the CYP2D subfamily as the low K_m component. Subsequently, it was shown that the homologous human CYP2D6 demethylenates MDMA with low K_m in a recombinant yeast system and that this isozyme contributes to human liver microsomal activity [18, 19]. However, the metabolic pathways have not been studied in detail in human liver microsomes, and other enzymes participating in the demethylenation and N-dealkylation reactions remain unidentified.

The aim of this study was, therefore, to systematically investigate the enzymology of the demethylenation and N-dealkylation pathways of ecstasy-related drugs in human liver microsomes. The three related drugs MDMA, MDE, and MDA were studied comparatively. The major enzymes contributing to the two pathways were identified, and the important role CYP2D6 plays in the demethylenation of all three drugs was further established. These data form a better basis to assess risk factors of entactogen abuse, such as genetic polymorphism and drug interactions.

MATERIALS AND METHODS

Chemicals

MDE, MDMA, MDA, and the metabolites *N*-ethyl-3,4-dihydroxyamphetamine and 3,4-dihydroxyamphetamine were synthesized as racemic mixtures as previously described [15, 20]. Furafylline was a kind gift from U. Fuhr (University of Cologne, Germany). Sulfaphenazole was obtained from Ciba-Geigy and ketoconazole from Janssen. Diethyldithiocarbamate, orphenadrine, and troleandomycin were purchased from Sigma. All other reagents were of the highest grade available from commercial sources.

Human Liver Microsomes and Recombinant Enzymes

Microsomes were prepared from a kidney donor liver bank and phenotyped *in vitro* as *CYP2D6* EM or PM using cumene hydroperoxide-mediated bufuralol 1′-hydroxylation and Western blotting as described [21]. Recombinant CYP2D6 was prepared in insect cells and reconstituted with NADPH:cytochrome P450 reductase as described [22]. All other CYPs were purchased from Gentest Corp. as "supersomes" co-expressed with NADPH:cytochrome P450 reductase and, where indicated, with cytochrome b_5. Information on protein concentrations and CYP450 contents was supplied by the manufacturer.

Incubation Conditions

To define optimal conditions for incubation and HPLC analysis, human liver microsomes (0.1–2.5 mg/mL) were incubated with MDE (0.1–2000 μM) and NADPH in the presence or absence of superoxide dismutase [14] for up to 1 hr. The final conditions, for which linearity of product formation with respect to time and protein had been shown, were as follows: 50 μg of microsomal protein was incubated in 100 mM sodium phosphate buffer, pH 7.4, containing MDE, MDMA, or MDA at the given concentration, 10 units of superoxide dismutase, and 1 mM NADPH in a final volume of 100 μL. Reactions were started by addition of NADPH and terminated by adding 10 μL of 70% perchloric acid. Incubation times were 15 min for N-dealkylation and 2 min for demethylenation, respectively, at 37°. Incubations with recombinant enzymes were performed under identical conditions at indicated substrate concentrations with 5 pmol of cytochrome P450 or 20 μg FMO-3. Reaction mixtures containing diethyldithiocarbamate, furafylline, or orphenadrine as chemical inhibitor were preincubated in the presence of NADPH for 5 min at 37°, and the reactions were started by the addition

of substrate. The other inhibitor substances were not preincubated. Bufuralol 1′-hydroxylation was determined as described [21].

Analysis of Metabolites

An HPLC method with fluorescence detection recently developed in our laboratory to quantify MDE and its metabolites in human plasma [23] was modified to analyze metabolites in *in vitro* incubations. To avoid an extraction step, protein was precipitated by the addition of perchloric acid. It was shown that all metabolites quantified in this study were stable under these conditions for at least 72 hr. The incubation mixtures were then centrifuged at 9300 *g* for 5 min, and the $HClO_4$ supernatants were directly injected into the HPLC system consisting of a LiChroCart Superspher 60 RP-select B column, 5 μm, 250 × 4 mm connected to a LaChrom fluorescence detector L-7480 (all HPLC equipment obtained from Merck). The mobile phase consisted of 20 mM potassium phosphate buffer, pH 3.0, and acetonitrile at varying concentrations (8% v/v for 3,4-dihydroxyamphetamine, 10% v/v for *N*-methyl-3,4-dihydroxyamphetamine, and 12% v/v for *N*-ethyl-3,4-dihydroxyamphetamine and MDA separation). The wavelengths for fluorescence detection were set at 286 nm (excitation) and 322 nm (emission) for detection of all metabolites. Under these conditions, retention times were 6.0 min for the dihydroxy metabolites and 17.5 min for MDA. Quantification was performed using the authentic substances as external standards except for *N*-methyl-3,4-dihydroxyamphetamine, which was quantified using *N*-ethyl-3,4-dihydroxyamphetamine as standard.

Antibodies and Western Blotting

Monoclonal antibody 114 was used for Western blot detection of CYP2D6, and previously characterized strongly inhibitory anti-LKM1 were used for CYP2D6 immunoinhibition studies [24]. At the concentration used in the experiments (1:200 of an ammonium sulfate fraction prepared from human serum), this preparation inhibited greater than 95% of MDE (5 μM) demethylenation. Antibodies to CYP1A2, CYP2C8, CYP2E1, and CYP3A4 were kindly provided by Dr. Philippe Beaune, Paris [25]. Under the conditions used, anti-CYP2C8 recognized both CYP2C8 and CYP2C9, while anti-CYP3A4 recognized both CYP3A4 and CYP3A5 in a single band. The anti-CYP2B6 antibody "WB-2B6" was purchased from Gentest Corp. Recombinant enzymes were co-analyzed on the same gels to identify the correct bands, because some of the antibodies recognized several bands. Secondary antibodies were obtained as standard reagents from common commercial sources. Western blotting was performed according to standard procedures using SDS–PAGE (10% polyacrylamide gels) with 25 μg of microsomal protein per lane. The ECL (enhanced chemiluminescence) system (Amersham Life Science) was used for detection. Band intensities on films were densitometrically analyzed with an Elscript 400 optical scanner (Hirschmann).

Mathematical Methods

Kinetic analyses were performed by non-linear least square regression analysis using GraphPad Prism 2.0 (GraphPad Software Inc.). In view of the unknown distribution of the data, the non-parametric Spearman rank correlation coefficient r_s was calculated to compare the data obtained with the human liver samples. Multiple regression analysis was performed using Instat (GraphPad Software Inc.).

RESULTS

Kinetic Studies in Human Liver Microsomes

Since earlier studies had not indicated pronounced stereospecific metabolism of MDMA, we used racemic mixtures throughout this study [18]. Detailed kinetic analyses were performed with liver microsomes previously characterized *in vitro* to be derived from an EM and a PM subject. This had been shown by the presence (EM) or absence (PM) of CYP2D6 activity, determined as cumene hydroperoxide-mediated bufuralol 1′-hydroxylation, and the presence or absence of CYP2D6 protein, as recognized by the specific monoclonal antibody 114 [21]. The demethylenation of all three amphetamines followed clearly biphasic Michaelis–Menten kinetics in EM liver microsomes, as shown in Eadie–Hofstee plots in Fig. 2. Over a substrate concentration range of 0.4–1200 μM, the kinetic data could be best modeled assuming a high- and a low-affinity demethylenase with K_m values in the low and upper micromolar range, respectively (Table 1). The high-affinity K_m values for the N-substituted MDMA and MDE were 4- to 5-fold lower than that for MDA. V_{max} of the low-affinity demethylenase was 3- to 5-fold higher than that of the high-affinity component of the same liver (Table 1). In contrast, PM microsomes completely lacked the high-affinity/low-capacity component, and the fitted kinetic parameters were in the same range as the low-affinity/high-capacity component of EM microsomes (Fig. 2A, Table 1).

The N-dealkylation of MDE and MDMA, resulting in production of MDA, was apparently monophasic in microsomes of EM and PM subjects, and the data were best fitted to a one-site binding model with K_m values in the upper micromolar range (Fig. 2B and Table 1). However, in one case (N-dealkylation of MDMA), a two-site binding model with K_{m1} = 82 μM and K_{m2} = 3.06 mM gave a slightly better fit, indicating that more than one enzyme may participate in this reaction. The V_{max} values for N-dealkylation reactions were between 7- and over 40-fold lower than those for the corresponding demethylenations (Table 1).

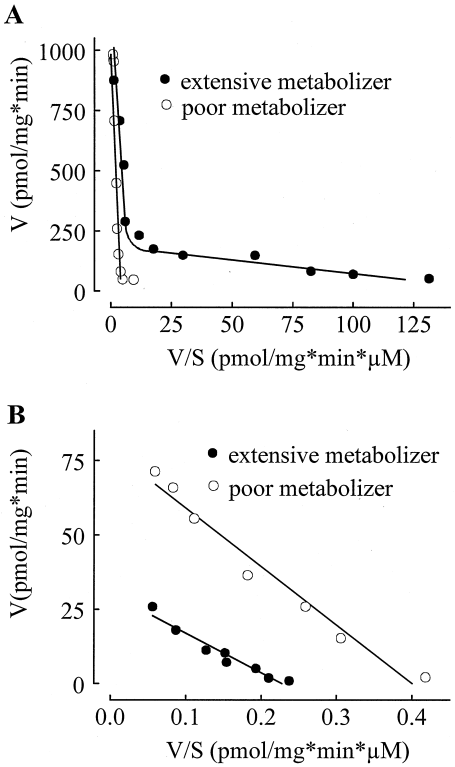

FIG. 2. Eadie–Hofstee plots for the formation of (A) N-ethyl-3,4-dihydroxyamphetamine from MDE and (B) MDA from MDE in liver microsomes from a CYP2D6 extensive and a CYP2D6 poor metabolizer. MDE (0.4–1200 μM) was incubated with microsomal protein. N-Ethyl-3,4-dihydroxyamphetamine (A) or MDA (B) was quantitated by HPLC and fluorescence detection. Data points represent the means of duplicate incubations. The calculated kinetic parameters are summarized in Table 1.

Investigation of High-Affinity Demethylenase

As expected, the high-affinity demethylenase observed in EM microsomes was highly sensitive to the potent CYP2D6 inhibitor, quinidine, and K_i values of 0.46, 0.20, and 0.12 μM were determined for the demethylenation of MDE, MDMA, and MDA, respectively. In contrast, furafylline (5 μM), sulfaphenazole (5 μM), diethyldithiocarbamate (20 μM), ketoconazole (10 μM), and troleandomycin (300 μM) had no or only minor effects on the demethylenation at 10-μM substrate concentration (data not shown). Anti-LKM1 autoantibodies, which potently and specifically inhibit CYP2D6, also decreased the demethylenation by over 95% with 5 μM MDE as substrate, whereas no significant inhibition was observed for the demethylenation in PM microsomes or for the N-dealkylation reaction (data not shown).

In microsomes of 12 human livers, formation of dihydroxy and N-dealkylated metabolites was quantified at low (5 μM) and high (200 μM) substrate concentrations, and activities were correlated to each other and to the relative content of several P450 isozymes determined by Western blotting (Table 2). At low substrate concentration, demethylenase activities were highly correlated to each other, to the relative CYP2D6 content, as well as to the CYP2D6-specific bufuralol 1′-hydroxylase activity (Table 2).

Investigation of Low-Affinity Demethylenase

To determine and compare CYP2D6-dependent and -independent demethylenase activities present in liver microsomes, *N*-ethyl-3,4-dihydroxyamphetamine formation from MDE was individually measured in the 12 microsomal samples in the absence and presence of anti-LKM1 at 200-μM substrate concentration (Fig. 3). The contribution by enzymes other than CYP2D6 varied between about 30% and almost 100% of the total microsomal activity at higher substrate concentration. To minimize effects related to variability, a pool of liver microsomes from three EM subjects was prepared and preincubated with anti-LKM1 autoantibodies to block CYP2D6 activity. Furafylline, a specific mechanism-based inhibitor of CYP1A2, was the most effective inhibitor of MDE demethylenation in this pool, followed by orphenadrine, proposed to be a selective inhibitor of CYP2B6 (Fig. 4). The CYP3A-selective inhib-

TABLE 1. Summary of apparent kinetic parameters for the demethylenation and N-dealkylation reactions in human liver microsomes

Demethylenation				N-dealkylation	
CYP2D6 extensive metabolizer					
MDA	MDMA	MDE		MDMA	MDE
11.6 ± 5.4	2.2 ± 1.6	2.6 ± 1.4	K_{m1} (μM)	—	—
167 ± 37	223 ± 54	241 ± 41	V_{max1} (pmol/mg · min)	—	—
1277 ± 287	377 ± 177	602 ± 185	K_{m2} (μM)	1111 ± 169	791 ± 66
825 ± 403	745 ± 85	1261 ± 130	V_{max2} (pmol/mg · min)	138 ± 12	34 ± 1.6
CYP2D6 poor metabolizer					
594 ± 54	1460 ± 293	476 ± 93	K_m (μM)	733 ± 25	356 ± 53
883 ± 37	2159 ± 274	2045 ± 195	V_{max} (pmol/mg · min)	104 ± 1.7	63 ± 3.5

A two-enzyme model was applied to the demethylenation, which resulted in clearly biphasic Eadie–Hofstee plots (Fig. 2). In this case, K_{m1} and V_{max1} refer to Michaelis–Menten parameters of the high-affinity component, whereas K_{m2} and V_{max2} refer to the low-affinity component. Values are means and standard errors (SE) obtained by non-linear least square regression analysis of duplicate data points obtained at substrate concentrations between 0.4 and 1200 μM.

TABLE 2. Comparison of various enzyme activities and relative contents for individual CYPs determined by Western blotting in a series of 12 human liver microsome samples

Parameter 1	Parameter 2	r_s
Demethylenation		
MDE (5 μM)	CYP2D6	0.89*
MDMA (5 μM)	CYP2D6	0.83*
MDA (5 μM)	CYP2D6	0.94*
MDE (5 μM)	MDMA demethylenation (5 μM)	0.94*
MDE (5 μM)	MDA demethylenation (5 μM)	0.97*
MDMA (5 μM)	MDA demethylenation (5 μM)	0.85*
MDE (5 μM)	Bufuralol-1′hydroxylation	0.98*
MDMA (5 μM)	Bufuralol-1′hydroxylation	0.91*
MDA (5 μM)	Bufuralol-1′hydroxylation	0.95*
Demethylenation, CYP2D6 inhibited by anti-LKM1		
MDE (200 μM)	CYP1A2	0.58†
MDE (200 μM)	CYP2B6	0.31
MDE (200 μM)	CYP2C8+9	0.10
MDE (200 μM)	CYP2D6	0.01
MDE (200 μM)	CYP2E1	0.44
MDE (200 μM)	CYP3A4+5	0.46
MDE (200 μM)	MDE demethylenation (5 μM)	0.10
N-dealkylation		
MDE (200 μM)	CYP1A2	0.48
MDE (200 μM)	CYP2B6	0.90*
MDE (200 μM)	CYP2C8+9	0.15
MDE (200 μM)	CYP2D6	0.37
MDE (200 μM)	CYP2E1	0.27
MDE (200 μM)	CYP3A4+5	0.50
MDE (200 μM)	MDMA N-dealkylation (200 μM)	0.97*

For correlations, the Spearman rank correlation coefficient r_s was calculated.
* $P < 0.001$.
† $P < 0.05$.

itors ketoconazole and troleandomycin exhibited less but still significant inhibition, which was much more pronounced with MDA and MDMA (51% and 43% inhibition with troleandomycin, respectively).

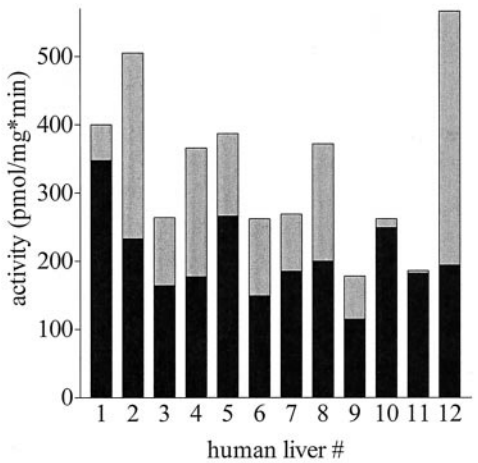

FIG. 3. MDE demethylenase activities of 12 human liver microsome samples. Enzymatic activities were determined at 200 μM MDE in the absence (complete bars) and presence (lower bar segment) of LKM1 autoantibodies to block greater than 95% of the activity of CYP2D6. Liver sample 11 was shown by Western blot analysis to be deficient in CYP2D6, while all other samples were shown to contain immunodetectable CYP2D6. Bars represent the means of duplicate incubations.

The principal catalytic ability of human P450s to demethylenate methylenedioxyamphetamines was investigated using a series of recombinant CYPs co-expressed in insect cell membranes with CYP reductase and in some cases with cytochrome b_5. Of 13 different recombinant enzyme preparations, CYP1A2, CYP3A4, and CYP1A1 showed the highest specific activities for MDE demethylenation at 200-μM substrate concentration, followed by CYP2D6 (Fig. 5). Other tested P450s had measurable but much lower activities. Interestingly, CYP3A4 was almost inactive in the absence of co-expressed cytochrome b_5. NADH (1 mM) added to microsomal reaction mixtures did, however, not increase activity for demethylenation. FMO-3, the major human hepatic flavin-containing monooxygenase form, had almost no measurable activity.

The MDE (200 μM) demethylenase activities determined in the panel of anti-LKM1-inhibited microsomes were moderately but significantly ($P < 0.05$) correlated to the relative levels of CYP1A2 (Table 2; Fig. 6A). The correlations to the contents of CYP3A4, CYP2B6, and CYP2E1 were still weaker and did not reach significance.

Investigation of N-dealkylation

Several of the inhibitors were found to inhibit MDE deethylation (Fig. 7). The most pronounced effect was again achieved by furafylline, followed by orphenadrine,

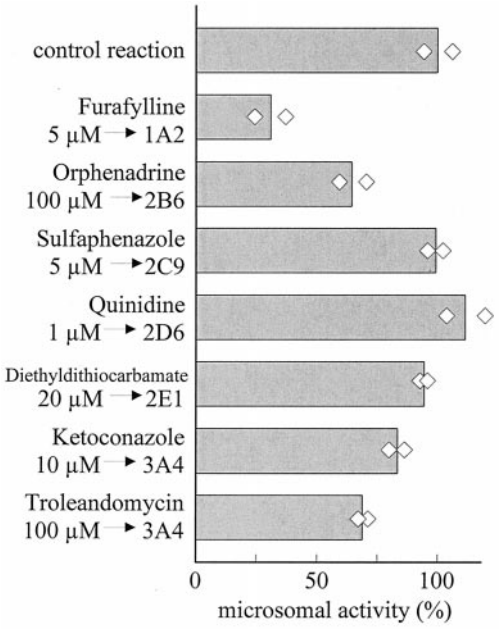

FIG. 4. Effect of CYP isoform-selective inhibitors on the low-affinity component of MDE demethylenase. Pooled liver microsomes from three extensive metabolizers were preincubated with anti-LKM1 autoantibodies to block CYP2D6. Activities were then determined with 200 μM MDE at the indicated inhibitor concentrations and expressed as percent of control activity (100%, no inhibitor present). The results of two independent incubations are represented by diamonds, and the means are represented by bars.

which inhibited between 25 and 63% at 100 and 500 μM, respectively (Fig. 7). Ketoconazole (5 μM) and troleandomycin (100 μM) caused less but still significant inhibition. In the recombinant system, CYP1A1, CYP1A2, and CYP2B6 displayed equally high turnover numbers for N-dealkylation of MDE (Fig. 5). All other enzymes, including

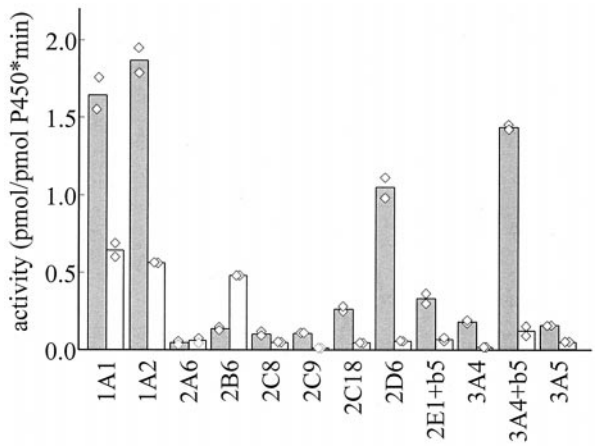

FIG. 5. Rates of demethylenation (filled bars) and N-deethylation (open bars) of MDE by recombinant human CYP isozymes. Five picomoles of recombinant human P450 isozyme co-expressed with NADPH:P450 reductase and, where indicated, with cytochrome b_5, was incubated with MDE (200 μM). N-Ethyl-3,4-dihydroxyamphetamine or MDA was quantitated by HPLC. The results of two independent incubations are represented by diamonds, and the means are represented by bars.

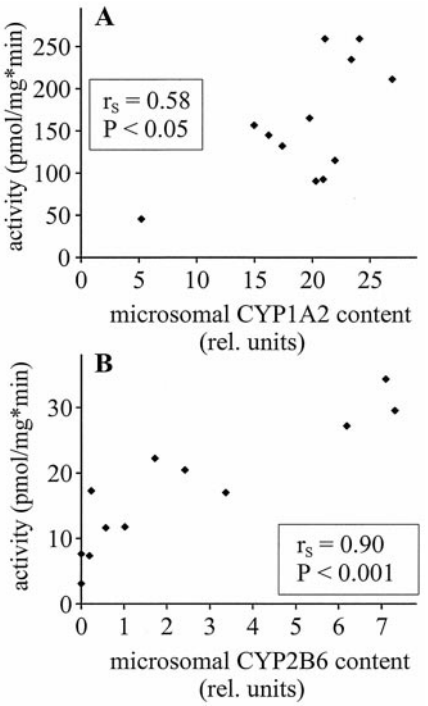

FIG. 6. (A) Comparison of low-affinity MDE demethylenase activity and relative content of CYP1A2 in 12 human liver microsome samples. Microsomes were preincubated with anti-LKM1 antibodies (dilution 1:200) to inhibit CYP2D6. Rates for formation of *N*-ethyl-3,4-dihydroxyamphetamine (pmol/mg × min) were determined at 200 μM DME and compared to relative levels of CYP1A2 determined by Western blotting. The Spearman rank correlation coefficient r_s was calculated (Table 2). Data points are means of duplicate incubations. (B) Comparison of N-dealkylase activity and relative content of CYP2B6 in 12 human liver microsome samples. Rates for MDA formation were determined at 200 μM MDE and compared to relative levels of CYP2B6 determined by Western blotting. The Spearman rank correlation coefficient r_s was calculated (Table 2). Data points are means of duplicate incubations.

FMO-3, had much lower activities. Except for CYP2B6, the turnover numbers for N-dealkylation were severalfold lower than for demethylenation and on average reflected the microsomal activity ratios (Table 1). Comparison of N-dealkylation activities with relative P450 contents in the same 12 liver samples used above revealed the best and the only significant correlation to the CYP2B6 content (r_s = 0.90; $P < 0.001$). CYP2B6 varied more than 15-fold among the 12 livers and was almost undetectable in two livers (Fig. 6B). The weaker correlations to CYP3A4 (r_s = 0.50; $P = 0.05$) and CYP1A2 (r_s = 0.48; $P = 0.06$) showed a trend towards significance, in agreement with partial contributions by these enzymes. However, when multiple regression analysis was performed, the only significant contribution to the total liver activity for N-deethylation was made by CYP2B6 ($P < 0.01$). There was a strong correlation between the microsomal N-dealkylation activities for MDE and MDMA, indicating that both are processed by the same enzymes in human liver (Table 2).

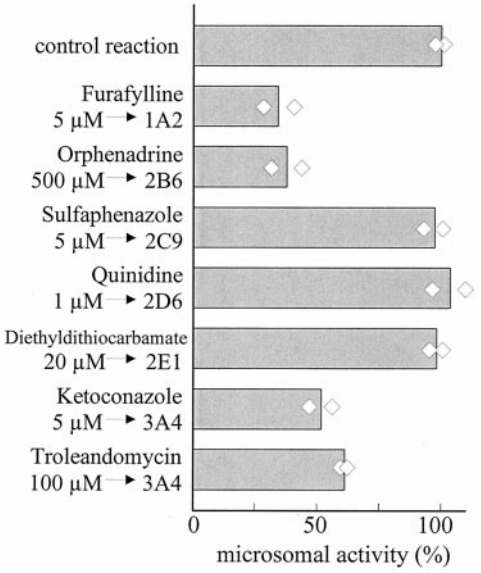

FIG. 7. Effect of CYP isoform-selective inhibitors on microsomal N-deethylation of MDE. Liver microsomes were incubated with 200 μM MDE and the indicated inhibitors. The resulting activity was expressed as percent of control activity (100%, no inhibitor present). The results of two independent incubations are represented by diamonds, and the means by bars.

DISCUSSION

Kinetic analysis of the demethylenation reaction revealed clearly biphasic kinetics in liver microsomes derived from a genetic *CYP2D6* extensive metabolizer individual with similar low K_m/low-capacity and high K_m/high-capacity components for all three drug derivatives. The apparent K_m values of the two N-substituted drugs in microsomes were almost identical to the K_m values of (+)-MDMA (1.72 μM) and (−)-MDMA (2.90 μM) previously determined in CYP2D6-expressing yeast microsomes [18]. Taken together, these first kinetic data on the metabolism of methylenedioxyamphetamines in human liver microsomes together with the additional experiments of this study suggest that CYP2D6 is the only low K_m component for the demethylenation of all three methylenedioxyamphetamines.

At higher substrate concentration, other enzymes with higher K_m contribute substantially to the total microsomal activity. This was shown by measuring MDE demethylenase activity at 200-μM substrate concentration in the microsome panel after complete inhibition of CYP2D6 with anti-LKM1 antibodies. Between 30 and 100% of the total microsomal activities were not affected by this treatment and must therefore be attributed to other enzymes. In fact, CYP2D6 appears to be relatively unimportant for the total activity at this substrate concentration, as indicated by the lack of correlation with the CYP2D6 content (Table 2). Taken together, the experimental results suggest that CYP1A2 constitutes the most important high K_m enzyme. Furafylline, a mechanism-based inhibitor with rather reliable selectivity for CYP1A2 [26], inhibited about 70% of the CYP2D6-independent demethylenation of MDE in a microsomal protein pool. Furthermore, CYP1A2 was the only CYP to which the activities of the 12 microsome samples were significantly correlated. The rather weak correlation obtained ($r_s = 0.58$; $P < 0.05$) reflects the contribution of additional isozymes.

In the recombinant system, CYP1A2 had the highest turnover number, although similar turnover numbers were also observed with recombinant CYP1A1, CYP2D6, and CYP3A4 co-expressed with cytochrome b_5. CYP1A1 is expressed at very low levels in liver [27], and we were in fact not able to clearly detect it in our samples. However, since it is readily inducible, it may be expressed at higher levels in some individuals. The fact that methylenedioxyamphetamines were shown to be efficient substrates for CYP1A1 and CYP1A2 in this study raises the question as to whether these drugs themselves may be able to induce CYP1A1 and/or CYP1A2. This possibility, which may have important toxicological implications, is further supported by the fact that these drugs share the methylenedioxyphenyl structure with safrole and isosafrole, which are preferential inducers of CYP1A2 in mice and rats [28]. CYP3A4, on the other hand, is the predominant liver P450 isozyme, and its expression levels in human liver are approximately 2- to 5-fold higher than those of CYP1A2 [29]. Based on the inhibition data with ketoconazole and troleandomycin, its contribution to the total low-affinity MDE demethylenase activity can be estimated to be 25% at most, i.e. less than half of the CYP1A2-dependent activity. Based on recombinant turnover numbers and relative expression levels, one would, however, expect roughly opposite proportions. This discrepancy is most likely explained by the strong dependence of P450 activity on the supporting enzymes CYP reductase and cytochrome b_5, one that has been observed for many substrates of CYP3A4, complicating the establishment of quantitative relationships between recombinant turnover numbers and microsomal activity. A relative overexpression of cytochrome b_5 in the recombinant system versus liver may lead to artificially high turnover numbers.

In previous investigations, the N-dealkylated metabolite MDA was first described as an *in vivo* metabolite of MDMA in the rat, where it appeared to be a major metabolite [13], whereas it was clearly shown to be a minor metabolite of MDE in humans [15, 23]. In animal studies, MDA was shown to be equally effective to MDMA regarding effects on the serotoninergic system [30] and interestingly, it was described as the major metabolite in rat brain, in particular in the male SD rat [31]. MDA formation may thus be important for drug effect and toxicity, in particular if it occurs *in situ* in the brain. Kinetic experiments suggested that there is no high-affinity enzyme for N-dealkylation of MDE and MDMA in human liver, and the low V_{max} values agree with the relatively minor *in vivo* metabolite formation [23]. Inhibition data suggested that CYP1A2, CYP3A4, and CYP2B6 may all participate in N-deethylation of MDE. A strong correlation between activity and Western blot detection of enzymes was, however, only seen for CYP2B6 content ($r_s = 0.90$; $P < 0.0001$; Fig. 6B and Table 2). Two

livers had almost no detectable CYP2B6, and the CYP2B6 content of the other livers varied more than 15-fold. This high variability agrees with previous reports [32]. In the recombinant system, CYP1A1/1A2 had similar activities to those of CYP2B6. The lack of a significant correlation to CYP1A1/1A2 in the livers could mean that CYP2B6 makes a larger contribution to the liver P450 content than CYP1A1/1A2. However, this remains to be investigated.

Individuals who lack functional CYP2D6 due to a poor metabolizer genotype or individuals under therapy with one of the potent CYP2D6 inhibitors [33] lack the high-affinity component for the major pathway of MDMA, MDE, and MDA metabolism. It thus appears a reasonable and likely possibility that the *CYP2D6* PM genotype constitutes a genetic risk factor predisposing users of methylenedioxyamphetamines to severe adverse drug effects. Conversely, as the major neurotoxic metabolites appear to be generated via catechol metabolites, PM individuals may be protected from neurotoxic consequences. However, the fact that additional enzymes are able to perform the same metabolic steps makes predictions more difficult. For example, the fraction of drug metabolized by low-affinity enzymes would be increased if the drug accumulated in specific compartments. Indeed, it was shown that MDMA concentrations in rat brain exceed the plasma concentration almost 10-fold [31]. In addition, because high-affinity CYP2D6 is easily saturated, the other enzymes may become more important in determining the plasma concentration of methylenedioxyamphetamine metabolites.

There are few *in vivo* kinetic data for MDMA or related drugs in humans available. In a recent study, plasma samples collected over 4 hr from volunteers who had ingested a single dose of 140 mg of MDE in the course of a clinical study [34] were analyzed for MDE and the metabolites *N*-ethyl-4-hydroxy-3-methoxyamphetamine and MDA [23]. We obtained blood samples from most of these individuals and performed *CYP2D6* genotyping [35]. One individual, referred to as 'P6' (see Figs. 4, 6, and 8 of [23]) was shown to be homozygous for the nonfunctional *CYP2D6*4* allele, whereas five other volunteers had genotypes compatible with the EM phenotype. Among these six volunteers, individual P6 had the lowest area under the curve for the demethylenated metabolite *N*-ethyl-4-hydroxy-3-methoxyamphetamine, namely 665 ng/mL*hr, compared to an average of 1506 ± 390 ng/mL*hr for the other five participants. The maximal MDE plasma concentrations reached during the study did not differ between P6 (1.35 μM) and the other participants (1.24 μM ± 0.24 μM).

These first *in vivo* data which relate plasma levels of MDE metabolites to the *CYP2D6* genotype thus demonstrate the significance of the *CYP2D6* polymorphism for the metabolism of MDE and likely also MDMA. However, they also show that considerable metabolism via demethylenation does occur in the absence of functional CYP2D6 in a PM individual, demonstrating that other enzymes must be active *in vivo*. Thus, the *CYP2D6* PM genotype may not by itself constitute a substantial risk factor for the adverse effects of MDMA and MDE. In fact, it was recently shown for the first time that three patients with fatal MDMA intoxication were all extensive metabolizers [36]. Our *in vitro* data suggest that in addition to CYP2D6 deficiency, low levels or inhibition by drug interaction of the low-affinity enzymes CYP1A2, CYP2B6, and CYP3A4 may be responsible for adverse drug effects. Indeed, a fatal drug interaction was reported between MDMA and ritonavir, a strong inhibitor of CYP3A4 and CYP2D6 [37]. It appears likely, therefore, that a number of different factors contribute to the risk of adverse and toxic effects of this group of drugs.

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