The Role of Human UDP-glucuronyltransferases on the Formation of the Methyleneoxymethamphetamine (Ecstasy) Phase II Metabolites $R$- and $S$-3-methoxymethamphetamine 4-O-glucuronides

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Role of Human UGT on MDMA Phase II Metabolism

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Abbreviations used are: UGT, UDP-glucuronyltransferase; P450, cytochrome P450; COMT, catechol-O-methyltransferase; SULT, sulphotransferase; ICM, insect cell microsomes; HLM, pooled human liver microsomes; RLM, rat liver microsomes; IS, internal standard; MDMA, 3,4-methylenedioxymethamphetamine; HMMA, 4-hydroxy 3-methoxymethamphetamine; DHMA, dihydroxymethamphetamine; M6G, morphine-6-β-D-glucuronide; UDPGA, uridine 5’-diphospho-glucuronic acid; LC, liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; CID, collision induced dissociation; IDA, information-dependent acquisition; S-HFBPCl, S-heptafluorobutyrylprolyl chloride; GC, gas chromatography; NICI, negative-ion chemical ionization; SIM, selected ion monitoring, SPE, solid phase extraction
Abstract

Different pharmacokinetic properties have been observed for the two enantiomers of the entactogen 3,4-methylenedioxy-methamphetamine (MDMA), most probably due to enantioselective metabolism. The aim of the present work was to study the involvement of human UDP-glucuronyltransferase (UGT) isoforms in the glucuronidation of the enantiomers of its major metabolite 4-hydroxy 3-methoxymethamphetamine (HMMA). First, the reference standards of \( R \)- and \( S \)-HMMA \( O \)-glucuronide were synthesized semi-preparatively using the enzymes of rat liver microsomes, followed by isolation with semi-preparative HPLC and identification using mass spectrometry and NMR. Racemic HMMA was then incubated using heterologously expressed human UGTs and pooled human liver microsomes, and the glucuronides were quantified by liquid chromatography-linear ion trap-mass spectrometry. UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 were involved in the glucuronidation of HMMA. UGT2B15, UGT2B17 and HLM revealed classical Michaelis-Menten kinetics, whereas UGT1A9 and UGT2B7 showed sigmoidal curves and the respective Eadie Hofstee plots indicated autoactivation kinetics. UGT2B15 showed the highest affinity and activity. UGT2B15, UGT2B17 and HLM were not considerably enantioselective, but showed slight preferences for \( S \)-HMMA. Marked enantioselectivity could only be observed for UGT1A9 with respect to the \( S \)-enantiomer, and for UGT2B7 with respect to the \( R \)-enantiomer. In conclusion the \( O \)-glucuronidation of HMMA \textit{in vivo} should not be expected to be enantioselective and the different pharmacokinetic properties may not be caused directly by glucuronidation.
Introduction

*R,S*-3,4-Methylenedioxy-methamphetamine \([R,S\text{-MDMA}, N\text{-Methyl-1-(3,4-}
\text{methylenedioxyphenyl)propane-2-amine}], 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). The *S*-enantiomer of MDMA is
known to be more potent than the *R*-enantiomer in producing the distinctive
subjective effects that are characteristic for ecstasy. However, it can induce severe
acute toxic symptoms such as tachycardia, hypertension, hyperthermia, and
hepatotoxicity and severe or even fatal intoxications have been described (Fallon et
al., 1999; Kalant, 2001).

Concerning chronic toxicity, data from animal experiments strongly suggest that
these compounds can cause irreversible damage to serotonergic 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 could not
be excluded that these findings were (in part) attributable to concomitant abuse of
other drugs of abuse (Gouzoulis-Mayfrank and Daumann, 2006). *R*- and *S*-MDMA
also differ in their dose-response curves for changes in serotonergic function and
neurotoxicity and their *in vivo* kinetics are known to be different (Fallon et al., 1999;
Kalant, 2001; Kraemer and Maurer, 2002; Peters et al., 2003; Pizarro et al., 2004;
Peters et al., 2005).
In vivo studies with racemic MDMA revealed two main metabolic pathways. As shown in Fig. 1, one major pathway includes O-demethylenation to 3,4-dihydroxymethamphetamine [DHMA, N-Methyl-1-(3,4-dihydroxyphenyl)propane-2-amine], followed by O-methylation catalyzed by the catechol-O-methyltransferase (COMT) mainly to 4-hydroxy-3-methoxymethamphetamine [HMMA, N-Methyl-1-(4-hydroxy-3-methoxyphenyl)propane-2-amine] and conjugation by UDP-glucuronyltransferases (UGTs) to the two diastereomers R- and S-3-methoxymethamphetamine 4-O-glucuronides (R-/S-HMMA O-glucuronide) or by sulfotransferases (SULTs) to 3-methoxymethamphetamine 4-O-sulfate (Maurer, 1996; Maurer et al., 2000; de la Torre et al., 2004). In urine samples of recreational MDMA users, mainly conjugated HMMA can be detected (Shima et al., 2008). However, 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 and was observed for the cytochrome P450 (P450)-mediated phase I metabolism and for the COMT-catalyzed methylation (Meyer et al., 2008a; Meyer and Maurer, 2009).

Recently, human UGT enzymes involved in the glucuronidation of racemic HMMA were investigated and human UGT2B15 was identified as the only isoform with adequate enzyme activity towards the described reaction (Shoda et al., 2009). However, the authors did not take into consideration that two different diastereomeric glucuronides, namely R- and S-HMMA O-glucuronides, should be formed.

Concerning all these points, the question arises whether the glucuronidation of HMMA is enantioselective too and contributes also to the pharmacokinetic difference between the respective MDMA enantiomers. Therefore, the two diastereomeric O-
glucuronides of HMMA have to be enzymatically synthesized as reference standards using rat liver microsomes (RLM). The aim of the present study was to elucidate the human UGT isoforms involved in the formation of $R$- and $S$-HMMA O-glucuronides and to obtain enantioselective enzyme kinetic data for the relevant UGT isoforms.

**Materials and Methods**

Racemic HMMA·HCl was obtained from Lipomed (Bad Saeckingen, Germany) and morphine-6-$\beta$-D-glucuronide (M6G) from Sigma Aldrich (Steinheim, Germany). Single HMMA glucuronides were synthesized in the author's laboratory as described below. Sodium bicarbonate was obtained from Fluka (Buchs, Switzerland), uridine 5'-diphospho-glucuronic acid trisodium salt (UDPGA), D-saccharic acid 1,4-lactone (saccharolactone), alamethicin, and Brij58 from Sigma Aldrich, ZIC-HILIC solid phase extraction (SPE) cartridges (1g, 6 ml) from Sequant (Marl, Germany). Water and acetonitrile of HPLC grade were obtained from Thermo Fisher Scientific (Dreiech, Germany). All other chemicals and reagents used were from Merck (Darmstadt, Germany). The following microsomes were from BD Gentest (Woburn, MA) and delivered by NatuTec (Frankfurt/Main, Germany): baculovirus-infected insect cell microsomes (ICM; Supersomes) containing 5.0 mg/ml of human cDNA-expressed UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, UGT2B17, wild-type baculovirus-infected ICMs (control Supersomes), and pooled human liver microsomes (HLMs; 20 mg microsomal protein/ml). 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 grade available. The derivatization reagent $S$-
heptafluorobutyrylprolyl chloride (S-HFBPCl) was synthesized in the authors laboratory according to (Peters et al., 2002)

**Preparation of Rat Liver Microsomes**

Microsomes were prepared from rat liver by differential ultra-centrifugation (De Duve, 1971). RLM were prepared from eight frozen (-80°C) livers of male Wistar rats (Ch. River, Sulzflleck, Germany). Protein concentrations of the microsomes were determined according to the method of Lowry (Lowry et al., 1951).

**Enzymatic Synthesis and Extraction of the $R$- and $S$-HMMA Glucuronides**

The bioassay consisted of 2.5 mM racemic HMMA·HCl, 2 mg/ml rat liver microsomes, 5 mM MgCl₂, 5 mM saccharolactone and 100 µg/mg protein Brij58 in a total volume of 10 ml 100 mM phosphate buffer (pH 7.4). The reaction was started with the addition of 5 mM UDPGA. After 4.5 h of incubation at 37°C, the glucuronidation was terminated with 10 ml of acetonitrile and the mixture centrifuged at 10,000 g for 20 min. The supernatant containing the $R$- and $S$-HMMA O-glucuronides was diluted 1:20 with acetonitrile containing 0.1% formic acid and was transferred to SPE cartridges (Sequant ZIC-HILIC, 1 g, 6 ml) previously conditioned triply with 5 ml of water and 5 ml of acetonitrile. After pass-through of the supernatant, the cartridges were washed triply with 5 ml of acetonitrile and with 5 ml of an acetonitrile water mixture (90:10, v/v). The cartridges were eluted three times with 2.5 ml of an acetonitrile/water mixture (50:50, v/v) and the acetonitrile part of the eluate was evaporated under reduced pressure at room temperature.

**Separation of $R$- and $S$-HMMA Glucuronide**
The two diastereomeric compounds \(R\)- and \(S\)-HMMA O-glucuronide were separated using a Hewlett Packard (HP, Agilent, Waldbronn, Germany) Series 1050 semi-preparative HPLC system consisting of a pump and a variable wavelength detector (\(\lambda = 230\) nm). The stationary phase consisted of a Phenomenex (Aschaffenburg, Germany) Chirex 3012 column (250 x 4.6, 5 µm) and the mobile phase of a mixture of 20% 10 mM ammonium formate buffer acidified with 0.1% formic acid and 80% acetonitrile acidified with 0.1% formic acid at a flow rate of 1 ml/min. The eluates were separated in aliquots of 50 µl and the fractions with the two glucuronides were collected. The HPLC fractions containing the single diastereomers were diluted 1:1 with acetonitrile and submitted to solid phase extraction using ZIC-HILIC cartridges as described above. The cartridges were eluted three times with 2.5 ml of water and the combined eluates of each glucuronide were lyophilized.

**Confirmation of Configuration**

The elution order of the diastereomeric glucuronides on the Chirex 3012 column was determined using 100 µl of a solution of the isolated first eluting HMMA glucuronide. After dilution with 900 µl of water the pH was adjusted to pH 5.2 with acetic acid and the mixture was incubated at 50°C for 1.5 h with 100 µl of a mixture (100 000 Fishman units per ml) of glucuronidase (EC No. 3.2.1.31) and arylsulfatase (EC No. 3.1.6.1) from *Helix Pomatia L*. Afterwards 10 µl of this solution as well as 10 µl of a 10 µM solution of racemic HMMA·HCl were worked-up as described previously by (Meyer and Maurer, 2009). Briefly, the analytes were derivatized with \(S\)-HFBPCl and analyzed by GC-MS using negative-ion chemical ionization (NICI). Selected-ion monitoring (SIM) was performed with the ions \(m/z\) 487 and 781 for derivatized HMMA. Retention time of the isolated glucuronide after conjugate cleavage and
derivatization was compared to the retention times of derivatized R- and S-HMMA which were previously described by (Meyer and Maurer, 2009).

Identity and Purity Confirmation of the Isolated Glucuronides

For identity check, a small amount of each diastereomer was dissolved in water and analyzed by liquid chromatography-mass spectrometry (LC-MS) as described below. For structure confirmation by 1H-NMR, solutions of S-HMMA glucuronide (4 mg/0.5 ml) and R-HMMA glucuronide (2 mg/0.5 ml) were prepared in D2O. 1H-NMR spectra (500 MHz) were recorded on a Bruker (Bremen, Germany) DRX 500 at 300 K. The chemical shifts were given in δ values (ppm) relative to acetone at δ 2.22.

For purity check of the respective diastereomers, a small amount of each glucuronide was dissolved in water and analyzed by HPLC-UV using an HP 1050 series HPLC system with UV detection at 230 and 280 nm using a Merck LiChroCART Superspher 60 RP Select B column (2.0 x 125 mm, 5 µm) and a Phenomenex Chirex 3012 (250 x 4.6 mm, 5 µm) column. The mobile phase consisted of 10 mM ammonium formate buffer, acidified with 0.1% formic acid and acetonitrile acidified with 0.1% formic acid. The isocratic composition was 98:2 (v/v) with a flow rate of 0.5 ml/min and 20:80 (v/v) with a flow rate of 1 ml/min, respectively. The injection volume was 15 µl for analysis at 230 nm and 25 µl for analysis at 280 nm.

Microsomal Incubations

Incubation mixtures (final volume: 50 µl) consisted of 100 mM phosphate buffer (pH 7.4), 5 mM MgCl2, alamethicin (50 µg/mg protein), 0.25 to 1 mg/ml UGT-containing microsomes and substrate at 37°C. The substrate was added after dilution of a 50 mM aqueous stock solution in the above-mentioned phosphate buffer. Reactions were started by addition of 5 mM UDPGA and terminated with 50 µl of acetonitrile.
Initial Screening Studies

Incubations were performed with 500 µM R,S-HMMA and 1 mg/ml UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 for 30 min. Further composition of the incubation mixtures is described under microsomal incubations.

Kinetic Studies

The kinetic constants of HMMA O-glucuronidation were derived from incubations with an incubation time of 25 min (recombinant UGTs) and 15 min (HLM). The protein concentrations were 0.25 mg/ml for UGT2B15, 0.5 for UGT1A9, UGT2B7, and UGT2B15, and 1 mg/ml for HLM. These concentrations were within the linear range of the glucuronide formation.

Enzyme kinetic constants were estimated by non-linear curve-fitting using GraphPad Prism 5.00 software (San Diego, CA). The normalized initial velocity data from the enzyme kinetic studies were fitted either to the Michaelis-Menten equation (equation 1) where \( v \) is the initial velocity of the reaction, \( S \) the substrate concentration, \( V_{\text{max}} \) the maximal velocity and \( K_m \) the substrate concentration at half \( V_{\text{max}} \) or to the Hill equation (equation 2) where \( K_m \) is the substrate concentration at 0.5 \( V_{\text{max}} \) and \( n \) the Hill coefficient.

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

The best kinetic model was selected, considering the randomness of the residuals, the standard errors of the estimates and the correlation coefficients. Additionally, Eadie-Hofstee plots were used to check for allosteric kinetics.
Sample preparation for microsomal incubations

After termination of the incubation, 10 µl of the internal standard (M6G, 5 µM) were added to each incubation mixture, the samples were vortexed, centrifuged (10,000 g, 10 min), 100 µl of the supernatants transferred to autosampler vials, and 10 µl-aliquots injected into the LC-MS system.

LC-MS Conditions

The analytes were separated using an Accela LC system (Thermo Fisher Scientific, TF, Dreieich, Germany) consisting of a degasser, a quaternary pump and an autosampler coupled to a TF LXQ Linear Ion Trap MS system equipped with a heated electrospray ionization (ESI) source. The collected data were processed by TF Xcalibur 2.0.7.

The LC conditions were as follows: Phenomenex Chirex 3012 column (250 x 4.6 mm, 5 µm); column temperature, 35 °C; isocratic mobile phase consisted of 30% 10 mM aqueous ammonium formate buffer containing 0.1 % (v/v) formic acid and 70% acetonitrile containing 0.1 % (v/v) formic acid; flow rate, 0.5 ml/min for identity confirmation and 1.0 ml/min for quantification. The total run times were 25 min or 12 min, respectively.

The MS conditions were as follows: ESI, positive mode; sheath gas, nitrogen at flow rate of 40 (arbitrary units, AU); auxiliary gas, nitrogen at flow rate of 25 AU; vaporizer temperature, 300 °C; source voltage, 3.00 kV; ion transfer capillary temperature, 380 °C; capillary voltage, 9.0 V; tube lens voltage, 75 V. For identity confirmation, collision induced dissociation (CID)-MS/MS experiments were performed on
precursor ions selected from MS\(^1\) using information-dependent acquisition (IDA): MS\(^1\) was performed in the full scan (FS) mode (m/z 100-400). MS\(^2\) was performed in the IDA mode: MS\(^2\) on the most intense signal from MS\(^1\) with normalized collision energies of 35%. Other settings were as follows: minimum signal threshold: MS\(^1\), 50 counts; isolation width, 2.00 u; activation Q, 0.25; activation time, 30 ms; dynamic exclusion mode, repeat counts 2, repeat duration, 15 s, exclusion list, 50, exclusion duration, 15 s.

For quantification, the MS\(^1\) was performed in the full scan mode (m/z 100-500). Daughter ion scans (m/z 50-400 for HMMA, m/z 125-400 for M6G, m/z 100-400 for R-/S-HMMA O-glucuronide) were performed using CID with normalized collision energies of 35% on respective precursor ions m/z 196, m/z 462, and m/z 372. The following ions in MS\(^2\) were used for quantification: m/z 165 for HMMA, m/z 286 for M6G, and m/z 196 for R-/S-HMMA O-glucuronide. Calibration curves were constructed plotting peak area ratios (S-HMMA glucuronide and R-HMMA glucuronide vs I.S.) of spiked calibrators vs their concentrations (0.01, 0.1, 1.0, 5.0, 10, 25 and 50 µM). For quantification, a weighted (1/X) linear regression model was used.

Results

Enzymatic Synthesis of R- and S-HMMA Glucuronides

Initial experiments showed that RLM were the most efficient microsomal source for enzymatic synthesis of R- and S-HMMA O-glucuronides (data not shown). Optimal incubation conditions were evaluated (data not shown) and shown to be best for: 5 mM MgCl\(_2\), 5 mM saccharolactone, 100 µg/mg protein Brij58, 2 mg/ml microsomal protein, 5 mM UDPGA, 2.5 mM racemic HMMA and an incubation time of 4.5 h. Under these conditions HMMA was completely converted to the respective
diastereomeric glucuronides. The two diastereomers were separated on a Phenomenex Chirex 3012 chiral column (Fig. 2A) and the elution order of R- and S-HMMA O-glucuronide were determined after enzymatic cleavage of the fraction I, derivatization with S-HFBPCl and GC-MS analysis. The retention time was compared to derivatized racemic HMMA (Fig. 2B) as described by (Meyer and Maurer, 2009), where the S-enantiomer elutes before the R-enantiomer. Fig. 2C shows the worked-up fraction I indicating that it corresponded to S-HMMA O-glucuronide. The final yield was 4 mg for S-HMMA O-glucuronide (87 %) and 2 mg for R-HMMA O-glucuronide (44 %). The purity of the products determined by HPLC-UV (230 and 280 nm) was >90%.

The purified glucuronides were identified using LC-MS as shown in Fig. 3. The chromatograms of ion m/z 372 (M+H⁺, Fig. 3A, 3B) showed S- and R-HMMA O-glucuronide at a retention time of 14.6 and 15.6 min, respectively. The isolated R-HMMA O-glucuronide contained less than 4 % of the S-diastereomer. For further structure confirmation, ¹H-NMR spectra of both diastereomers were recorded with the following results: R-HMMA O-glucuronide: ¹H-NMR (500 MHz, D₂O) δ 7.150 (1H, d, J=8.3, H-5), 7.006 (1H, d, J=1.9, H-2), 6.882 (1H, dd, J=8.3, 1.9, H-6), 5.117 (1H, d, J=7.4, H-1´´), 3.880 (3H, s, OCH₃), 3.841 (1H, m, H-3´´), 3.680-3.560 (3H, m, H-2´´, H-4´´, H-5´´), 3.527 (1H, m, H-2´), 3.011 (1H, dd, J=14.0, 6.6, H-1´a), 2.889 (1H, dd, J=14.0, 7.6, H-1´b), 2.689 (3H, s, NCH₃), 1.282 (3H, d, J=6.7, H-3´); S-HMMA O-glucuronide: ¹H-NMR (500 MHz, D₂O) δ 7.153 (1H, d, J=8.3, H-5), 7.001 (1H, d, J=1.9, H-2), 6.885 (1H, dd, J=8.3, 1.9, H-6), 5.117 (1H, d, J=7.4, H-1´´), 3.880 (3H, s, OCH₃), 3.842 (1H, m, H-3´´), 3.680-3.560 (3H, m, H-2´´, H-4´´, H-5´´), 3.523 (1H, m, H-2´), 3.017 (1H, dd, J=14.0, 6.5, H-1´a), 2.879 (1H, dd, J=14.0, 7.8, H-1´b), 2.691 (3H, s, NCH₃), 1.275 (3H, d, J=6.7, H-3´). Significant differences in the chemical shifts for the R- and S-diastereomers could not be observed under the used
conditions. The 1H-NMR spectra were in agreement with those published by (Shima et al., 2007) after chemical synthesis of the mixture of $R$- and $S$-HMMA O-glucuronide.

**LC-MS for Diastereoselective Quantification**

The applied LC-MS conditions for quantification provided separation of HMMA, M6G, $R$- and $S$-HMMA O-glucuronide. The chosen target ions of the respective daughter spectra 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 internal standard). The method showed good linearity in a range of 0.01 – 50 µM $R$- and $S$-HMMA O-glucuronide ($R^2 > 0.998$). Matrix effect studies, comparing the peak areas of the glucuronides in neat standard solutions with those in spiked incubation mixtures containing the same concentrations of the glucuronides, gave no indication for ion suppression or enhancement (n=5).

**Initial activity screening**

As shown in Fig. 4, O-glucuronidation of racemic HMMA was catalyzed by UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT2B4, UGT2B7, UGT2B15, and UGT2B17.

**Kinetic Studies**

Twelve HMMA concentrations ranging from 0.01 to 20 mM were used in the kinetic analysis of UGTs and HLM. The $K_m$, $V_{max}$, $V_{max}/K_m$ values and the Hill coefficients $n$ are listed in Table 1. The data for UGT2B15, UGT2B17 and HLM followed the expected classical hyperbolic Michaelis-Menten kinetics as shown in Fig. 5. In contrast, UGT1A9 and UGT2B7 were best described by the Hill equation indicating
sigmoidal kinetics. Marked enantioselectivity ($V_{max}/k_m(S\text{-HMMA})/V_{max}/k_m(R\text{-HMMA}) > 1.5$ or $< 0.67$) was observed for UGT1A9 towards $S$-HMMA and UGT2B7 towards $R$-HMMA.

As shown in Fig. 7, HMMA glucuronidation in the range of 1-10 µM by UGT2B7, UGT2B17 and HLM was not considerably enantioselective, but there was a slight preference for the $S$-diastereomer. No preference could be observed for UGT2B15 and only UGT1A9 revealed a relevant selectivity for glucuronidation of $S$-HMMA.

**Discussion**

Reference standards of the HMMA glucuronides needed for enzyme kinetic studies were not commercially available. Therefore, the two diastereomers of HMMA O-glucuronide were synthesized enzymatically by RLM. Such bioassays for glucuronide synthesis are a versatile alternative to classical chemical synthesis, especially for mg quantities and have been used previously for a wide range of substrates (Soars et al., 2002; Uutela et al., 2009). HMMA O-glucuronide was previously synthesized by chemical synthesis by Shima et al. (Shima et al., 2007) and by enzymatic synthesis as well (Shoda et al., 2009), but the two diastereomers were not isolated separately. The final yield using chemical synthesis was 6%, which is somewhat low compared to the enzymatic synthesis presented by Shoda et al. (Shoda et al., 2009) with 71% and to the yields in this study with 87% for the $S$-diastereomer and 44% for the $R$-diastereomer.

The incubation conditions chosen for the initial screening of 12 human UGT isoforms were applicable for checking the general involvement of particular isoforms. The microsomes were not allowed to sit on ice for pre-incubation with alamethicin, because preliminary experiments showed no difference for this treatment as already described by Oleson and Court (Oleson and Court MH, 2008). In contrast to Shoda
et al. (Shoda et al., 2009), UGT1A10 was not found to be involved in the HMMA glucuronidation. One reason for this observation might be the uncommonly high substrate concentration used by Shoda et al. (Shoda et al., 2009). In the study presented here, enzyme kinetic data not only for UGT2B15, but also for UGT1A9, UGT2B7 and UGT2B17 were recorded. Because of their very low activity, kinetics of UGT1A1, UGT1A3, UGT1A8 and UGT2B4 could not be studied. Different concentrations of UDPGA (1.0-7.5 mM) were tested and 5 mM was a saturating concentration for all isoenzymes. Unfortunately, common protein concentration could not be chosen for all isoforms because the glucuronide formation by UGT1A9, UGT2B7 and UGT2B17 was too low to obtain glucuronide concentrations suitable for reliable quantification. Duration and protein content of all incubations were within the linear range of glucuronide formation (data not shown) and less than 20% of substrate was metabolized in all incubations.

The kinetic data for UGT2B15, UGT2B17 and HLM followed classical Michaelis-Menten plots (Fig. 5). In contrast, UGT1A9 and UGT2B7 revealed sigmoidal curves and the respective Eadie Hofstee plots (Fig. 6) indicated autoactivation kinetics (Hill coefficients are given in Table 1). Such autoactivation kinetics have been previously described for glucuronidation in HLM (Hutzler and Tracy, 2002), but also for recombinant UGTs, e.g. UGT2B7 towards 4-methylumbelliferone and 1-naphthol (Uchaipichat et al., 2008) and UGT1A10 towards dopamine glucuronidation (Itaaaho et al., 2009).

The $K_m$ values for the formation of $R$- and $S$-HMMA O-glucuronide by UGT2B15 were in a similar range as previously described for the mixture of both diastereomers (Shoda et al., 2009). In contrast, the respective $V_{max}$ values were approximately five fold higher in our study which could be explained by different incubation times and different activities of the used enzymes. According to our studies, the metabolite
formation was linear only up to 30 min, whereas Shoda et al. (Shoda et al., 2009) used a considerably longer incubation time (120 min). Measuring outside the linear range may lead to false estimation of the metabolite formation. UGT2B15 turned out to have the highest affinity as well as the highest capacity of all isoforms tested (Table 1). \( V_{\text{max}} \) values for UGT2B15 are approximately 10-fold higher than for the other isoforms. The respective \( K_m \) values for all isoforms seem to be very high compared to other glucuronidation reactions (Kiang et al., 2005; Mazur et al., 2009) and are not expected to be reached after intake of recreational user doses of MDMA leading to plasma concentrations of MDMA in the range of 1 \( \mu \text{M} \) (194 \( \mu \text{g/l} \)) (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 \( \mu \text{M} \) and higher in severe intoxications (Peters et al., 2003; Schifano, 2004). Therefore the glucuronidation activities of all relevant recombinant UGTs and HLM were interpolated in a range of 1-10 \( \mu \text{M} \) substrate concentration (Fig. 7), to consider the activities within a relevant concentration range showing metabolite formation activities comparable to previously published data for other substrates.

Comparing the \( V_{\text{max}}/K_m \) values (Table 1) to values described for other glucuronidations, these were in similar ranges (Court MH et al., 2002; Ohno et al., 2008; Itaaho et al., 2009; Mazur et al., 2009). The higher this value, the better the overall effectiveness of the respective reaction should be. This fact sounds reasonable, as the catalytic efficiency value is getting higher with increasing affinity (low \( K_m \)) and increasing velocity (high \( V_{\text{max}} \)).

Enantioselective metabolism is the most likely explanation for the enantioselective pharmacokinetics of MDMA. Enantioselectivity was observed for \( N \)-demethylation and demethylenation by cytochrome P450 2C19 (CYP2C19), demethylenation by CYP2D6 (Meyer et al., 2008b) and for the COMT-catalyzed methylation of DHMA to
HMMA, with a preference for the S-enantiomers (Meyer and Maurer, 2009). The study presented here is the first providing enantioselective enzyme kinetic data for MDMA phase II metabolism, the O-glucuronidation of HMMA.

In the case of glucuronidation marked enantioselectivity ($V_{\text{max}}/k_m(S\text{-HMMA})/V_{\text{max}}/k_m(R\text{-HMMA}) > 1.5$ or $< 0.67$) was only observed for UGT1A9 towards S-HMMA and UGT2B7 towards R-HMMA. When looking at the interpolated values in the range of 1-10 µM, no considerable enantioselectivity by UGT2B7, UGT2B17 and HLM was detected, but there was a slight preference for S-HMMA. No preference could be observed for UGT2B15 and only UGT1A9 revealed a relevant selectivity for glucuronidation of S-HMMA. Interestingly the marked enantioselectivity for UGT2B7 towards R-HMMA seemed not to be relevant in the low concentration range, in which S-HMMA was glucuronidated even with a slightly higher activity than R-HMMA. With respect to the presented data, the O-glucuronidation of HMMA in vivo should not be expected to be enantioselective. Nevertheless, the S-enantiomer of MDMA was eliminated at a higher rate than its R-enantiomer (Fallon et al., 1999; Kalant, 2001; Kraemer and Maurer, 2002; Peters et al., 2003; Pizarro et al., 2004; Peters et al., 2005). Considering the results of the presented study, this pharmacokinetic differences can mainly be explained by enantioselective demethylenation to DHMA and methylation to HMMA (Meyer et al., 2008c; Meyer and Maurer, 2009) or additionally by possible enantioselective sulfation.

UGT2B15 was the most abundant isoform involved in the glucuronidation of HMMA and is known to be polymorphically expressed. Two distinct allelic variants due to a single nucleotide polymorphism are known, UGT2B15*1 and UGT2B15*2, whereas UGT2B15*1 is less frequent in Caucasians compared to other populations such as Asian, Hispanic, or African (0.45 to 0.64, respectively) (Guillemette, 2003). For S-oxazepam, a specific substrate for UGT2B15, a five-fold higher conjugating activity
was described for the *1 allele (Court MH et al., 2002) whereas for the glucuronidation of androgens an increased activity of the *2 allele was described (Levesque et al., 2001). As UGT2B15 was the main isoform involved in the O-glucuronidation, possible interindividual differences in HMMA glucuronidation in vivo can be expected and should be further investigated.

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References


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(UGT2B7) provides evidence for multiple substrate binding and effector sites. *Mol Pharmacol* **74**:1152-1162.

Legends to the Figures

FIG. 1. Metabolic formation of the $R$- and $S$-HMMA-$\text{O}$-glucuronides

FIG. 2. A) LC separation and UV detection ($\lambda = 230$ nm) of the diastereomeric HMMA-$\text{O}$-glucuronides
B, C) GC-MS ($m/z$ 481) analysis of the diastereomers of $S$-HFBPCI-derivatized HMMA (B) and of the $S$-HFBPCI-derivatized fraction I after enzymatic cleavage (C).

FIG. 3. LC-MS detection of the isolated $S$-HMMA glucuronide (left column) and the $R$-HMMA glucuronide (right column): chromatograms of ion $m/z$ 372 (3A and 3B); MS$^1$ fullscan mass spectra (3C and 3D) and MS$^2$ mass spectra of $m/z$ 372 (3E, 3F) formed by the neutral loss of glucuronic acid

FIG. 4. Human UGT isoforms involved in the $\text{O}$-glucuronidation of HMMA under the described incubation conditions. Dark grey bars represent $S$-HMMA $\text{O}$-glucuronide, light grey bars represent $R$-HMMA $\text{O}$-glucuronide, respectively. Data points represent means of duplicate measurements.

FIG. 5. Enzyme kinetics of HMMA $\text{O}$-glucuronidation for UGT1A9, UGT2B7, UGT2B15, UGT2B17 and HLM. UGT1A9 and UGT2B7 were fitted to the Hill equation. UGT2B15, UGT2B17 and HLM were fitted to the Michaelis-Menten equation. Data points represent means and ranges (error bars) of duplicate measurements. The solid curves represent formation of $S$-HMMA glucuronide and the broken curves represent formation of $R$-HMMA glucuronide from racemic HMMA.
Fig. 6. Eadie-Hofstee plots for kinetic profiles of UGT1A9 and UGT2B7. The solid curves represent S-HMMA glucuronide and the broken curves represent R-HMMA glucuronide.

Fig. 7. Calculated activities in the range of common plasma concentrations after recreational doses of MDMA (1-10 µM) for UGT1A9, UGT2B7, UGT2B15, UGT2B17 and HLM. The solid curves represent formation of S-HMMA glucuronide and the broken curves represent formation of R-HMMA glucuronide from racemic HMMA.
Table 1. Kinetic Data for the O-Glucuronidation of racemic HMMA

<table>
<thead>
<tr>
<th></th>
<th>S-HMMA O-Glucuronide</th>
<th>R-HMMA O-Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formation</td>
<td>Formation</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>UGT 1A9</td>
<td>0.6 ± 0.02</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>UGT 2B7</td>
<td>0.6 ± 0.03</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>UGT 2B15</td>
<td>4.4 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>UGT 2B17</td>
<td>0.5 ± 0.02</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>HLM</td>
<td>30.1 ± 2.0</td>
<td>5.8 ± 0.9</td>
</tr>
</tbody>
</table>

units are: $V_{max}$, nmol/min/mg; $K_m$, mM
Figure 1

R-/S-MDMA → R-/S-DHMA → R-/S-HMMA → S-HMMA O-glucuronide → R-HMMA O-glucuronide

CYP, COMT, UGT
Figure 5

**UGT 1A9**

**UGT 2B7**

**UGT 2B15**

**UGT 2B17**

**HLM**

---

**Figure 5**

**Metabolite formation (nmol/mg/min)**

**R-/S-HMMA [mM]**
Figure 7

- **UGT 1A9**
  - Metabolite formation vs. R-/S-HMMA [mM]
  - S and R data points

- **UGT 2B7**
  - Metabolite formation vs. R-/S-HMMA [mM]
  - S and R data points

- **UGT 2B15**
  - Metabolite formation vs. R-/S-HMMA [mM]
  - S/R data points

- **UGT2B17**
  - Metabolite formation vs. R-/S-HMMA [mM]
  - S and R data points

- **HLM**
  - Metabolite formation vs. R-/S-HMMA [mM]
  - S and R data points