4-Hydroxy-3-methoxymethamphetamine Glucuronide as a Phase II Metabolite of 3,4-Methylenedioxymethamphetamine: Enzyme-Assisted Synthesis and Involvement of Human Hepatic Uridine 5′-Diphosphate-Glucuronosyltransferase 2B15 in the Glucuronidation

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3,4-Methylenedioxymethamphetamine (MDMA), one of the most popular illicit recreational drugs, is metabolized primarily into 4-hydroxy-3-methoxymethamphetamine (HMMA) by drug-metabolizing enzymes. HMMA is further metabolized by phase II enzymes to give the glucuronide or sulfate which is excreted into urine. In the present study, enzyme kinetic studies with various microsomes showed that rat liver microsomes pretreated with Aroclor 1254 were most suitable for the enzyme-assisted synthesis of the glucuronide (HMMA-Gluc). This method selectively produced the β-anomer of HMMA-Gluc in a very high, isolated yield (71%), and with a purity that was sufficient for use in an analysis of MDMA intake and for enzyme kinetic studies. We also identified, by an LC-MS method, the human uridine 5′-diphosphate-glucuronosyltransferase (UGT) isoforms that catalyze the glucuronidation of HMMA. Among 12 isoforms of human recombinant UGT expressed in insect cells, UGT2B15 was the only isoform that showed adequate enzymatic activity in catalyzing HMMA glucuronidation with K_m and V_{max} values of 3.8 mM and 1.6 nmol/min/mg protein, respectively. The finding that UGT2B15 is capable of HMMA glucuronidation suggests this isoform may have an important *in vivo* role in human MDMA metabolism.

Key words 3,4-methylenedioxymethamphetamine; enzyme-assisted synthesis; glucuronide; uridine 5′-diphosphate-glucuronosyltransferase

The abuse of amphetamine-like designer drugs, which at present is a serious public health problem, is increasing among young adults. Although originally examined as an adjunct in psychotherapy, 3,4-methylenedioxymethamphetamine (MDMA), known in drug slang as "Ecstasy," has recently become the most popular recreational drug worldwide.[1—3)]

The metabolic pathways of MDMA have been elucidated and over a dozen metabolites have been identified in animals and humans. The major MDMA metabolism pathway involves demethylation to 3,4-dihydroxymethamphetamine (DHMA) followed by *O*-methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA) as shown in Chart 1. For unequivocal proof of MDMA use, detection of these metabolites along with MDMA from a urine sample analysis is required. HMMA exists as a major metabolite in urine,[4—8)] whereas only a few studies concerning MDMA conjugates as phase II metabolites have been reported. The HMMA level in urine has been reported to increase after acid/enzymatic hydrolysis, suggesting that the majority of MDMA metabolites are excreted into urine as the glucuronide or sulfate of HMMA.[9)] If these conjugates were more readily available, they could be used as analytical standards to obtain further unequivocal proof of MDMA use. The synthesis of HMMA glucuronide (HMMA-Gluc) was reported by Shima *et al.*,[10)] but the yield by chemical synthesis was only 6%. For analysis of MDMA metabolites and for enzyme kinetic studies of the glucuronidation process, a more practical method to prepare HMMA-Gluc is needed. In this study, we describe the first enzyme-assisted synthesis of HMMA-Gluc using Aroclor 1254-induced rat liver microsomes. This method was easily performed and stereoselectively produced the β-anomer conjugate in very high yield. In addition, using the synthetic HMMA-Gluc, we investigated the glucuronidation activities of human uridine 5′-diphosphate-glucuronosyltransferase (UGT) isoforms toward HMMA. In a series of UGT1A and 2B isoforms, UGT2B15 was found to catalyze

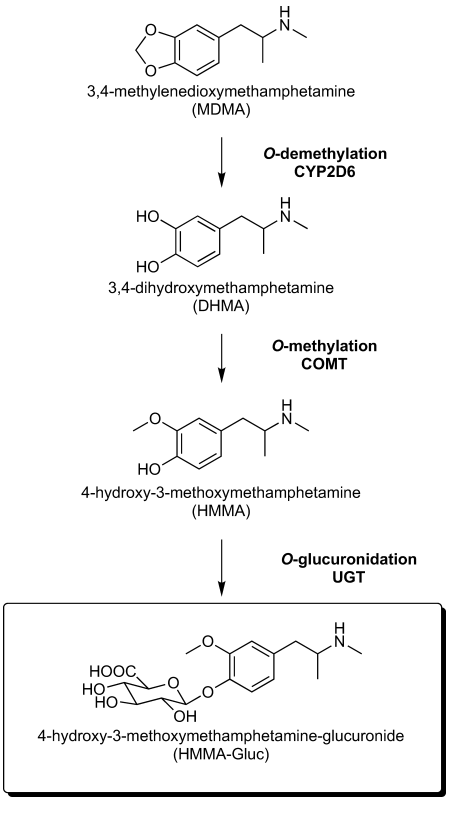

Chart 1. Principal Metabolic Pathway of MDMA in Humans

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the glucuronidation of HMMA.

Experimental

General Information Uridine 5′-diphosphoglucuronic acid (UDPGA) and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents and solvents were purchased from Wako Pure Chemical (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo, Japan), and Kanto Chemical (Tokyo, Japan), and were used without purification. Male Sprague-Dawley rat liver microsomes (Lot No. JJS) and Aroclor 1254-induced Male Sprague-Dawley rat liver microsomes (Lot No. ADM) were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). Pooled human liver microsomes (Lot No. 70196) and microsomes from baculovirus-insect cells expressing UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17 were purchased from BD Gentest (Woburn, MA, U.S.A.). Control experiments were carried out using microsomes from insect cells infected with wild type baculovirus purchased from BD Gentest.

Analytical HPLC was performed using a CBM-20A system controller, LC-20A pump, SPD-M20A UV/Vis photodiode array detector, and CTO-10AC column oven (Shimadzu, Kyoto, Japan) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6×250 mm (Shiseido, Tokyo, Japan). The mobile phases were A: 0.1% trifluoroacetic acid (TFA)/H_2O and B: 0.1% TFA/CH_3CN. Preparative HPLC was performed using a SSC-6600 gradient controller, SSC-3465 pump, SSC-5410 UV/Vis detector and SSC-3465 column oven equipped with a SenshuPak PEGASIL ODS column, 5 μm 20×250 mm (Senshu Kagaku, Tokyo, Japan). The mobile phases were A: 0.1% TFA/H_2O and B: 0.1% TFA/CH_3CN. LC-MS was performed using a Dual λ Absorbance Detector 2487, micromass ZQ and an Alliance model 2695 (Waters, Milford, MA, U.S.A.) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6×250 mm (Shiseido). The mobile phases were composed of A: 0.1% HCOOH/H_2O and B: CH_3CN. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian AS 400 Mercury spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d_4 (δ scale). High resolution mass spectra were obtained on a LTQ Orbitrap (Thermo Fischer Scientific, Waltham, MA, U.S.A.).

Synthesis of HMMA The synthesis of HMMA was carried out according to previous reports,[11,12] with slight modifications. A solution of 5.0 g of 4-hydroxy-3-methoxyphenylacetone in anhydrous methanol (50 ml) was added to a solution of 12 ml (117 mmol) of 40% methylamine in methanol solution, followed by the addition of 2.65 g (42 mmol) of $NaBH_3CN$ in several portion. The solution was adjusted to pH 6 with 2.0 M HCl and stirred at room temperature for 24 h under N_2. The mixture was then poured into 100 ml of water, adjusted to pH 2 with concentrated HCl, and stirred at room temperature for 1 h. After evaporation to remove methanol, the aqueous layer was washed with ether, basicified by the portionwise addition of NaOH until pH>12, and extracted twice with ether. The organic layer was dried over $MgSO_4$, concentrated *in vacuo*, and 2.0 M HCl in ether was added until efficient precipitation was observed. The precipitate was collected and recrystallized from ethanol to afford HMMA as a white solid (HCl salt, 3.44 g, yield 53%). mp 217—218 °C, ^{1}H-NMR (CD_3OD) δ: 1.01 (d, J=6.4 Hz, 3H), 2.34 (s, 3H), 2.46 (dd, J=7.0, 12.8 Hz, 1H), 2.66—2.77 (m, 2H), 3.81 (s, 3H), 6.60 (dd, J=2.0, 8.0 Hz, 1H), 6.72—6.74 (m, 2H), ^{13}C-NMR (CD_3OD) δ: 18.9, 33.5, 43.3, 56.3, 57.7, 113.8, 116.4, 122.7, 131.4, 146.4, 149.1, MS (ESI) 196 $[M+H]^+$, 165 $[M+H-NHCH_3]^+$, HR-MS (ESI) Found 196.1334, Calcd for $C_{11}H_{18}O_2N^+$ 196.1332.

Enzyme-Assisted Synthesis of HMMA-Gluc Ten milliliters of a buffer solution containing 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 5 mM UDPGA, and 10 mM HMMA was stirred in a 37 °C water bath, and the reaction was started by the addition of 200 μl of Aroclor 1254-induced rat liver microsomes (24 mg protein/ml) and stirred continuously for 20 h. The reaction was stopped with 5 ml of 10% $HClO_4$. The precipitated proteins were removed by centrifugation (3000 rpm, 10 min, 4 °C), and the supernatant was filtered. The filtrate was purified by preparative HPLC, and after evaporation of the fraction containing the product, HMMG-Gluc was obtained as a white solid (13.2 mg, yield 71% from UDPGA). ^{1}H-NMR (D_2O) δ: 1.27 (d, J=6.4 Hz, 3H), 2.69 (s, 3H), 2.87 (dd, J=7.2, 14.0 Hz, 1H), 3.00 (dd, J=7.2, 14.0 Hz, 1H), 3.49—3.54 (m, 1H), 3.62—3.69 (m, 3H), 3.88 (s, 3H), 3.99 (d, J=9.2 Hz, 1H), 5.14 (d, J=7.2 Hz, 1H), 6.86 (dd, J=8.4, 2.0 Hz, 1H), 6.99 (d, J=2.0 Hz, 1H), 7.14 (d, J=8.4 Hz, 1H), ^{13}C-NMR (D_2O) δ: 15.0, 30.2, 38.5, 56.0, 56.5, 71.2, 72.6, 74.6, 75.2, 100.6, 114.0, 116.9, 122.3, 131.7, 144.5, 149.2, 172.0, MS, (ESI) 372 $[M+H]^+$, 196 $[M+H\text{-glucro}]^+$, 165 $[M+H\text{-glucro-}NHCH_3]^+$, 370 $[M-H]^-$, HR-MS (ESI) Found 372.1659, Calcd for $C_{17}H_{26}O_8N^+$ 372.1653.

Assay for HMMA Glucuronidation Using Microsomes Glucuronidation of HMMA by three kinds of microsomes was determined by HPLC. The incubation mixture (50 μl) contained 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 20 mM UDPGA, microsomes and HMMA. Concentrations of microsomal proteins were 0.48 mg protein/ml for Aroclor 1254-induced rat liver microsomes, 0.48 mg protein/ml for non-induced rat liver microsomes, and 0.5 mg protein/ml for human liver microsomes. HMMA concentrations varied from 1.0 to 40 mM. Incubations were performed in a water bath at 37 °C, for 10 min with Aroclor 1254-induced and non-induced rat liver microsomes, and for 30 min with human liver microsomes. The enzyme assays were terminated by the addition of 50 μl of 10% $HClO_4$, briefly vortexed, and then centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants were injected into the HPLC. The absorbance of HMMA-Gluc at 275 nm was detected, and the peak area was determined for kinetic analysis. The apparent K_m and V_{max} were estimated by analyzing Michaelis–Menten plots using KaleidaGraph ver. 4.0 software (Synergy Software, Reading, PA, U.S.A.).

UGT Assay for HMMA Glucuronidation Enzyme assay mixtures (50 μl final volume) contained 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 25 μg/ml alamethicin, 5 mM UDPGA, 5 mM HMMA, and 1 mg protein/ml of microsomes expressing UGT isoforms or control microsomes. Incubation was performed for 120 min at 37 °C and was terminated by the addition of 50 μl of 10% $HClO_4$. The mixture was briefly vortexed, then centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants were analyzed by LC-MS. The mass spectrometer was operated in the selected ion monitoring mode using the $[M+H]^+$ ion, m/z 372, for HMMA-Gluc.

The kinetic parameters for UGT2B15 processing of HMMA were estimated by analyzing Michaelis-Menten plots using KaleidaGraph ver. 4.0 software.

Results

Enzyme-Assisted Synthesis of HMMA-Gluc In the case of catechol derivatives, large scale (mg) syntheses of their glucuronides have been accomplished by an enzyme-associated method using rat liver microsomes pretreated with Aroclor 1254, a mixture of polychlorinated biphenyls, as biocatalyst.[13] We applied this method to the synthesis of HMMA-Gluc because of the structural similarities between HMMA and catechol. Concentrations of UDPGA and HMMA were 5 mM and 10 mM, respectively and alamethicin, a pore-forming peptide, was added to the reaction system to activate UGT in the liver microsomes without affecting CYP activity.[14]

A representative chromatogram of the reaction mixture is shown in Fig. 1. During the course of the reaction, peak C at 7.2 min was found to increase with a concomitant decrease in HMMA, peak D, at 9.2 min. The purification of peak C was therefore carried out by preparative HPLC after protein precipitation. After elimination of the solvent, a white solid was obtained. From analysis of ^{1}H-NMR, ^{13}C-NMR, and MS spectra, the structure of the product was determined to be HMMA-Gluc. The advantage of enzyme-assisted synthesis is that the formation solely of biologically relevant stereo- and

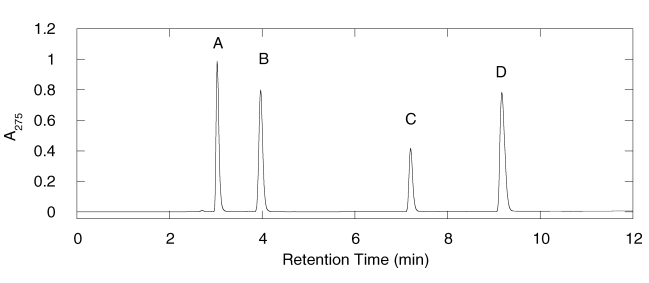

Fig. 1. HPLC Chromatogram after Enzymatic Synthesis

A and B, by-products derived from microsomes or UDPGA; C, HMMA-Gluc; D, HMMA.

regioisomeric products can be expected. In fact, the stereochemistry of the anomeric proton H1′, identified by its characteristic chemical shift at 5.15 ppm, was found by analysis of the coupling constant between H1′ and H2′ (7.2 Hz) to be, as expected, in the β-configuration. Although purely chemical syntheses of HMMA-Gluc have been low yielding, this enzyme-assisted synthesis was found to stereoselectively produce HMMA-Gluc in very high yield (71%).

Kinetic Analysis of Glucuronidation in Microsomes UGT activities in microsomes are expected to differ with respect to species and microsomal enzyme inducer. To study the biocatalytic efficiency of various microsomes toward glucuronidation, kinetic parameters for HMMA glucuronidation were determined using Aroclor 1254-induced and non-induced rat liver and human liver microsomes. All three types of microsomes catalyzed HMMA glucuronidation and the apparent kinetic parameters are shown in Table 1. The apparent V_{max} of Aroclor 1254-induced rat liver microsomes was 64.7 nmol/min/mg protein, two fold higher than from non-induced rat liver microsomes. Human liver microsomes showed a lower apparent V_{max} as compared with both rat liver microsomes. K_m values of 1.9—7.6 mM were obtained for all three microsomes which, while low, are sufficient for the purpose of enzymatic synthesis. These results suggest that Aroclor 1254-induced rat liver microsomes are a suitable biocatalyst for an enzyme-assisted synthesis of HMMA-Gluc.

Human UGT Isozyme Catalysis of HMMA Glucuronidation It is known that the metabolism of MDMA involves *N*-demethylation by CYP2D6 to DHMA. DHMA can undergo subsequent *O*-methylation mediated by catechol *O*-methyltransferase (COMT) to HMMA as shown in Chart 1. The hydroxyl group of HMMA can be further metabolized by phase II enzymes to produce conjugates, the glucuronide/sulfate, which are excreted into urine. Although the majority of MDMA metabolites are conjugates, there have been no reports on the UGT isoforms involved in HMMA glucuronidation. Therefore, enzyme analyses were performed on the 12 kinds of human microsomes that express UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17). Initially, an HPLC method to quantitate the amount of HMMA-Gluc in the enzyme reaction mixture was tried; however, it was difficult to detect the UV absorbance from HMMA-Gluc because of overlap from impurity peaks derived from insect cell microsomes (data not shown). Therefore, an LC-MS method, in selected ion monitoring mode set for HMMA-Gluc at *m*/*z* 372, was used. Results from the LC-MS study are shown in Fig. 2. In the case of the UGT1A series, HMMA-Gluc was hardly detectable, even for the 1A1, 1A6 and 1A9 isozymes which are known to catalyze the glucuronidation of hydroxyl groups in phenolic compounds.[15] In contrast, production of HMMA-Gluc was observed in the UGT2B series, with UGT2B15 being especially productive. The V_{max} and K_m values for human UGT2B15 were determined to be 1.6 nmol/min/mg protein and 3.8 mM, respectively, showing that human UGT2B15 exhibits high glucuronidation efficiency with HMMA.

Table 1. Kinetic Parameters for HMMA Glucuronidation by Liver Microsomes

	Aro[a]	Rat[b]	Human[c]
V_{max} (nmol/min/mg protein)	64.7	30.6	16.7
K_m (mM)	6.7	1.9	7.6

a) Aroclor 1254-induced rat liver microsomes. *b*) Non-induced rat liver microsomes. *c*) Pooled human liver microsomes.

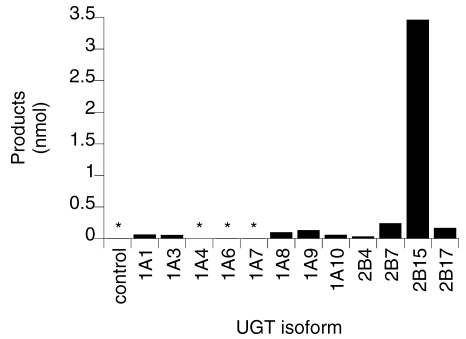

Fig. 2. Glucuronidation of HMMA by Microsomes from Insect Cells Expressing Human UGT Isoforms

∗ Not detected.

Discussion

MDMA metabolism involves demethylation to DHMA catalyzed by CYP2D6, followed by *O*-methylation to HMMA, catalyzed by COMT. Although some HMMA is detected in urine samples as a metabolite of MDMA, most of it is conjugated by phase II enzymes to give a glucuronide or sulfate which is excreted into urine.[9,10] If metabolites of MDMA were readily available as authentic standards, the detection of these metabolites along with HMMA or MDMA in urine samples would constitute a powerful analytical method for unequivocal proof of MDMA use. Unfortunately, the conjugate metabolites are not commercially available and are difficult to synthesize. In this report, we focused on HMMA-Gluc as a major urinary metabolite of MDMA and developed a useful method based on enzyme-assisted synthesis using rat liver microsomes for its production. Liver microsomes from Aroclor 1254-induced rats were used as a highly active source of mammalian UGT. After purification by protein precipitation and preparative HPLC, the structure of HMMA-Gluc was characterized by ^{1}H-NMR and mass spectrometry. This enzymatic method was highly stereoselective, producing the β-anomer. Pure HMMA-Gluc was isolated in very high yield (71%, 13.2 mg), in amounts sufficient for the analysis of MDMA consumption and for enzyme kinetics studies.

An LC-MS method was employed for identification of glucuronidation activities of human UGT isoforms toward HMMA. High levels of enzymatic activity responsible for the formation of HMMA-Gluc from HMMA were only observed in UGT2B15 which is an isoform that catalyzes the glucuronidation of flavonoids and androgenic and estrogenic steroids.[16] A number of endogenous phenolic compounds, drugs, and hydroxylated metabolites of drugs are substrates for the UGT1A series, but interestingly, HMMA is not glucuronidated by any of its members.

Oxazepam, a commonly used 1,4-benzodiazepine anxiolytic drug, is known to be cleared primarily by glucuronidation catalyzed by UGT2B15.[17] The glucuronidation of oxazepam by human subjects is gender dimorphic, arising from a genetic polymorphism for UGT2B15. Such an effect of UGT2B15 polymorphism on gender differences may be con-

sidered in the context of MDMA activity, *i.e.*, it is known that increasing doses of MDMA produce more hallucinogen like perceptual alterations, particularly in women. As in the case of oxazepam, this gender difference might be attributable to the involvement of UGT2B15 in the glucuronidation of HMMA.

In conclusion, we have demonstrated a large scale preparation of HMMA-Gluc by enzyme-assisted synthesis. Kinetic parameters indicated that using Aroclor 1254-induced rat liver microsomes was superior to using non-induced rat liver microsomes or human liver microsomes. In addition, we showed HMMA is glucuronidated to HMMA-Gluc by human UGT2B15. Further studies, utilizing enzyme-assisted synthesis of other illegal drug metabolites that are difficult to prepare by chemical synthesis, for example, psilocin, are now in progress.

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