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Title page

Studies on para-methoxymethamphetamine (PMMA) metabolite pattern and influence of CYP2D6 genetics in human liver microsomes and authentic samples from fatal PMMA intoxications

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Running title page

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	Discussion:	1353

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d) List of nonstandard abbreviations used in the paper (alphabetical order):

2C-B, 4-bromo-2,5-dimethoxyphenethylamine; COMT, catechol-O-methyl-transferase; CYP P450, cytochrome P450; di-OH-A, 3,4-dihydroxyamphetamine (dihydroxyamphetamine, α-methyldopamine); di-OH-MA, 3,4-dihydroxymethamphetamine (dihydroxymethamphetamine, N-methyl-α-methyldopamine); EM, extensive metabolizer; HCl, hydrochloride; HLM, human liver microsomes; HM-A, 4-hydroxy-3-methoxyamphetamine; HM-MA, 4-hydroxy-3-methoxymethamphetamine; HPLC, high performance liquid chromatography; IS, internal standard; LC-MS, liquid chromatography mass spectrometry; MAO-A, monoamine oxidase type A; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MQ water, Milli-Q water; NADPH, nicotinamide adenine dinucleotide phosphate; OH-A, 4-hydroxyamphetamine; OH-MA, 4-hydroxymethamphetamine; pHLM, pooled human liver microsomes; PM, poor metabolizer; PMA, para-methoxyamphetamine (4-methoxyamphetamine); PMMA, para-methoxymethamphetamine (4-methoxymethamphetamine); QC samples, quality control samples; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase; UHPLC-MS-MS, ultra-high performance liquid chromatography tandem mass spectrometry; UM, ultrarapid metabolizer

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Abstract

Para-methoxymethamphetamine (PMMA) has caused numerous fatal poisonings worldwide and appears to be more toxic than other ring-substituted amphetamines. Systemic metabolism is suggested to be important for PMMA neurotoxicity, possibly through activation of minor catechol metabolites to neurotoxic conjugates. The aim of this study was to examine the metabolism of PMMA in humans, and for this purpose we used human liver microsomes (HLM) and blood samples from three cases of fatal PMMA intoxication. We also examined the impact of CYP2D6 genetics on PMMA metabolism using genotyped HLM isolated from CYP2D6 poor, population average and ultrarapid metabolizers. In HLM, PMMA was metabolized mainly to 4-hydroxymethamphetamine (OH-MA), while low concentrations of para-methoxyamphetamine (PMA), 4-hydroxyamphetamine (OH-A), dihydroxymethamphetamine (di-OH-MA) and oxilofrine were formed. The metabolite profile in the fatal PMMA intoxications were in accordance with the HLM study, with OH-MA and PMA being the major metabolites, while OH-A, oxilofrine, HM-MA and HM-A were detected in low concentrations. A significant influence of CYP2D6 genetics on PMMA metabolism in HLM was found. The catechol metabolite di-OH-MA has previously been suggested to be involved in PMMA toxicity. Our studies show that the formation of di-OH-MA from PMMA was 2-7 times lower than from an equimolar dose of the less toxic drug MDMA, and do not support the hypothesis of catechol metabolites as major determinants of fatal PMMA toxicity. Altogether, the present study revealed the metabolite pattern of PMMA in humans and demonstrated a great impact of CYP2D6 genetics on human PMMA metabolism.

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Introduction

Para-methoxymethamphetamine (4-methoxymethamphetamine, PMMA) is a toxic serotonergic designer drug which is structurally and pharmacologically closely related to para-methoxyamphetamine (4-methoxyamphetamine, PMA) and MDMA (3,4-methylenedioxymethamphetamine, “ecstasy”) (Fig. 1). Since the 1990s, PMMA has occasionally appeared on the illicit drug market in many countries, as powder or tablets purported to be “ecstasy” or “amphetamine”. PMMA is unpopular among drug users, due to its mainly unpleasant effects and high toxicity. It is regarded as more toxic than MDMA and methamphetamine, and has a narrow margin of safety (Steele et al., 1992; EMCDDA, 2003). During the last five years, PMMA is associated with at least 131 fatal and 31 nonfatal poisonings worldwide (WHO, 2015). In Norway, 27 fatal PMMA-related intoxications were registered in an outbreak during 2010-2012 (Vevelstad et al., 2012; Vevelstad et al., 2016a).

The pharmacological actions of PMMA are mainly related to increased serotonergic and noradrenergic synaptic transmission in the central nervous system (Simmler et al., 2014). PMMA is a potent inhibitor of serotonin and noradrenaline uptake transporters, and also induces release of serotonin and noradrenalin. At high concentrations dopaminergic transport is also affected, however, the potency of PMMA for inhibition of dopamine uptake is low with a dopamine/serotonin inhibition ratio of 0.04 in vitro (Simmler et al., 2014; Liechti, 2015). Based on previous studies demonstrating inhibitory activity of PMA on the degrading enzyme monoamine oxidase A (MAO-A), PMMA is also presumed to exert potent inhibition of this enzyme, a characteristic contributing to drug toxicity (Green and El Hait, 1980; Freezer et al., 2005; Stanley et al., 2007). PMMA toxicity is mainly related to the PMMA dose, and PMMA blood concentrations above 2.8 µM are considered lethal (Chen et al., 2012; Vevelstad et al., 2012; Kronstrand et al., 2015; WHO, 2015; Vevelstad et al., 2016b). PMMA toxicity usually manifests as serotonin syndrome. Intoxication symptoms range from mild

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hyperthermia, neuromuscular hyperactivity and confusion, to fatal hyperthermia, convulsions, coma, respiratory distress, hypoglycemia, cardiac arrest and multiple organ failure (Chen et al., 2012; Vevelstad et al., 2012; Nicol et al., 2015). Fatal PMMA intoxications have also been associated with PMMA concentrations in the low 'recreational range'. In the Norwegian outbreak of 27 fatal PMMA-related intoxications, two cases had PMMA blood concentrations of 0.1 and 0.2 µM, respectively, with no other toxicological contributor to death (Vevelstad et al., 2012; Vevelstad et al., 2016b). Similar concentration levels have occasionally been reported also in other PMMA outbreaks (Nicol et al., 2015). This may indicate that certain individuals are particularly susceptible to PMMA toxicity.

In a previous study we found no pharmacogenetic dispositions for PMMA toxicity in humans (Vevelstad et al., 2016a). However, several reports have postulated that the neurotoxicity of similar ring-substituted amphetamines such as MDMA is related to hepatic drug metabolism by the polymorphically expressed CYP (cytochrome P450) 2D6 enzyme (Esteban et al., 2001; Monks et al., 2004). In particular, it has been proposed that the minor catechol metabolites dihydroxymethamphetamine (di-OH-MA, N-methyl-α-methyldopamine) and dihydroxyamphetamine (di-OH-A, α-methyldopamine) are involved (Carvalho et al., 2004a; Carvalho et al., 2004b; Jones et al., 2005; Milhazes et al., 2006; Carmo et al., 2007). Di-OH-MA is not neurotoxic in itself (Zhao et al., 1992; Monks et al., 2004), but is a precursor of conjugated GSH/N-acetylcysteine compounds that have been implicated in serotonergic neurotoxicity and neurodegeneration (Molliver et al., 1986 ; Schmidt and Taylor, 1988; Hiramatsu et al., 1990; McCann and Ricaurte, 1991; Paris and Cunningham, 1992; Miller et al., 1995; Chu et al., 1996; Miller et al., 1996; Bai et al., 1999; Esteban et al., 2001; Carvalho et al., 2004b; de la Torre and Farre, 2004; Monks et al., 2004; Jones et al., 2005; Carmo et al., 2006; Perfetti et al., 2009). Di-OH-MA, which is well known as the main

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intermediate metabolite of MDMA (Segura et al., 2001; de la Torre et al., 2004), is reported to be formed in rats after administration of PMMA (Staack et al., 2003).

The high toxicity of PMMA, as compared to MDMA, requests more research on PMMA's metabolite pattern and pharmacogenetics in humans. The existing knowledge on the metabolism of PMMA is limited to two microsomal studies (Staack et al., 2004b; Lai et al., 2015) and experimental studies in rats (Staack et al., 2003; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b; Palenicek et al., 2011). The aim of this study was to examine the metabolism of PMMA in human liver microsomes (HLM) and in authentic blood samples from fatal PMMA intoxications. We also examined the impact of CYP2D6 genetics on PMMA metabolism by using genotyped HLM isolated from CYP2D6 poor metabolizers (PM), population average (pHLM) and ultrarapid metabolizers (UM).

Materials and methods

Drugs and chemicals

PMMA-HCl was purchased from Cayman Chemicals (Ann Arbour, MI, USA) and MDMA-HCl from Chiron (Trondheim, Norway) and used for incubation solutions.

The ^{13}C-labeled internal standards (IS) $^{13}C_6$ amphetamine, $^{13}C_6$ methamphetamine, $^{13}C_6$ MDMA, $^{13}C_6$ MDA, $^{13}C_6$ PMMA, $^{13}C_6$ PMA and $^{13}C_6$ 4-bromo-2,5-dimethoxyphenethylamine ($^{13}C_6$ 2C-B) were purchased from Chiron (Trondheim, Norway). Stock solutions of IS were prepared in methanol, and a mixture of IS was prepared in MQ water in concentrations of 1 to 5 µM depending on IS.

To make analytical calibrators and QC samples, amphetamine was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and Cerillant (Round Rock, TX, USA); 4-

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hydroxymethamphetamine (OH-MA) from Sigma-Aldrich (Saint Louis, MO, USA); di-OH-MA from Cayman Chemicals (Ann Arbour, MI, USA); PMMA, 4-hydroxy-3-methoxyamphetamine (HM-A) and 4-hydroxy-3-methoxymethamphetamine (HM-MA) from Lipomed (Arlesheim, Switzerland); methamphetamine and PMA from Cerillant (Round Rock, TX, USA) and Lipomed (Arlesheim, Switzerland); MDMA from Cerillant (Round Rock, TX, USA) and Chiron (Trondheim, Norway); 3,4-methylenedioxyamphetamine (MDA) from Cerillant (Round Rock, TX, USA) and Alltech (Deerfield, IL, USA); and 4-hydroxyephedrine (oxilofrine) and 4-hydroxyamphetamine (OH-A) from National measurement institute (Sydney, NSW, Australia). Formic acid was purchased from VWR (Oslo, Norway). Nicotinamide adenine dinucleotide phosphate (NADPH) regeneration solution A and B were purchased from Corning BV (Woburn, MA, USA). LC-MS grade methanol was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and AnalaR® ammonium formate from BDH Laboratory Supplies (Poole, England). De-ionized water was obtained from a Milli-Q UF Plus water purification system (Millipore, Bedford, MA, USA). The enzymes β-glucuronidase (type LII, from Patella vulgate 1,000,000–3,000,000 units/g solid) and sulfatase (type H-1 from Helix Pomatia, ≥10,000 units/g solid) were obtained from Sigma–Aldrich (St. Louis, MO).

All chemicals were of ≥ 98% purity. All compounds were stored according to supplier recommendations. Analytical calibrators and QC samples were prepared in 1 mM ascorbic acid.

Human liver microsomes

Pooled human liver microsomes (pHLM, XTreme 200 Pool) and single donor human liver microsomes (HLM) that were genotyped for CYP2D6 content and activity were purchased from XenoTech and delivered by Tebu-bio (Roskilde, Denmark). The pHLM had

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been prepared from 200 donors of balanced gender (20 mg microsomal protein/mL, 400 - 500 pmol total CYP450/mg protein, stored in a 250 mM sucrose solution). Four different lots were used in our studies (#1210223, #1210347, #1110258 and #1410230). From single donors of both genders, HLM classified as “No CYP2D6 activity, NA” were used to represent CYP2D6 poor metabolizers (PM), and HLM classified as “CYP2D6 high activity, HA” were used to represent CYP2D6 ultrarapid metabolizers (UM). Further information about the pooled and the single donor HLM, such as CYP2D6 allelic variant, gender, ethnicity, age, cytochrome content and CYP2D6 enzyme activity, is shown in Supplemental Table 1. The microsomes were aliquoted and stored at -80°C.

Microsomal incubations

A solution of 1 mM MDMA was prepared by dissolving the compound in phosphate buffer (100 mM, pH 7.4). PMMA was dissolved in ethanol (116 mM) and further diluted to 1 mM in phosphate buffer (100 mM, pH 7.4). The maximum ethanol concentration during incubation was 0.086%. 10 µL of HLM (pHLM, CYP2D6 PM or CYP2D6 UM; final concentration 2 mg protein/mL) was mixed with 80 µL of NADPH regeneration system solution (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$). This mixture was preincubated at 37±1°C for 10 min. The reactions were initiated by adding 10 µL of ice-cold 1 mM PMMA or MDMA solution (final drug concentration 100 µM, final volume 100 µL), followed by incubation at 37±1°C in a shaking water bath for 0, 15, 30, 60, 120 and 240 min. The incubations were terminated by adding 10 µL of ice-cold 1.2 M formic acid (final concentration 0.1 M) and vortexing the samples. Immediately thereafter, 10 µL of a mixture of ^{13}C labeled internal standards was added. The samples were vortexed for 30 seconds, centrifuged for 15 min at 4°C and 14,500 × g. The supernatant was transferred to autosampler vials and analysed for PMMA, MDMA, methamphetamine, amphetamine, PMA, OH-MA, OH-A, di-OH-MA, MDA, HM-MA, HM-A and oxilofrine. Due to saturated detector response for 4-OH-MA using this procedure, aliquots of 10 µL were transferred to new auto sampler vials and diluted 100 times before ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) analysis. For each time point, 3-4 separate experiments were performed, using microsomes from 3-4 lots of pHLM or 2-3 CYP2D6-genotyped single donor HLM (except for PMMA 60 min in CYP2D6 poor metabolizers; n=2).

A substrate concentration of 100 µM PMMA and MDMA was chosen to enable detection of metabolites present at low concentrations. The PMMA concentrations measured in fatal intoxications are usually 3-40 µM in blood (Lin et al., 2007; Chen et al., 2012; Lurie et al., 2012; Nicol et al., 2015; Vevelstad et al., 2016a), and probably 2-6.5 times higher in liver tissue based on findings for similar drugs like PMA and methamphetamine/amphetamine (Felgate et al., 1998; McIntyre et al., 2013). This suggests that PMMA concentrations of 100 µM in liver tissue may be representative for fatal PMMA intoxications. Similar drug concentrations and liver/blood ratios have also been reported in fatal MDMA intoxications (Elliott, 2005). Moreover, 100 µM PMMA corresponds to a substrate/HLM protein ratio of 50 nmol PMMA/mg protein, which is slightly below the reported Km for PMMA in pHLM (Staack et al., 2004b).

Instrumental analysis in the HLM studies

PMMA, PMA, OH-MA, OH-A, MDMA, MDA, methamphetamine, amphetamine, di-OH-MA, HM-MA, H-MA and oxilofrine were analyzed by a Waters Aquity UPLC system (Waters, Milford, MA, USA). Separation was performed on an Aquity HSS T3 column (2.1 x 100 mm, 1.8 µm particles) with column temperature of 65°C and gradient elution at a flow

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rate of 0.5 mL/min with 100% methanol (mobile phase A) and 10 mM aqueous ammonium formate, pH 3.1 (mobile phase B). The gradient had an initial composition of 2.5% A, increasing to 32.5% A during 6 min, switched to 100% A at 6.01 min and held for 1 min. The gradient returned to initial conditions consisting of 2.5% A at 7.01 min. The total cycle time was 9 min. The injection volume used was 3-5 µL using partial loop injection with a needle overfill flush of 3 µL. Weak wash was performed with 0.6 mL methanol:water (10:90) and strong wash with 0.2 mL methanol:water (90:10). Mass spectrometric analysis was carried out with a Waters Quattro Premier XE tandem mass spectrometer with electrospray interface. Positive ionization was performed in multiple reaction monitoring (MRM) mode. Additional data regarding the MRM transitions, cone voltage (CV) and collision energy (CE) for the measurement of the analytes and the internal standards are given in Supplemental Table 2. The respective $^{13}C_6$-analogues were used as internal standards for PMMA, PMA, MDMA, MDA and methamphetamine. For the other compounds, $^{13}C_6$ 2C-B was used as internal standard. Between assay precision and accuracy based on the results from six different days, for low, medium and high QC samples run together with the microsomal samples, is shown in Table 1, together with the method lower limit of quantification and calibration range. The extraction recovery and matrix effects (ME) of analytes and internal standards were determined using the method described by Matuszewski et al (Matuszewski et al., 2003). Extraction recovery and ME was evaluated for low and high QC samples, while the internal standards were evaluated with the concentrations used for HLM analysis. A ME below 100% indicates ion suppression, while ME above 100% indicates ion enhancement. Five different lots of pooled and single donor human liver microsomes were tested, and compared to three replicates of the neat spiked solution for each level. Recovery and ME for all compounds are shown in Supplemental Table 3.

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PMMA metabolites in fatal PMMA-related intoxications

Post-mortem femoral blood samples from three fatal PMMA intoxications were analyzed for PMMA and PMMA metabolites. Methamphetamine, amphetamine, MDMA and MDA have several metabolites in common with PMMA. The criteria for inclusion were therefore the presence of PMMA and the absence of methamphetamine, amphetamine and MDMA (analytical cutoffs 0.015, 0.007 and 0.025 mg/L, respectively) in the blood sample (Oiestad et al., 2011; Vevelstad et al., 2016a). Our screening panel does not include MDA, as MDA in our case work is usually found only in low concentrations as a metabolite of MDMA. The quantitative analyses for PMMA and metabolites were performed by UHPLC-MS/MS as described previously (Vevelstad et al., 2016a). The following PMMA metabolites were determined in post-mortem blood: 4-hydroxymethamphetamine (OH-MA), 4-hydroxyamphetamine (OH-A), PMA, 4-hydroxy-3-methoxyamphetamine (HM-A), 4-hydroxy-3-methoxymethamphetamine (HM-MA) and oxilofrine (4-hydroxyephedrine). Analysis of these metabolites was also performed after enzymatic hydrolysis of the blood samples with β-glucuronidase and sulfatase. Analysis of di-OH-MA was not successful in post-mortem blood samples, probably due to limited sensitivity, problems with linearity and low stability of this reactive compound (Hiramatsu et al., 1990; Perfetti et al., 2009). CYP2D6 genotyping was performed as described previously (Vevelstad et al., 2016a). The study was approved by the Regional Committee for Medical and Health Research Ethics and by the Higher Prosecution Authority.

Data and statistical analysis

Data given in Figure 2-4 are presented as the mean ± Standard Error of the Mean (S.E.M.). For the data given as percent of control (e.g. percent of the measured initial drug

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concentration, or percent of the concentration in pooled HLM at a given time point), the relative S.E.M. is given. The 'PMMA metabolic ratio' was calculated as the total molar concentration of all metabolites in blood divided by the PMMA concentration.

Statistical tests were performed using SPSS, version 23 (SPSS Inc., Chicago, IL, USA). Data were compared using one-way analysis of variance (ANOVA) followed by the Tukey posthoc test for multiple comparisons. P values less than 0.05 were considered as statistically significant. The figures were made using SigmaPlot software version 12.3 (Systat Software, Inc. San Jose, CA) and ChemSketch.

Results

Metabolism of PMMA in pooled human liver microsomes (pHLM)

The metabolism of PMMA was studied in pHLM, representing the average Caucasian population (Supplemental Table 1). PMMA was metabolized more rapidly in pHLM compared to the reference substance MDMA (Fig. 2). After 30, 60, 120 and 240 min of incubation at 37°C, 19.4 ± 1.5, 29.8 ± 4.0, 34.9 ± 3.7 and 51.1 ± 2.9% of the initial PMMA concentration was metabolized, respectively (Fig. 2A). For comparison, only 13.7 ± 3.0% of MDMA was metabolized after 240 min under the same conditions of incubation (Fig. 2C).

In pHLM, PMMA was mainly metabolized to OH-MA (Fig. 2A). After 240 min of incubation, the levels of OH-MA constituted 44.7 ± 4.4% of the initial PMMA concentration. The PMMA metabolites PMA, OH-A and di-OH-MA were formed in smaller amounts, constituting 1.5 ± 0.1, 0.8 ± 0.1 and 0.5 ± 0.03% of the initial PMMA concentration, respectively, after 240 min of incubation (Fig. 2B). The formation of di-OH-MA plateaued after approx. 60 min of incubation. No HM-MA, HM-A, methamphetamine or amphetamine

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was detected, while trace amounts of oxilofrine were formed from PMMA after 120 – 240 min of incubation with PMMA.

In pHLM, MDMA was metabolized to di-OH-MA and MDA, which constituted 3.5 ± 0.1 and 2.6 ± 0.4 %, respectively, of the initial MDMA concentration after 240 min of incubation (Fig. 2D). For di-OH-MA, the formation rate declined after 15 min of incubation. At these incubation conditions, no formation of OH-MA, HM-MA, HM-A or oxilofrine from MDMA was observed.

Impact of CYP2D6 genetics on PMMA metabolism and metabolite formation in human liver microsomes (HLM)

The metabolism of PMMA in pHLM was compared with the metabolism of PMMA in genotyped HLM classified as CYP2D6 UM or CYP2D6 PM, using MDMA as a reference substance. A significant impact of CYP2D6 genotype on the metabolism of PMMA in HLM was found (Fig. 3A). After 240 min of incubation, 77.4 ± 7.7, 51.1 ± 2.9 and 21.5 ± 5.9% of the initial PMMA concentration was metabolized in CYP2D6 UM, pooled and PM HLM, respectively. Compared with the concentration of PMMA in pHLM, the concentration was significantly lower in UM (46.4 ± 15.8% of pHLM, $p<0.05$), and significantly higher in PM (167.7 ± 12.6% of pHLM, $p<0.01$), after 240 min of incubation (Fig. 3A). The formation of all detected metabolites was higher in UM and lower in PM, compared to pHLM, during the entire incubation period, although the differences were not statistically significant at all time points (Fig. 3B-E). The PMMA metabolic ratio (total PMMA metabolite/PMMA concentration) increased gradually up to a maximum of 0.1, 1.0 and 2.9 after four hours of incubation in PM, pHLM and UM, respectively. The formation of di-OH-MA increased steadily during the first 60 min of incubation in UM and pHLM (Fig. 3E). The levels formed in UM were 3.5 – 9.8 times higher than in pHLM ($p<0.05$ at 120 min), while only trace amounts of di-OH-MA were formed in PM. The maximum levels formed of di-OH-MA constituted 2.4 ± 0.9, 0.5 ± 0.05 and 0.01 ± 0.01% of the initial PMMA concentration in CYP2D6 UM, pHLM and PM, respectively.

For comparison, the reference drug MDMA (Fig. 4A) was only slightly metabolized in HLM by all CYP2D6 genotypes, and there was no significant impact of CYP2D6 genotype on the formation of MDA. The formation of the main intermediate metabolite di-OH-MA was significantly affected by CYP2D6 genotype (Fig. 4C). After 240 min of incubation, the concentration of di-OH-MA in UM was 166.2 ± 16.6% of pHLM ($p<0.01$), while the concentration in PM was 72.3 ± 9.5% of pHLM ($p>0.05$) (Fig. 4C). The maximum levels of di-OH-MA constituted 5.2 ± 0.5, 3.5 ± 0.1 and 2.2 ± 0.3% of the initial MDMA concentration in CYP2D6 UM, pHLM and PM, respectively.

PMMA metabolites in fatal PMMA intoxications

The metabolite profile of PMMA was investigated in post-mortem blood samples from three fatal PMMA intoxications, since the in-vivo metabolite profile in humans has not been previously published. The fatalities represented males aged 25-51 years. The inclusion criteria were presence of PMMA and absence of methamphetamine, amphetamine and MDMA in blood, since the latter drugs have several metabolites in common with PMMA. The individual PMMA and metabolite concentrations are presented in Table 2. The PMMA concentrations in the post-mortem samples were 6.9, 7.4 and 26.4 µM, and the main metabolites were OH-MA and PMA, representing 81-100 % of the total metabolite concentrations measured both in non-hydrolyzed and hydrolyzed blood samples. The concentrations of OH-MA and HM-MA increased 7-12 times after hydrolysis. Individual #3, displaying a very high PMMA concentration, had minor levels of free OH-A and HM-MA, while also oxilofrine and HM-A

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was detected in low amounts after hydrolysis. The PMMA metabolic ratio (total PMMA metabolite/PMMA concentration) was below 1.0 in all fatalities. CYP2D6 genotyping showed that individual #1 had CYP2D6*1/*1 genotype, predicting a CYP2D6 extensive (EM) phenotype, while individuals #2 and #3 had CYP2D6 *1/*4, predicting the CYP2D6 intermediate (IM) metabolizer phenotype.

Discussion

The serotonergic ring-substituted drug para-methoxymethamphetamine (PMMA) is considered more toxic than classic ring-substituted amphetamines like MDMA, despite similar chemical structure (Fig. 1) (Steele et al., 1992; WHO, 2015). In the present study, we have characterized the metabolite pattern of PMMA in humans by using HLM and blood samples from fatal PMMA intoxications. Further, we have used genotyped HLM isolated from CYP2D6 poor, population average and ultrarapid metabolizers to examine the impact of CYP2D6 genetics on human PMMA metabolism.

Our studies in pHLM, representing the average Caucasian population, showed that 19.4% of PMMA was metabolized after 30 min of incubation, increasing to 51.1% after 240 min. In comparison, only 13.7% of MDMA was metabolized after 240 min, demonstrating that in these incubation conditions, PMMA was metabolized more rapidly compared to MDMA. Our results are in accordance with previous pHLM studies, which have reported 20-25% of PMMA being metabolized within 20-25 min (Staack et al., 2004b; Lai et al., 2015). No information is available on the pharmacokinetics of PMMA in humans, while the plasma half-life of PMMA in rats is 1 hour (Rohanova and Balikova, 2009a; Palenicek et al., 2011).

The main PMMA metabolite formed in our HLM study was OH-MA (pholedrine), constituting 87% of the metabolized PMMA, while the minor metabolites PMA, OH-A and di-OH-MA constituted only 1.0 – 2.9% after four hours of incubation. In the fatal PMMA

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intoxications, which all exhibited PMMA levels within the lethal range above 2.8 µM (Chen et al., 2012; Vevelstad et al., 2012; Kronstrand et al., 2015; WHO, 2015; Vevelstad et al., 2016b), the detected metabolites were in general consistent with the results in HLM. The formation of the metabolite PMA was, however, more pronounced in vivo as compared to the in vitro HLM experiments. In blood samples from the fatalities, PMA represented 30-100% of the total metabolite concentration, while it represented only up to 10% in the HLM. Additionally, in one of the fatalities who displayed a high PMMA concentration (26.4 µM), the metabolites HM-MA and HM-A were detected. These metabolites were not expected to be formed in the HLM study, since the indispensable methyl donor S-adenosyl methionine (SAM) was not added to the incubation solution (Helmlin et al., 1996; Kuwayama et al., 2009; Kuwayama et al., 2012). The proposed pathway for the metabolism of PMMA in humans is illustrated in Fig.5.

By comparing the metabolism in CYP2D6 UM, pHLM and PM HLM, we found a significant influence of CYP2D6 genetics on the metabolism of PMMA and the formation of most of the metabolites. The scarce influence of CYP2D6 genetics on PMA formation is in keeping with the literature, concluding that N-dealkylation of amphetamines mainly occurs via other CYP enzymes, like CYP2B6 (Kreth et al., 2000; Maurer et al., 2000b). Regarding the three PMMA fatalities, no inferences could be drawn on the influence of CYP2D6 genetics on PMMA metabolism in vivo, due to the limited number of cases, unknown time intervals between PMMA intake and death, and post-mortem drug redistribution. The low concentrations of PMMA metabolites observed in the blood samples may, however, indicate that death occurred within a few hours after PMMA exposure. This hypothesis is in keeping with the available case information and the existing literature on PMMA toxicity (Chen et al., 2012; Vevelstad et al., 2012; Vevelstad et al., 2016a).

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PMMA is associated with hundreds of fatal poisonings worldwide and appears to be more toxic than MDMA. The mechanism for the high toxicity of this ring-substituted amphetamine is however unknown. Previous reports have postulated that systemic metabolism is crucial for the serotonergic neurotoxicity of ring-substituted amphetamines, possibly by conjugation of minor catechol metabolites with GSH/N-acetylcysteine to form potent neurotoxic conjugates (Molliver et al., 1986 ; Schmidt and Taylor, 1988; Hiramatsu et al., 1990; McCann and Ricaurte, 1991; Paris and Cunningham, 1992; Miller et al., 1995; Chu et al., 1996; Miller et al., 1996; Bai et al., 1999; Esteban et al., 2001; Carvalho et al., 2004b; de la Torre and Farre, 2004; Monks et al., 2004; Jones et al., 2005; Carmo et al., 2006; Perfetti et al., 2009). To our knowledge, the present study is the first to demonstrate the formation of the catechol di-OH-MA, as well as HM-MA, HM-A and oxilofrine, in humans or in incubations with HLM after PMMA exposure. Our study showed that the formation of di-OH-MA from PMMA was 2-7 times lower than from an equimolar dose of the less toxic drug MDMA, which does not support the hypothesis of catechol metabolites as major determinants of fatal PMMA toxicity. Rapid redosing of PMMA, due to its weak and delayed euphoric effects compared to MDMA (Lin et al., 2007; Westin and Brede, 2011; Chen et al., 2012; Vevelstad et al., 2012; Nicol et al., 2015), could theoretically lead to accumulation of di-OH-MA conjugates in the brain, as is reported after redosing of MDMA in rats (Erives et al., 2008). However, further studies are needed to investigate if this is applicable also to PMMA.

Former studies have suggested that the neurotoxicity of ring-substituted amphetamines is dependent on CYP2D6 genetics (Esteban et al., 2001; Monks et al., 2004). A previous study in our laboratory did not reveal any significant correlation between CYP2D6 genotype and fatal PMMA toxicity in humans (Vevelstad et al., 2016a). Accordingly, previous studies regarding MDMA have not found evidence for a major influence of CYP2D6 genotype on

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drug toxicity (O'Donohoe et al., 1998; Gilhooly and Daly, 2002; de la Torre et al., 2012). Others have suggested that the impact of CYP2D6 genetics on the formation of potentially toxic metabolites would be restricted to the first few hours after MDMA or PMMA intake, because amphetamines are CYP2D6 inhibitors. The CYP2D6 enzyme is irreversibly inactivated within 2 hours after intake of a recreational dose of MDMA (O'Mathuna et al., 2008), and hence, all subjects may be phenocopied to the CYP2D6 PM phenotype shortly after MDMA intake (Yang et al., 2006; O'Mathuna et al., 2008; Perfetti et al., 2009). In our HLM study, there was a minimal turnover of MDMA compared to PMMA, probably because MDMA exerts a much more potent inhibition of CYP2D6 (Ki 0.6 µM) compared to PMMA/PMA (Ki~24 µM) (Wu et al., 1997; de la Torre et al., 2012). Genetic polymorphisms in the enzyme catechol-O-methyltransferase (COMT) may also be relevant for susceptibility to neurotoxicity by ring-substituted amphetamines, since catechol metabolites are rapidly O-methylated by COMT (Perfetti et al., 2009).

Studies of drug metabolism in HLM are useful for revealing qualitative metabolite patterns and to evaluate the importance of different CYP enzymes and genotypes, but do not accurately resemble the biotransformation in hepatocytes or human liver (Brandon et al., 2003). Further, in the present HLM study we analysed for unconjugated metabolites only. However, the high substrate recovery observed in our study indicates that the formation of glucuronide/sulfate/thiol conjugates was low, which is in accordance with a previous HLM study (Lai et al., 2015). Regarding the fatal PMMA intoxications, we did not detect di-OH-MA in the post-mortem blood samples. This is probably due to the highly unstable nature of catechol compounds, which detection depends on rapid analysis of fresh biological samples (Hiramatsu et al., 1990; Helmlin et al., 1996; Maurer et al., 2000a; Staack et al., 2003; Carvalho et al., 2004a; Perfetti et al., 2009; Vevelstad et al., 2016a). A low concentration of

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di-OH-MA has been measured in rats administered PMMA, however, this analysis was performed in fresh urine samples (Staack et al., 2003; Rohanova and Balikova, 2009a).

In conclusion, the major PMMA metabolite formed in HLM was OH-MA, while di-OH-MA, PMA, OH-A and oxilofrine were minor metabolites. The metabolite pattern in HLM was, in general, in accordance with the findings in post-mortem blood samples from three fatal PMMA intoxications. CYP2D6 genetics had a significant influence on PMMA metabolism in HLM. The catechol di-OH-MA was demonstrated as a metabolite of PMMA in HLM, but the formation was 2-7 times lower than from MDMA at equimolar doses. Taken together, our findings in HLM and in fatal PMMA intoxications do not support the hypothesis of catechol metabolites and CYP2D6 as major determinants of fatal PMMA toxicity in humans. Further investigations are necessary to elucidate the high toxicity of PMMA.

Authorship contributions

Participated in research design:	Vevelstad, Bogen, Øiestad, Arnestad
Conducted experiments:	Vevelstad, Bogen, Nerem, Øiestad
Contributed new reagents or analytic tools:	-
Performed data analysis:	Vevelstad, Bogen, Øiestad, Nerem
Wrote or contributed to the writing of the manuscript:	Vevelstad, Bogen, Øiestad, Arnestad

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Figure legends

Fig. 1.

Molecular structure of the ring-substituted amphetamines PMMA, PMA and MDMA.

Fig. 2.

Concentration-time profiles of PMMA (A,B) and MDMA (C,D) and their respective major (left) and minor (right) metabolites, in pooled human liver microsomes (pHLM). pHLM were incubated with PMMA or MDMA (100 µM) at 37°C for 240 min. Each symbol and error bars denote the mean ± S.E.M. of 3-4 experiments (except for PMMA 60 min; N=2).

Fig. 3.

Impact of CYP2D6 genotype on the concentration-time profile of PMMA (A) and the metabolites OH-MA (B), PMA (C), OH-A (D) and di-OH-MA (E), respectively, in HLM. CYP2D6 UM, pooled (pHLM) or PM HLM were incubated with PMMA (100 µM) at 37°C for 240 min. Each symbol and error bars denote the mean ± S.E.M. of 3-4 experiments (except for PMMA 60 min in CYP2D6 PM; N=2). *P < 0.05; ***P* < 0.01; ***P < 0.001 compared with pHLM. †P < 0.05; ††P < 0.01; †††P < 0.001 for UM compared with PM.

Fig. 4.

Impact of CYP2D6 genotype on the concentration-time profile of MDMA (A) and the metabolites MDA (B) and di-OH-MA (C), respectively, in HLM. CYP2D6 UM, pooled (pHLM) or PM HLM were incubated with MDMA (100 µM) at 37°C for 240 min. Each symbol and error bars denote the mean ± S.E.M. of N=3-4 experiments. *P < 0.05; **P < 0.01 compared with pHLM. †P < 0.05; ††P < 0.01; †††P < 0.001 for UM compared with PM.

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Fig. 5.

Proposed pathway for the metabolism of PMMA in humans. The figure is based on the present study in HLM and in blood samples from fatal PMMA intoxications, and on previously published studies in rodents and humans. The major enzymes presumed to be involved in PMMA metabolism are included in cursive. (Maurer et al., 2000a; Easton et al., 2003; Staack et al., 2003; de la Torre and Farre, 2004; Staack et al., 2004a; Staack et al., 2004b; Staack and Maurer, 2005; Kuwayama et al., 2009; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b; Palenicek et al., 2011).

PMMA, para-methoxymethamphetamine; OH-MA, 4-hydroxymethamphetamine; di-OH-MA, dihydroxymethamphetamine; HM-MA, 4-hydroxy-3-methoxymethamphetamine; PMA, para-methoxyamphetamine; OH-A, 4-hydroxyamphetamine; di-OH-A, dihydroxyamphetamine, HM-A, 4-hydroxy-3-methoxyamphetamine, HLM, human liver microsomes; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase.

TABLES

Table 1

Method performance: lower limit of quantification (LLOQ), calibration range, between assay precision (RSD) and accuracy (Bias)

Analyte	LLOQ (µM)	Calibration range (µM)	QC low			QC medium			QC high		
			Mean	RSD	Bias	Mean	RSD	Bias	Mean	RSD	Bias
PMMA	0.025	1-150	2.1	11 %	3 %	23	20 %	13 %	100	10 %	-1 %
PMA	0.01	0.01-3	0.028	23 %	-6 %	0.71	25 %	-12 %	7.1	20 %	-12 %
MDMA	0.05	1-150	4.6	12 %	13 %	44	15 %	9 %	111	9 %	11 %
MDA	0.025	0.025-7.5	0.069	19 %	-6 %	1.8	10 %	-10 %	18	22 %	-10 %
Methamphetamine	0.05	1-150	4.1	10 %	3 %	39	12 %	-2 %	101	8 %	1 %
Amphetamine	0.05	0.05-15	0.070	19 %	-4 %	1.8	6 %	-9 %	18	7 %	-8 %
OH-MA	0.1	0.1-100	0.13	18 %	-9 %	3.6	13 %	-9 %	37	17 %	-8 %
OH-A	0.01	0.01-1.7	0.013	31 %	-15 %	0.41	14 %	0 %	3.5	17 %	-15 %
Di-OH-MA	0.01	0.01-3	0.030	20 %	2 %	0.79	13 %	-1 %	6.6	14 %	-17 %
Oxilofrine	0.01	0.01-1.5	0.015	17 %	-3 %	0.41	12 %	-2 %	2.6	16 %	-36 %
HM-MA	0.01	0.01-1.5	0.015	19 %	-3 %	0.40	22 %	0 %	3.6	13 %	-11 %
HM-A	0.005	0.005-0.76	0.0077	19 %	10 %	0.20	8 %	2 %	2.0	14 %	-2 %

Table 2

Individual blood concentrations of PMMA and metabolites in three fatal PMMA intoxications[a]

	#1		#2		#3	
Concentration (µM)	Unbound	Hydrolyzed	Unbound	Hydrolyzed	Unbound	Hydrolyzed
PMMA	6.9		7.4		26.4	
PMA	0.02		0.2		2.5	
OH-MA	0	0.09	0.4	2.7	1.9	23.1
OH-A	0	0.03	0	0.05	0.3	1.1
Oxilofrine	0	0	0	0.01	0	0.02
HM-MA	0	0	0	0	0.03	0.3
HM-A	0	0	0	0	0	0.02

PMMA, para-methoxymethamphetamine; PMA, para-methoxyamphetamine; OH-MA, 4-hydroxymethamphetamine; OH-A, 4-hydroxyamphetamine; HM-MA, 4-hydroxy-3-methoxymethamphetamine; HM-A, 4-hydroxy-3-methoxyamphetamine.

[a] Fatal PMMA intoxications in which no methamphetamine, amphetamine or MDMA was detected in femoral blood, since these drugs have several metabolites in common with PMMA

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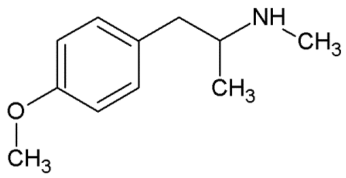
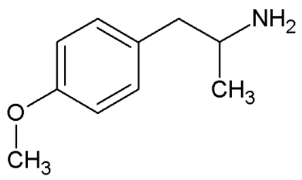
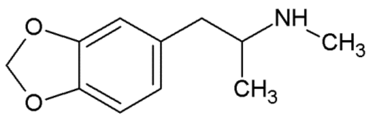


Para-methoxymethamphetamine (PMMA)



Para-methoxyamphetamine (PMA)



Methylenedioxymethamphetamine (MDMA)

Figure 1

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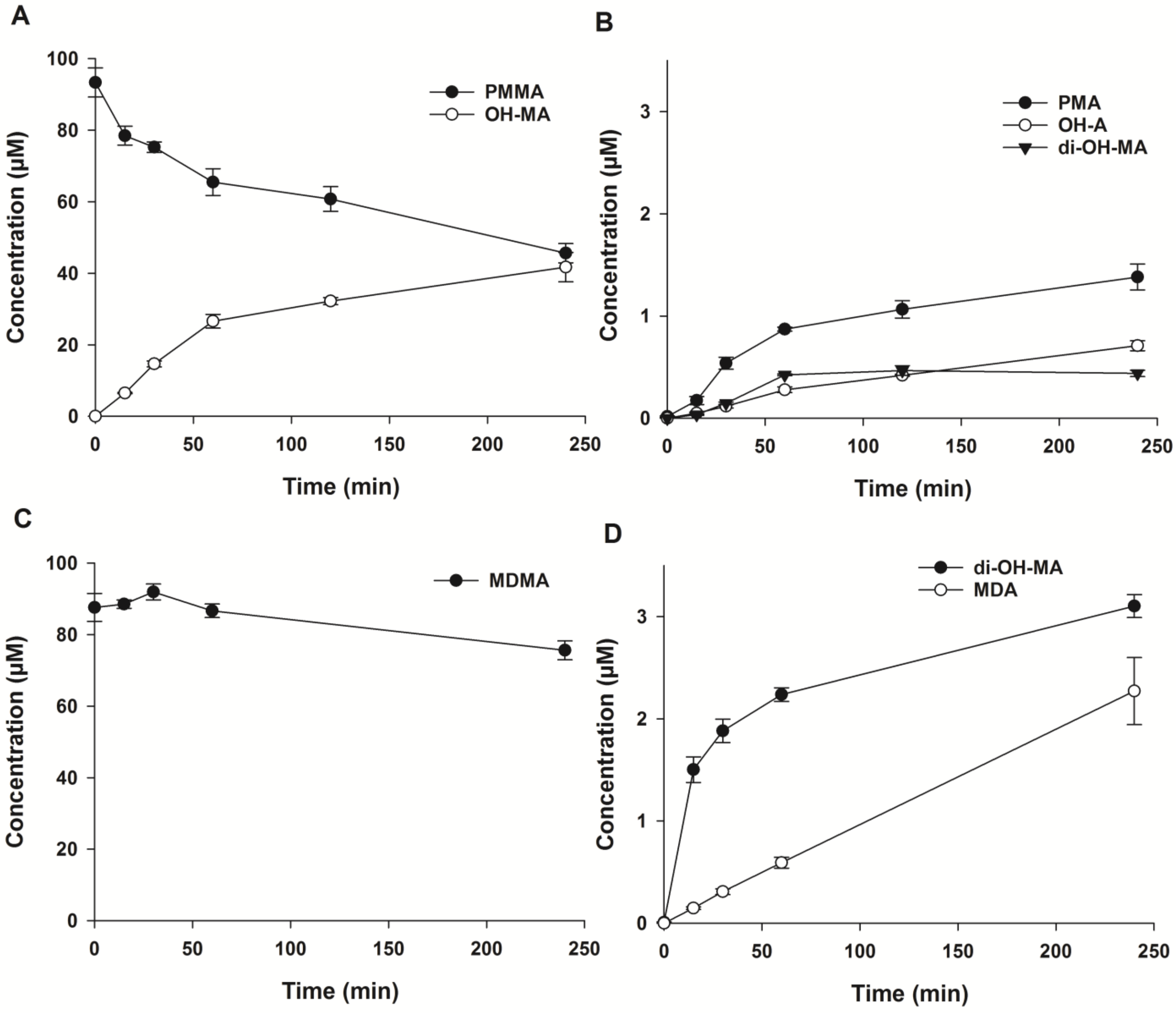

Figure 2

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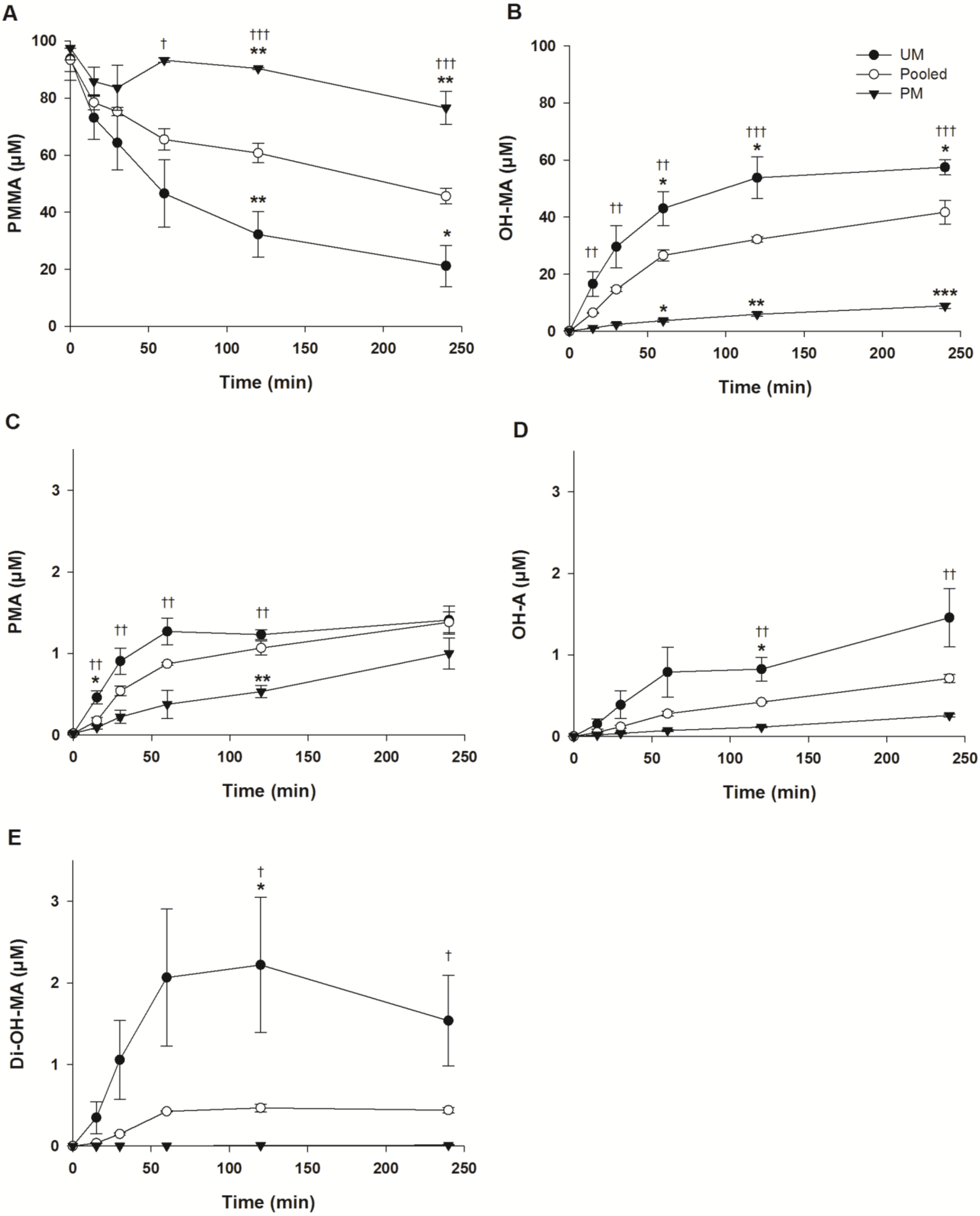

Figure 3

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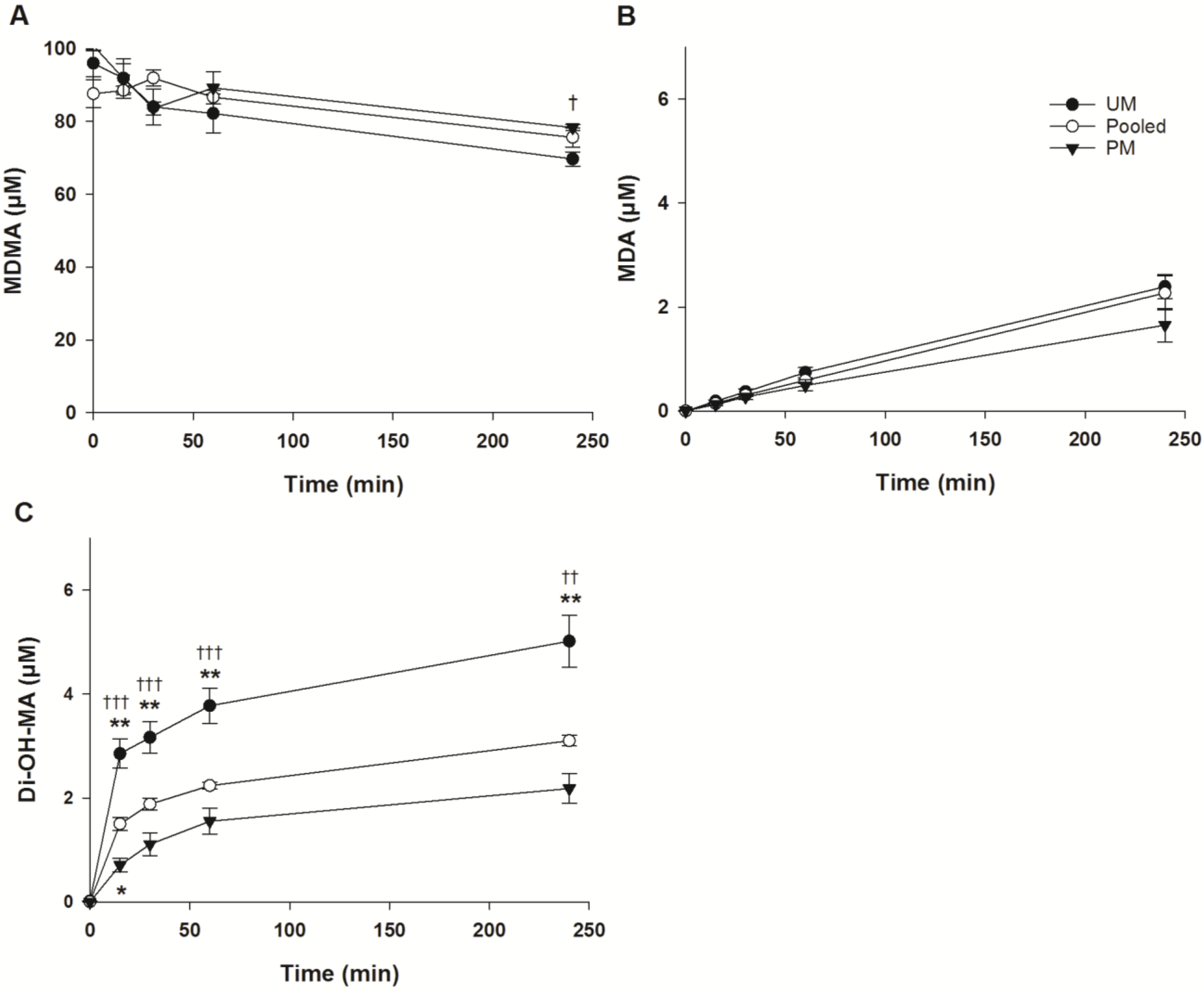

Figure 4

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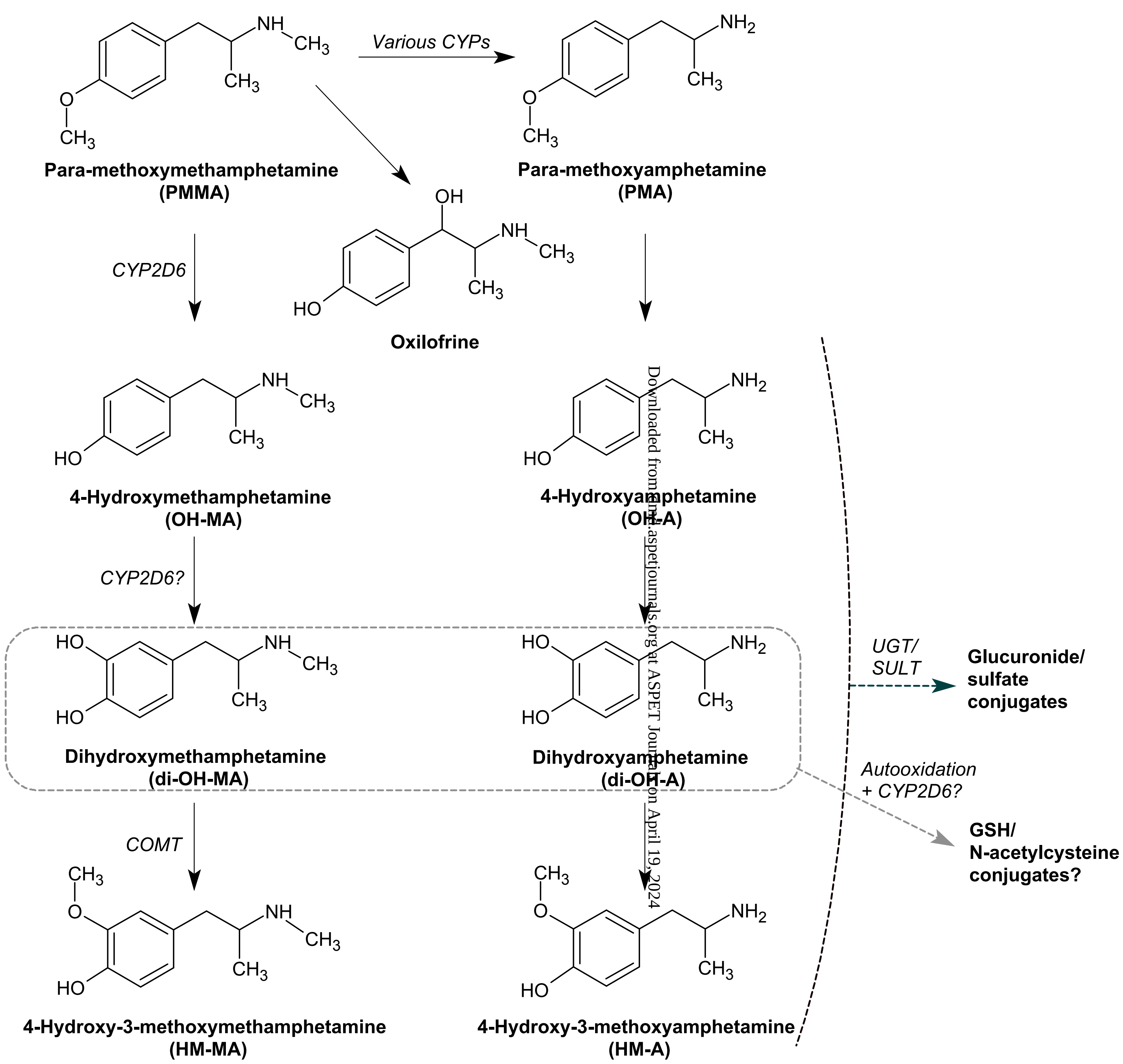

Figure 5

Journal title

Drug Metabolism and Disposition

Title

Studies on para-methoxymethamphetamine (PMMA) metabolite pattern and influence of CYP2D6 genetics in human liver microsomes and authentic samples from fatal PMMA intoxications

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SUPPLEMENTAL TABLE 1

Characteristics of the pooled and the individual donor HLM[a]

CYP2D6 classification	Lot/ID	CYP2D6 activity[b]	CYP2D6 alleles	Gender	Ethnicity	Age	CYP content[c]
Pooled	1210223 1210347 1110258 1410230	259 ± 5 - 294 ± 39		50:50	Caucasian[d]	[e]	394-478
PM	499	58.9 ± 3.3	*4/*4	Female	Caucasian	55	386
	486	55.2 ± 1.3	*4/*5	Female	Caucasian	49	503
	502	10.8 ± 0.9	*4/*4	Male	Caucasian	49	160
UM	289	957 ± 19	*1/*2x2	Female	Caucasian	60	487
	432	342 ± 4	*2x2/*41 or *2/*41x2	Male	African American	60	360

ID, Individual number of individual donors; PM (marketed as “CYP2D6 No activity”), CYP2D6 poor metabolizers; pooled (marketed as “XTreme 200”), pool of human liver microsomes from 200 individuals; UM (marketed as “CYP2D6 High activity”), CYP2D6 ultrarapid metabolizers.

[a] *All data provided by and published with consent from the supplier XenoTech*

[b] *CYP2D6 activity rate (pmol/mg protein/min). According to XenoTech: “Marker substrate reaction for this rate was dextrometorphan O-demethylation. Values for enzyme activities are mean ± standard deviation of three or more determinations.”*

[c] *Cytochrome P450 content (pmol/mg protein)*

[d] *84-88% Caucasians, the others mainly Hispanic*

[e] *All ages*

SUPPLEMENTAL TABLE 2

Instrumental parameters

Analyte	Target ion/qualifier ion transition	Cone voltage (V)	Collision energy (eV)
PMMA	180.2>121.1	15	18
	180.2>149.1	15	12
PMA	166.1>121.1	15	18
	166.1>149.1	15	12
MDMA	194.12>133.07	20	14
	194.12>163.08	20	14
MDA	180.1>135.04	15	10
	180.1>163.08	15	10
Methamphetamine	150.1>91.1	15	20
	150.1>119.1	15	15
Amphetamine	136.1>91.1	14	20
	136.1>119.1	14	15
OH-MA	166.1>107.1	18	20
	166.1>135.1	18	13
OH-A	152.1>107.1	15	18
	152.1>135.1	15	10
Di-OH-MA	182.1>123.1	15	18
	182.1>151.1	15	20
Oxilofrine	182.0>149.0	20	30
	182.0>164.0	20	15
HM-MA	196.1>137.1	22	26

	196.1>165.1	22	13
HM-A	182.1>137.1	22	26
	182.1>165.05	15	15
$^{13}C_6$ PMMA	186.2>127.1	15	18
	186.2>155.1	15	12
$^{13}C_6$ PMA	172.1>127.1	15	18
	172.1>155.1	15	12
$^{13}C_6$ MDMA	200.1>139.1	20	14
	200.1>169.1	20	14
$^{13}C_6$ MDA	186.1>141.1	15	15
	186.1>169.1	15	10
$^{13}C_6$ methamphetamine	156.1>97.1	15	20
	156.1>125.1	15	15
$^{13}C_6$ amphetamine	142.1>97.1	14	20
	142.1>125.1	14	15
$^{13}C_6$ 2C-B	266.1>140.0	20	15
	266.1>249.0	15	15
	266.1>251.0	20	15

SUPPLEMENTAL TABLE 3

Extraction recovery and matrix effects of quality control (QC) samples and internal standards (IS) in human liver microsomes[a]

	Recovery (%)	Matrix effect (%)
PMMA	98	102
PMA	86	115
MDMA	99	102
MDA	96	106
Methamphetamine	99	102
Amphetamine	94	109
OH-MA	98	104
OH-A	96	130
Di-OH-MA	94	96
Oxilofrine	92	71
HM-MA	94	109
HM-A	99	123
$^{13}C_6$ PMMA	84	104
$^{13}C_6$ PMA	83	104
$^{13}C_6$ MDMA	84	102
$^{13}C_6$ MDA	86	102
$^{13}C_6$ methamphetamine	86	102
$^{13}C_6$ amphetamine	85	104
$^{13}C_6$ 2C-B	79	104

[a] Data represent the mean of six experiments at high and low QC concentration levels and of IS, respectively, using 5 different lots of pooled and single donor human liver microsomes