Para-methoxymethamphetamine (PMMA): A study of risk factors for fatal intoxication with emphasis on metabolite pattern and CYP genetics

PhD thesis
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“Everything is a poison and nothing is without poison; only the dose makes that a thing is no poison”.

— Theophrastus Bombastus von Hohenheimen (Paracelsus)

“Ever tried
Ever failed
No matter
Try again
Fail again
Fail better”

— Samuel Beckett
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyl-transferase</td>
</tr>
<tr>
<td>CYP P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>di-OH-A</td>
<td>3,4-dihydroxyamphetamine (dihydroxyamphetamine, α-methylamphetamine)</td>
</tr>
<tr>
<td>di-OH-MA</td>
<td>3,4-dihydroxymethamphetamine (dihydroxymethamphetamine)</td>
</tr>
<tr>
<td>DFS</td>
<td>Department of Forensic Sciences, Oslo University Hospital, Oslo</td>
</tr>
<tr>
<td>EM</td>
<td>extensive metabolizer</td>
</tr>
<tr>
<td>HLMs</td>
<td>human liver microsomes</td>
</tr>
<tr>
<td>HM-A</td>
<td>4-hydroxy-3-methoxyamphetamine</td>
</tr>
<tr>
<td>HM-MA</td>
<td>4-hydroxy-3-methoxymethamphetamine</td>
</tr>
<tr>
<td>Ki</td>
<td>inhibitory potency, the substrate concentration required to give half-maximal inhibition</td>
</tr>
<tr>
<td>MAO-A</td>
<td>monoamine oxidase type A</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-methylenedioxyamphetamine</td>
</tr>
<tr>
<td>MR</td>
<td>CYP2D6 metabolic ratio (OH-MA/PMMA)</td>
</tr>
<tr>
<td>NET</td>
<td>noradrenaline transporter</td>
</tr>
<tr>
<td>OH-A</td>
<td>4-hydroxyamphetamine</td>
</tr>
<tr>
<td>OH-MA</td>
<td>4-hydroxymethamphetamine</td>
</tr>
<tr>
<td>pHLM</td>
<td>pooled human liver microsomes</td>
</tr>
<tr>
<td>PM</td>
<td>poor metabolizer</td>
</tr>
<tr>
<td>PMA</td>
<td>para-methoxyamphetamine (4-methoxyamphetamine)</td>
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<tr>
<td>PMMA</td>
<td>para-methoxymethamphetamine (4-methoxymethamphetamine)</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>SULT</td>
<td>sulfotransferase</td>
</tr>
<tr>
<td>UGT</td>
<td>uridine diphosphate glucuronosyltransferase</td>
</tr>
<tr>
<td>UHPLC-MS/MS</td>
<td>ultra-high performance liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>UM</td>
<td>ultrarapid metabolizer</td>
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Our interest in the synthetic designer drug para-methoxymethamphetamine (PMMA, 4-methoxymethamphetamine) and other novel psychoactive designer drugs started suddenly in July 2010. A cluster of unnatural deaths in young adults in Norway caught our attention because they were apparently related to amphetamine/methamphetamine use, which are not particularly toxic drugs. Supplementary analyses were performed and revealed the presence of the toxic drug PMMA in the blood. During the following months, an outbreak emerged in Norway of more than 30 fatal PMMA-related intoxications. Although public warnings were rapidly issued, the year 2010 marked the beginning of the novel psychoactive designer drug era in Norway and Europe, with 50–100 more or less toxic novel drugs of abuse popping up annually in Europe.

We were surprised by the high toxicity and limited toxicological knowledge of PMMA compared with the widely abused analogue drugs methamphetamine/amphetamine and MDMA (3,4-methylenedioxymethamphetamine). In particular, we noticed a partial overlapping between fatal and non-fatal PMMA concentrations in the blood, and some subjects in the latter group were judged as not impaired despite having rather high PMMA concentrations. This suggested the potential involvement of protective or disposing toxicity factors. In light of my recently acquired research experience regarding drug interactions on and inherited variability in drug-metabolizing cytochrome P450 enzymes, we consecutively forwarded the blood samples from some fatal and non-fatal PMMA cases for CYP genotyping, with interesting results. These preliminary studies and the quest for a greater knowledge about toxicological risk factors for a fatal outcome of PMMA intake evolved into the present PhD work.
1 Introduction

As a background for understanding the present toxicological study of the methamphetamine derivative PMMA, a brief overview will be given of the knowledge that was available at the beginning of our study in regards of the following topics: normal brain function and monoamine signaling, actions and metabolism of amphetamine-type drugs, and PMMA pharmacology.

1.1 Normal brain function and monoamine signaling

The normal signaling between neurons in the brain is mediated by chemical neurotransmitters such as the generally stimulating glutamate or the inhibiting GABA (gamma-aminobutyric acid). The monoamine neurotransmitters dopamine, noradrenaline and serotonin are involved in the regulation of higher brain functions such as motivation, reward, movement, emotions, energy, thought control, body temperature, memory, and sleep (Fig. 1). The monoamines have in common an amino group (-NH₂) connected to an aromatic phenyl ring by a two-carbon ethyl chain (-CH₂-CH₂-). There are two groups of monoamine neurotransmitters, phen-ethyl-amines (dopamine and noradrenaline) and tryptamines (serotonin). Phenethylamine as such is a natural amphetamine analogue with psychostimulant effects (Mantle et al., 1976), whereas tryptamine is a natural neuromodulator (Yu et al., 2003a) (Fig. 1).
Figure 1. Molecular structures of the monoamine neurotransmitters dopamine, noradrenaline, and serotonin, and of the natural analogues phenethylamine and tryptamine.

In the brain there are separate dopaminergic, noradrenergic, and serotonergic neurons (Eiden and Weihe, 2011). To elicit a normal signal between neurons, synaptic vesicles loaded with monoamines in the presynaptic neuron are rapidly fused with the cell membrane, and the neurotransmitter is released into the synaptic cleft between the neurons (Wimalasena, 2011). The monoamines bind to specific monoamine receptors on the postsynaptic neuron and evoke a cascade of responses. Within milliseconds after release, the neurotransmitter actions are terminated by reuptake into the presynaptic neuron, where the neurotransmitters are recycled into vesicles or degraded by the enzyme monoamine oxidase A (MAO-A). There are separate reuptake transporters for each monoamine, including the serotonin transporter (SERT), noradrenaline transporter (NET), and dopamine transporter (DAT), whereas the monoamine reuptake transporter VMAT2 in the synaptic vesicle membrane is common for all of the monoamines (Partilla et al., 2006). MAO-A is located in the outer mitochondrial membrane, and it inactivates the monoamines. This enzyme has a high affinity for serotonin, noradrenalin, and adrenalin, whereas dopamine is metabolized both by MAO-B and MAO-A enzymes. Thus, the VMAT2 and the MAO enzymes are key regulators for the rapid termination of synaptic signaling and the maintenance of normal brain chemistry and function. The intracellullar enzyme catechol-O-methyl-transferase (COMT) also contributes to the termination of neurotransmission signaling by methylation of
The main actions of monoamine neurotransmission under natural conditions are summarized in Fig. 2. Dopamine mediates reward, motivation to repeat a behavior, and dependency. Dopaminergic neurons are located in the midbrain, and dopamine is released into the brain’s reward pathways when we succeed, are in love, have sex, etc. Noradrenergic neurons are located in locus ceruleus, and noradrenaline is released into many parts of the central nervous system when we face fearful situations in order to mediate arousal and energy production in preparation for a fright, fight, or flight response (Sofuoglu and Sewell, 2009). Serotonin is considered to be “involved in everything but responsible for nothing” (Jacobs and Fornal, 1999). Serotonergic neurons are located in the brain stem, and serotonin is released in many parts of the brain and contributes to the regulation of mood, sleep, thought control, muscle function, and body temperature, and might also be released by exercise and sunlight (Sansone and Sansone, 2013).

**Figure 2.** Main actions of monoamine neurotransmission under natural conditions, and under the influence of amphetamines.
1.2. Actions of amphetamine-type drugs

Amphetamine, methamphetamine, MDMA, and the structural derivatives of these drugs are all termed amphetamines and have a close structural resemblance to the natural monoamine neurotransmitters. When present in the brain and body, the amphetamines have in common that they severely and persistently increase the neurotransmission of one or more of the monoamines, and the monoamine levels in the synaptic clefts may increase up to tens or even hundreds of times compared with natural conditions (Robertson et al., 2009) (Fig. 2 right side). Highly increased dopamine levels induce euphoria and drug dependency, whereas highly increased noradrenaline levels induce a severe stress response in the brain, sympathetic nervous system and body with increased heart rate and blood pressure, fright, agitation, and dilated pupils (Sofuoglu and Sewell, 2009). Highly increased serotonin levels induce convulsions, coma, and hyperthermia, and this is referred to as serotonin syndrome (section 1.2.1). Amphetamines with agonist effects on serotonin receptors might also induce hallucinations and psychosis (Sun-Edelstein et al., 2008).

The increased monoaminergic neurotransmission by amphetamines is mediated by one or more of the following mechanisms (Matsumoto et al., 2014; Rickli et al., 2015):

A. Reduced synaptic and vesicular reuptake of transmitters, by inhibition of the presynaptic transporters DAT, NET, and/or SERT and the vesicular transporter VMAT2.

B. Increased synaptic release and increased cytoplasmic levels of transmitters, by reversed action of the presynaptic and vesicular transporters and by direct diffusion through the cell membrane. This occurs with small drug molecules that act as substrates for the transporters and thus are co-transported into the cells and vesicles together with ions, which induces depolarization of the membranes and reverse monoamine transport (Cameron et al., 2015; De Felice, 2016).

C. Inhibition of the MAO-A enzyme, resulting in severely increased levels and persistent effects of monoamines (Clark et al., 1964; Freezer et al., 2005; Matsumoto et al., 2014; Smythies et al., 1967; Vuori et al., 2003).

D. Direct agonist effects of the drug on postsynaptic serotonin receptors, mainly 5-HT2A/2C.
Most amphetamines are indirect sympathomimetics that act with diverse potencies mainly through mechanisms A and B with or without C, while some novel psychostimulants also act as direct sympathomimetics with agonist effects on postsynaptic receptors according to mechanism D (Rickli et al., 2015). Large drug molecules like 3,4-methylenedioxypyrovalerone (MDPV), however, become “trapped” in the transporters like a sock in a vacuum cleaner and act as inhibitors only and not as substrates. Accordingly, most amphetamines are transported into the neurons like false transmitters and they become trapped intracellularly in the cytoplasm and vesicles. Based on structure-activity considerations, it is possible to predict the major effects of novel amphetamines in humans.

1.2.1. **Serotonin syndrome**

Serotonin syndrome may develop rapidly within hours, and the clinical manifestations range from mild to life-threatening or fatal depending on the degree of serotonin overstimulation (Fig. 3). The typical triad of symptoms encompasses neuromuscular excitation, from tremors in the lower extremities to convulsions; changed mental status, from anxiety to coma; and autonomic excitation, from nausea to a body temperature above 43°C. The symptoms are mediated by overstimulation of serotonin receptors both centrally and peripherally, particularly overstimulation of 5-HT$_{2A}$ receptors (Sun-Edelstein et al., 2008), but when the serotonin level is highly increased the receptor specificity is reduced resulting in noradrenergic and dopaminergic overstimulation as well.
1.3. Metabolism of amphetamine-type drugs

Amphetamines are lipid soluble and can penetrate the blood-brain barrier. They are metabolized in the body to form water-soluble and less toxic compounds that are excreted mainly by the kidneys. Metabolism of amphetamines involves oxidation, reduction or hydrolysis mediated by hepatic cytochrome P450 (CYP) enzymes, by removing methyl groups (-CH3, demethylation) or adding hydroxyl groups (-OH, hydroxylation). Hydroxylated metabolites are further conjugated with glucuronide, sulfate, or glutathione molecules before excretion, and this is mediated by UGT (uridine diphosphate glucuronosyltransferase), SULT (sulfotransferase), or glutathione S-transferase enzymes (Easton et al., 2003; Kraemer and Maurer, 2002; Monks et al., 2004). The CYP enzyme isoforms 2D6, 2C9, and 2C19 metabolize 80% of common drugs (de la Torre et al., 2012). The catalytic activity of CYP enzymes, called the CYP “phenotype”, is determined by inheritance, i.e. the CYP “genotype”, and by environmental factors like drugs, food compounds, or tobacco smoke, which can instantaneously inhibit or gradually induce CYP enzyme activity. There is a 10–20-fold inter-individual genetic variability in the activity of these enzymes, and
variability in CYP genotypes and phenotypes explains much of the diversity regarding drug efficacy and toxicity at a specific drug dose. Amphetamines are not metabolized by the MAO-A enzyme, which has a low substrate affinity for this enzyme. This is probably due to the presence of a methyl group on the alpha-carbon of the side chain of amphetamines that protects the drugs from being metabolized by this enzyme (Iversen, 2007).

1.3.1. \textit{CYP2D6}

The CYP2D6 enzyme accounts for only 1–2\% of the total hepatic CYP proteins but catalyzes the metabolism of approximately 20–30\% of all prescribed drugs (O'Mathuna et al., 2008; Yeo et al., 2003). Amphetamines as a class interact with CYP2D6 enzymes with variable affinities both as substrates and inhibitors (Wu et al., 1997). CYP2D6 is mainly localized in the liver but to a minor extent also in the intestines, kidneys, and the brain (Kirchheiner et al., 2011; Siegle et al., 2001). The CYP2D6 enzyme exhibits a marked genetic polymorphism, and early observations in the 1970s of an individual with deficient formation of 4-hydroxyamphetamine (OH-A) from the amphetamine derivative para-methoxyamphetamine (PMA) contributed to this discovery (Kitchen et al., 1979). More than 90 CYP2D6 gene alleles have been described, and subjects can be classified into four inherited phenotypes of enzyme activity: CYP2D6 poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), or ultrarapid metabolizers (UM) (Zanger et al., 2004). In Caucasian populations, the frequencies are approximately 7\%, 40\%, 50\%, and 1–3\%, respectively (Bertilsson, 1995; Zanger et al., 2004) (http://www.cypalleles.ki.se/cyp2d6.htm). The CYP2D6 PM genotype is caused by deleted or mutated and non-functional CYP2D6 alleles leading to the absence of the enzyme or a functionally inactive enzyme, whereas gene duplications or hyperfunctional alleles cause the CYP2D6 UM phenotype with increased enzyme expression (Lovlie et al., 2001). Due to drug interactions with the CYP2D6 enzyme, genetic CYP2D6 EM individuals with two normal alleles may phenotypically become PM when exposed to a potent CYP2D6 enzyme inhibitor like MDMA (ecstasy), and it takes about 10 days for the body to synthesize new active enzyme (O'Mathuna et al., 2008; Perfetti et al., 2009; Yang et al., 2006). The female Dark Agouti rat is widely used as an animal model for the CYP2D6 PM phenotype in humans, whereas males of other rat strains such as Sprague Dawley or Wistar serve as models for the CYP2D6 EM phenotype (Schulz-Utermoehl et al., 1999).
1.3.2. Other CYPs

CYP isoenzymes of the 2C and 3A subfamilies also have the capacity to metabolize ring-substituted amphetamines, which might be of importance in CYP2D6 PM individuals and might contribute to inter-individual differences in drug action (Kreth et al., 2000; Yamada et al., 2005). The CYP2C9 enzyme accounts for about 20% of the total hepatic CYP protein content and metabolizes approximately 15% of clinically used drugs (Wang et al., 2009), whereas CYP3A4/3A5 account for about 15-30% of the hepatic CYP content and metabolize about 30-40% of CYP-metabolized medications (Klein and Zanger, 2013).

1.4. PMMA

PMMA (para-methoxymethamphetamine) is an illegal drug belonging to the phenethylamine class of amphetamines (Fig. 4). At the beginning of the present study, the pharmacological and toxicological properties of PMMA in humans were still largely uncharacterized. PMMA was reported to have weak MDMA-like effects or mainly unpleasant effects and a higher toxicity than the widely abused MDMA and methamphetamine.

![Molecular structures of PMMA, methamphetamine, and MDMA.](image)

**Figure 4.** Molecular structures of PMMA, methamphetamine, and MDMA.

1.4.1. Chemistry of PMMA

PMMA is a derivative of methamphetamine or MDMA with one methoxy-group (-OCH₃) substituted in the para-position (4-position) of the phenyl ring (Fig. 4). Ring-substitution in the para-position is reported to add serotonergic and hallucinogenic properties to amphetamine drugs (Matsumoto et al., 2014; Simmler et al., 2014; Smythies et al., 1967).
PMMA was first synthesized in 1938 (Hildebrandt G., German Patent 665.793, unpublished), but it never had any medical use owing to its high toxicity. It is a white or yellowish powder and is typically found as a hydrochloride salt. It is invariably sold as tablets or powder purported to be “amphetamine” or “ecstasy”, and thus the users are unaware that they are taking this toxic drug. Tablets with PMMA are often sold with various logos (Fig. 5). PMMA is partially metabolized to PMA, the para-methoxylated derivative of amphetamine. PMA has similar or more potent effects compared with PMMA. PMMA and PMA are associated with the slang names “Dr. Death” and “Killer” (Cimbura, 1974). PMMA pills typically contain 20–100 mg PMMA (EMCDDA, 2003), while users on the Internet report using 50–230 mg of uncertain purity. The molecular weight of PMMA free base is 179.3 g/mol. PMMA has one chiral center and appears as two optical enantiomers, R(−) and S(+) PMMA. Findings in rats suggest that the S(+) enantiomer of PMMA is the more active compound (Young et al., 1999).

Figure 5. PMMA powder (left, printed with permission from A. Westin, St. Olavs hospital) and tablets with the Superman logo, of which some contained PMMA and some contained MDMA (right, printed with permission from the National Criminal Investigation Service, Norway).

1.4.2. Metabolism of PMMA

There are no controlled experimental study reports on the in vivo pharmacokinetics of PMMA in humans. The existing knowledge is limited to experimental studies in rats (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b;
Staack et al., 2003) and two studies in human liver microsomes (HLMs) (Lai et al., 2015; Staack et al., 2004b).

The time disposition and kinetic profile of PMMA in blood and tissues (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b) and the metabolism pattern in urine (Staack et al., 2003) was studied in male Wistar rats, a model of the human CYP2D6 EM phenotype. PMMA was administered in doses from 5 to 40 mg/kg by the subcutaneous or gastric route, and the highest dose imitated a state of non-fatal intoxication comparable to a human recreational dose of 100 mg PMMA (Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). The highest PMMA dose was rapidly absorbed with a peak plasma PMMA concentration of 22 μM half an hour after dosing, whereas the appearance of metabolites was delayed and peaked in plasma two hours after administration (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). After administration of a low dose, the PMMA concentrations in brain and serum peaked simultaneously, whereas the peak in the brain was delayed 30 minutes compared to the peak in serum after the high dose and was achieved one hour after intake (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). PMMA was extensively metabolized in rats with a plasma half-life of one hour, a volume of distribution of 6.4 L/kg, and a plasma clearance of 4.4 L/hour (Rohanova and Balikova, 2009a). PMMA accumulated in the tissues with concentrations greatly exceeding those in the blood. The highest brain/plasma concentration ratio of 16 occurred eight hours after PMMA administration (Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). Of the tissues studied, the highest PMMA concentrations were detected in the lungs > brain > liver after one hour and in plasma after 30 minutes (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). The penetration through the blood-brain barrier was more efficient for PMMA/PMA than for the water-soluble hydroxylated metabolites 4-hydroxymethamphetamine (OH-MA) and OH-A, and the maximum level of OH-MA constituted only 11% of the maximum PMMA in the brain (Honecker and Coper, 1975; Lewander, 1971; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). In studies using human liver microsomes and baculovirus-infected insect cell microsomes, Staack found that CYP2D6 was the only CYP enzyme catalyzing the metabolism of PMMA to OH-MA (Staack et al., 2004b).
1.4.3. **Effects and toxicity of PMMA**

For unknown reasons, PMMA (Fig. 6) appears to be more toxic than structurally similar drugs such as MDMA, and it has a narrower margin of safety (Steele et al., 1992). Toxicity is for both drugs mainly related to increased serotonergic neurotransmission (Staack et al., 2004a). There are no controlled experimental study reports on the clinical effects of PMMA in humans, and the existing knowledge is limited to published user reports, rodent studies, in vitro studies, and observational studies of PMMA intoxications, supported by structure-activity considerations as mentioned above.

![Figure 6. Seizure of PMMA (printed with permission from L. O. Kristiansen, Moss police station).](image)

**User reports**

PMMA is thought to induce mainly unpleasant effects, whereas the desired euphoria, mental stimulation, and hallucinations are weak or absent. In a self-dosing experiment in the 1980s, the American pharmacologist Alexander Shulgin – dubbed the “godfather of Ecstasy” – ingested 110 mg of PMMA. He experienced unpleasant effects like eye muscle disturbances, compulsive yawning, high pulse, and elevated blood pressure, which lasted for four hours, but no central stimulant effects (Shulgin and Shulgin, 1990). Drug users
administering PMMA as confirmed by analysis of tablets or powder reported no “high”, and instead reported unpleasant effects or intense bodily discomfort such as eye muscle disturbances, mental restlessness, palpitations, sweating, nausea, hallucinations, hyperthermic seizures, and one day of “blackout” (Brunt et al., 2012; Westin and Brede, 2011). The mean PMMA content in the tablets was 44 mg (range 5–130 mg) (Brunt et al., 2012). According to an Internet report, ingestion of a “PMMA” dose of 150 mg resulted in severe negative physical effects, whereas another subject found 215 mg to be similar to taking MDMA (EMCDDA, 2003). Several of the five volunteers reported sedating effects of PMMA.

**Rodent studies**

PMMA induces weaker stimulation in rats than PMA (Glennon et al., 1988; Glennon et al., 2007; Glennon et al., 1997; Young et al., 1999). A PMMA dose of 30 mg/kg produces no significant stimulation in the rats (Glennon et al., 1988), whereas 40 mg/kg imitates a state of non-fatal intoxication in humans, and 40-80 mg/kg triggers stimulation and hyperactivity in the rats (Palenicek et al., 2011; Rohanova and Balikova, 2009a; Rohanova and Balikova, 2009b). Half of the rats die at PMMA doses of 53–63 mg/kg (Glennon et al., 1988) or 80–100 mg/kg (Steele et al., 1992). Accordingly, the fatal dose is only 2–4 times higher than the behaviorally active dose, and although there is a scientific controversy as to the power of extrapolating from rodents to humans (de la Torre and Farre, 2004; Shanks et al., 2009), a similarly narrow margin between activity and toxicity might exist in humans (Steele et al., 1992). The corresponding dose margins for MDMA seem to be wider (Peroutka, 1990). Repeated dosing of PMMA causes lasting detrimental effects on serotonergic neurons (Steele et al., 1992).

**In vitro studies**

During the course of our study, new knowledge was published by other authors regarding the mechanisms of actions for PMMA in humans. The main actions are increased serotonergic and noradrenergic synaptic transmission in the central nervous system and the body, mediated by mechanisms A, B, and C as presented in section 1.2 (Liechti, 2015; Matsumoto et al., 2014; Simmler et al., 2014). Similar to MDMA, PMMA is a potent inhibitor
of serotonin and noradrenaline reuptake transporters, and half-maximal inhibition was achieved at a PMMA concentration of 1-2 μM. PMMA is also a substrate for these two transporters and induces release of serotonin and noradrenaline through the transporters, whereas the potency for inhibiting the dopamine reuptake transporter is very low with a DAT/SERT ratio of 0.04 (Liechti, 2015; Simmler et al., 2014). A low DAT/SERT ratio is associated with increased toxicity and a reduced potential for addiction and reinforcement (Wee et al., 2005) in comparison with the highly addictive drug methamphetamine, which has a DAT/SERT ratio above 10. PMMA might also induce release of dopamine, but only at high PMMA concentrations (Simmler et al., 2014). Owing to the potent inhibition of the MAO-A enzyme, dopaminergic neurotransmission will also be somewhat increased by PMMA, which might explain why the drug can induce a slight euphoria (Matsumoto et al., 2014). PMMA is not a serotonin receptor agonist, as demonstrated by no relevant binding to 5-HT_{1A}, 5-HT_{2A}, or 5-HT_{2C} receptors (Simmler et al., 2014). This fits well with the weak or absent hallucinogenic effects of PMMA. For comparison, classical hallucinogens like mescaline activate 5-HT_{2} receptors (Glennon et al., 1992; Glennon et al., 1984).

**Observational studies**

Prior to our study, there were at least 39 fatal PMMA-related intoxications registered in the world. The first reported PMMA-related death occurred in Spain in 1993 (Lora-Tamayo et al., 1997), followed by one in Austria in 2000 (EMCDDA, 2003), three in Denmark, one in Germany, and one in Norway in 2003 (Becker et al., 2003; Johansen et al., 2003; Refstad, 2003), eight in Taiwan in 2006 (Lin et al., 2007), and 24 in Israel in 2007–2008 (Lurie et al., 2012). Serotonergic signs and symptoms were the main clinical features of these deaths (EMCDDA, 2003). At the present time, a total of 131 analytically confirmed deaths involving PMMA have been reported globally, 96 of which were in Europe, 27 in Canada, and 8 in Taiwan (WHO, 2015).

**Structure-activity considerations**

At the beginning of our project, PMMA was believed to be a potent inhibitor of the MAO-A enzyme based on structural similarity to PMA (Green and El Hait, 1980; Scorza et al., 1997).
The toxicity of amphetamines is demonstrated to be severely increased when MAO-A is inhibited (Clark et al., 1964; Hewton et al., 2007; Smythies et al., 1967; Vuori et al., 2003).
2 Study aims

The general aim of this doctoral thesis was to study the risk factors for a fatal outcome of PMMA intoxication with an emphasis on PMMA metabolite pattern and CYP genetics.

The specific aims were:

I. To characterize the clinical manifestations of fatal PMMA intoxication in humans (papers I and II).

II. To investigate potential risk factors for a fatal outcome of PMMA intoxication (papers I and II).

III. To study if there is an association between CYP genotype/phenotype, PMMA metabolism, and risk of fatal PMMA intoxication in humans (paper II).

IV. To examine the PMMA metabolite pattern and the influence of CYP2D6 genetics in HLMs and authentic blood samples from fatal PMMA intoxications (paper III).
3 Methodological considerations

This section discusses only the methodological considerations that are not or are insufficiently dealt with in the individual papers.

3.1 Study design

Papers I and II were observational retrospective studies conducted in a consecutive case series, and paper II had a case-control design. Paper III was an experimental in vitro study combined with an observational retrospective study.

3.2 Data and sample collection and definitions

This thesis includes all cases of fatal PMMA-related intoxications registered in Norway from July 2010 to December 2012, and the materials were provided from the Norwegian Forensic Toxicology Database (Biobank) at the Department of Forensic Sciences, Oslo University Hospital (DFS). Until January 2017, DFS was organized as the Division of Forensic Science at the Norwegian Institute of Public Health in Oslo, Norway. DFS has the national responsibility in Norway for forensic toxicology analysis in biological specimens in all cases of suspected criminal offences in Norway, as well as in unnatural deaths where DFS serves 93% of the Norwegian population. St. Olavs hospital in Trondheim, which serves the remaining Mid-Norwegian population in regards of unnatural deaths, registered two fatal PMMA-related intoxications in 2011. However, there was no blood available for further analysis, and these cases could therefore not be included in the present work.

In paper I, all fatal PMMA intoxications and non-fatal PMMA abuse cases registered in Norway during the first six months of the outbreak were included, from July 2010 to January 2011. The non-fatal cases mainly represented drivers suspected of drug or alcohol-impaired driving. Relevant case information registered in the DFS databank was used, supplemented by thorough reviews of the forensic autopsy reports in regards of demographic data, circumstances of death, circumstances of the suspected criminal offence (mainly driving
under the influence of drugs/alcohol), comorbidity, results from routine toxicological analysis, and the pathologist’s conclusion as to the cause of death.

Paper II included all of the fatal PMMA intoxications registered in Norway in 2010–2012 for which blood was available for analysis. Deaths involving violence or potent opioids were not included because these factors are in general major contributors to death. As references, we included the non-fatal PMMA abuse cases registered during the same time period. Non-fatal cases with a PMMA blood concentration lower than 0.5 μM were not included because pharmacological effects are not expected at such levels. Analysis of PMMA and PMMA metabolites, and CYP genotyping, was performed in blood, and relevant case information and autopsy reports were reviewed. As references for the CYP genotype distribution in the Norwegian population, we also included and performed CYP genotyping in blood samples from 100 healthy living blood donors. The blood donors claimed to be in good health with no use of illicit drugs. As references for natural deaths not related to drug use, we also included previously published CYP genotype data from 205 natural death cases kindly provided by the authors from a previous publication (Zackrisson et al., 2004). Regarding CYP2C19, genotype data were available for 211 natural death cases. These previously published deaths occurred in Sweden in 2002, and intoxication, suicide, or other unnatural causes of death were ruled out by the forensic pathologist. The great majority of the Swedish population is Caucasian, similar to Norwegians.

Paper III was an experimental in vitro study in pooled or CYP2D6-genotyped HLMs. This was combined with a study of PMMA and metabolite concentrations in three fatal PMMA intoxications not involving any drugs that have metabolites in common with PMMA.

Additional results represented a complete toxicological and CYP2D6 genetic overview of all registered PMMA-related deaths in Norway from July 2010 to December 2012, except for two PMMA-related deaths registered and analyzed at St. Olavs Hospital in 2011. Autopsy reports were not available at this time point, owing to recent changes in the Norwegian ethics regulations for research on human data. The PMMA blood concentrations presented in this overview were measured as a part of the initial routine forensic toxicology analysis on
request by the police, and therefore some of the PMMA concentrations are not quite identical with those that were later analyzed in our project and presented in papers I–III.

3.3. Toxicological analysis

In connection with paper I, we developed and validated methods for routine screening analysis and quantitative determination of PMMA and PMA using UHPLC-MS/MS, as described in paper I.

The routine method for analysis of PMMA and PMA described in Paper I did not include any other metabolites than PMA, and for papers II and III we developed a second analytical method using UHPLC-MS/MS. Besides PMMA and PMA, this method also included OH-MA, OH-A, dihydroxymethamphetamine (di-OH-MA), 4-hydroxy-3-methoxymethamphetamine (HM-MA), 4-hydroxy-3-methoxyamphetamine (HM-A), and oxilofrine. The method was validated both for whole blood (paper II) and HLMs (paper III). In paper II, the analysis was performed both before and after cleavage of conjugates by hydrolysis, i.e. for unbound and total concentrations. We used gentle enzymatic hydrolysis with β-glucuronidase and sulfatase for two hours at 60°C. In the HLM study in paper III, analysis was performed without hydrolysis. This decision was made on the basis of resources and the toxicological aspect of our study because the in vivo toxicity and efficacy of drugs are determined by the unbound fraction surrounding the drug target.

Screening and quantification of other drugs was performed with routine methods at Oslo University Hospital, as described in papers I and II.

Analysis of the catechol di-OH-MA was not successful in our postmortem blood samples, despite repeated attempts using a variety of analytical approaches such as adding ascorbic acid to the samples as an antioxidant, and testing different methods of hydrolysis. This is in line with previous reports and is probably caused by the highly unstable nature of catechol compounds, the detection of which depends on rapid analysis of fresh samples (Carvalho et al., 2004a; Helmlin et al., 1996; Hiramatsu et al., 1990; Maurer et al., 2000a; Perfetti et al., 2009; Staack et al., 2003; Vevelstad et al., 2016a). A low concentration of di-OH-MA has
previously been measured in rats administered PMMA, and this analysis was performed in fresh urine samples (Staack et al., 2003)

3.4. CYP genotyping

In all fatal and non-fatal cases with available blood, CYP genotyping was performed at Oslo University Hospital. Genotyping included the most common clinically relevant sequence variants of CYP2D6, CYP2C9, CYP2C19, and CYP3A5 using real-time PCRs with melting curve analysis. Analysis of CYP2D6 gene copy numbers was performed using quantitative real-time PCR, except for the fatal PMMA-related intoxications that were analyzed by pyrosequencing at the National Board of Forensic Medicine (NBFM) in Sweden. Pyrosequencing has proven to be a valid method for analysis of CYP2D6 gene copy numbers and CYP genotyping in postmortem blood samples (Druid et al., 1999; Soderback et al., 2005). In our reference group of natural deaths, all pharmacogenetic analyses were performed using pyrosequencing at NBFM because these represented a part of previously published work (Zackrisson et al., 2010).

An important lesson learned from our study was that analysis of CYP2D6 gene copy numbers using quantitative real-time PCR was unsuitable for postmortem blood samples. Compared with pyrosequencing, the real-time PCR highly overestimated the CYP2D6 gene copy numbers, probably owing to matrix effects related to the postmortem nature of the specimens. Our findings emphasize the need for thorough optimization and validation of quantitative gene copy number assays for the analysis of postmortem sample materials.

3.5. Calculations

In paper II, the CYP2D6 metabolic ratio (OH-MA/PMMA) in blood was calculated as an estimate of the individual CYP2D6 phenotype using the total (hydrolyzed) concentration of OH-MA, i.e. the sum of unbound, glucuronidated, and sulfated metabolite. PMMA is not conjugated.
In order to overcome the challenge of comparing drug or metabolite concentrations measured in blood from living subjects with those measured in postmortem blood samples, comparison was done by means of molar concentration ratios and not by absolute concentrations (papers I and II, see section 3.6).

3.6. **Postmortem drug concentrations**

Postmortem concentrations of drugs and metabolites are never the same as those at the time of death. This is due to redistribution of the compounds between tissues and the blood, and to drug metabolism, synthesis, and degradation after death as part of the putrefaction process (Hilberg et al., 1999). Amphetamines are likely to undergo postmortem redistribution with increasing drug concentrations in the blood after death, because they are basic compounds for which the concentrations in body organs are up to many times higher than in the blood (Yarema and Becker, 2005). Peripheral blood typically drawn from a femoral vein is the most representative blood matrix for postmortem drug analysis, in order to estimate the antemortem drug concentration (Hilberg et al., 1999). All but one of the postmortem blood samples in our study represented peripheral blood.

3.7. **Studies in human liver microsomes**

Because the biotransformation of toxic drugs in humans cannot be studied in vivo for ethical reasons, in vitro models must be used. In our study we used commercially available HLMs, which provide the most popular in vitro model for predicting the human biotransformation of drugs owing to favorable cost, availability, and simplicity of use (Brandon et al., 2003; Yokchue and Anderson, 2015). HLMs consist of vesicles of hepatocyte endoplasmic reticulum prepared by differential centrifugation and contain almost solely CYP and UGT enzymes (Brandon et al., 2003). Studies in HLMs allow for qualitative estimations of drug metabolism in humans and for comparisons between substrates and CYP genotypes (Brandon et al., 2003; Zhang et al., 2015). There is, however, no competition with other enzymes that are normally present in the cytosol of intact liver cells, resulting in a higher biotransformation rate in HLMs compared to the human in vivo situation (Brandon et al., 2003). In our study, HLMs genotyped for CYP2D6 content and activity were purchased from XenoTech. Pooled
HLMs (pHLMs) prepared from 200 donors of balanced gender were used to represent the general population. HLMs from single donors classified as “No CYP2D6 activity” were used to represent CYP2D6 PM, and HLMs classified as “CYP2D6 high activity, HA” were used to represent CYP2D6 UM. For each time point, we performed three or four separate experiments and used microsomes from three or four lots of pHLMs or from two or three individual HLM donors.

3.8. Ethical considerations

The regulatory framework for research on biological material from deceased individuals was severely restricted in Norway during the project period. For papers I and II, the Regional Committee for Medical and Health Research Ethics and the Higher Prosecution Authority approved access to autopsy reports, whereas no person-sensitive data were available at the time of paper III or for the overview of the entire outbreak of fatal PMMA intoxications in Norway presented as Additional results. The latter overview of PMMA concentrations and CYP2D6 genotypes would have been more informative if we could have also presented the circumstances of death, including signs and symptoms of PMMA intoxication, the time interval between ingestion and death, the route of drug intake, and the pathologist’s conclusion as to cause of death.
4 Summary of each paper

4.1. Paper I

“The PMMA epidemic in Norway: comparison of fatal and non-fatal intoxications”

The purpose of this study was to characterize the clinical manifestations of fatal PMMA intoxication in humans and to investigate the risk factors for a fatal outcome. This was accomplished by comparing the available clinical and toxicological information in fatal PMMA intoxications (n = 12) and non-fatal PMMA abuse cases (n = 22). The fatal PMMA blood concentrations were generally high and were far above the levels in non-fatal cases (Fig. 7). In one of the non-fatal cases, the PMMA blood concentration was higher than the previously suggested toxic level of 2.8 μM (star symbol in Fig. 7) (EMCDDA, 2003). All deaths were classified as accidental. Two thirds of the fatal cases were found dead, many of whom presumably died in their sleep, whereas death was witnessed in one third of the cases with symptoms consistent with severe serotonergic and catecholaminergic overstimulation. This study showed that the risk factors associated with a fatal outcome of PMMA intoxication were high doses or repeated intakes of PMMA, the drug being disguised as amphetamine or ecstasy, concomitant use of other psychostimulant or potent sedative drugs, a high total concentration of psychostimulants in the blood, and being asleep or left unattended. The overlap between fatal and non-fatal PMMA concentrations might indicate that there are currently unknown individual factors that are predisposing or protective in view of fatal outcome.
**Figure 7.** PMMA concentrations in blood (median, 25th–75th percentiles, minimum/maximum observations, outliers, and extreme outliers). The dotted line marks the previously suggested toxic level.

### 4.2. Paper II

*“Is toxicity of PMMA (para-methoxymethamphetamine) associated with cytochrome P450 pharmacogenetics?”*

On the basis of the findings in paper I, which suggested the involvement of individual predisposing or protective factors, we wanted to investigate if fatal toxicity of PMMA was associated with PMMA metabolism and CYP pharmacogenetics. The PMMA and metabolite concentrations in blood and the frequency distribution of clinically relevant CYP gene variants were compared in fatal PMMA intoxications (n = 17) and non-fatal PMMA abuse controls (n = 30), using non-abusers (n = 305) as references for the CYP genotype distribution.
in the background population. This study showed that the CYP2D6 enzyme and genotype are important for the metabolism of PMMA into the major metabolite OH-MA. We found, however, that other enzymes are also involved in this biotransformation. In the fatal PMMA intoxications, the relative formation of OH-MA from PMMA, as an estimate of the CYP2D6 phenotype, was significantly lower and not correlated with the CYP2D6 genotype, compared with the non-fatal cases where there was a clear correlation between the CYP2D6 phenotype and genotype (Fig. 8). This indicates that most deaths occurred at an earlier stage of PMMA metabolism compared to the non-fatal cases, or that there was a more pronounced CYP2D6 enzyme inhibition in the fatalities owing to the high concentrations of PMMA and amphetamine/methamphetamine, which are all weak, dose-dependent CYP2D6 enzyme inhibitors. We could not identify any genetic CYP2D6, CYP2C9, CYP2C19, or CYP3A5 predictive markers for fatal toxicity of PMMA. There was, however, an overrepresentation of genetic CYP2D6 PMs in the non-fatal PMMA abuse cases. This paper revealed that fatal toxicity of PMMA was associated with a low degree of PMMA metabolism by the CYP2D6 enzyme, but we found no association with any particular CYP2D6, CYP2C9, CYP2C19, or CYP3A5 genotype.

**Figure 8.** Correlation between the CYP2D6 genotype, and the CYP2D6 phenotype estimated as the CYP2D6 metabolic ratio (MR = OH-MA/PMMA), in fatal PMMA intoxications (left) and non-fatal PMMA abuse cases (right). CYP2D6 poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM), and ultrarapid (UM) metabolizers.
4.3. Paper III

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

This study examined the PMMA metabolite pattern and involvement of CYP2D6 genetics in humans. For this purpose we used pHLMs and CYP2D6-genotyped HLMs, and blood samples from fatal PMMA intoxications (n = 3). In pHLMs, PMMA was metabolized mainly to OH-MA, whereas low concentrations of PMA, OH-A, di-OH-MA, and oxilofrine were formed. A similar metabolite profile was found in the fatalities, but the metabolites HM-MA and HM-A were also detected in low concentrations. The proposed pathway for the metabolism of PMMA in humans is given in Fig. 9. There was a significant influence of CYP2D6 genetics on PMMA metabolism in HLMs (Fig. 10). The formation of all detected metabolites was higher in CYP2D6 UM and lower in PM compared to pHLMs. The catechol metabolite di-OH-MA has previously been suggested to be involved in PMMA toxicity. Our study showed that the formation of di-OH-MA from PMMA was two to seven times lower than from an equimolar dose of the less toxic drug MDMA. This study revealed the metabolite pattern of PMMA in humans and demonstrated a significant impact of CYP2D6 genetics on human PMMA metabolism, but our findings do not support the hypothesis of catechol metabolites as major determinants of fatal PMMA toxicity.
**Figure 9.** Proposed pathway for the metabolism of PMMA in humans. The figure is based on the present study in HLMs and fatal PMMA intoxications and on previously published studies in rodents and humans.
Figure 10. Impact of CYP2D6 genotype on the concentration-versus-time profile of PMMA in HLMs. CYP2D6 ultrarapid metabolizers (UM), pooled metabolizers (Pooled), and poor metabolizers (PM).

4.4. Additional results

“Update on the PMMA (paramethoxymethamphetamine) outbreak in Norway”

An overview of the toxicological and CYP2D6 genotype findings in the entire Norwegian outbreak of fatal PMMA intoxications was presented as a poster in Australia 2016 (Vevelstad et al., 2016b). In total, 27 fatal PMMA-related intoxications were registered in Norway during 2010 (n = 10), 2011 (n = 16), and 2012 (n = 3). The PMMA concentrations in blood were generally in the lethal range, with a median of 9.0 μM, range 0.1–26.6 μM (1.6 mg/L (0.02–4.8 mg/L)) (Fig. 11). Of the eight fatalities with a sub-toxic PMMA concentration lower than 2.8 μM (left dotted bar in Fig. 11), six could be attributed to morphine/heroin, whereas no obvious toxicological contributor to death was identified in the other two. The PMA concentrations reflected metabolism of PMMA, constituting 2–19% of the parent drug concentration. The frequency distribution of the CYP2D6 UM, EM, IM and PM genotypes was 8%, 40%, 40% and 12%, respectively, which is as expected in a Caucasian population (n = 25). This work showed that fatal PMMA intoxication was strongly associated with PMMA overdosing and polydrug abuse, but that some individuals might be particularly susceptible to sub-toxic PMMA concentrations. No association was found between the CYP2D6 genotype and fatal PMMA intoxications.
Moreover, all but one of the 27 fatal PMMA intoxication cases were pronounced dead at the scene by health care professionals (not previously published data). One subject was still alive but comatose and hyperthermic at first contact with health care, but developed cardiac arrest in the emergency room and was pronounced dead after three days of intensive care treatment, as described in paper I.
5 Discussion of major findings

5.1. Clinical manifestations of fatal PMMA intoxication

As described in paper I, the available information regarding the antemortem clinical manifestations and events was fragmentary for most of the fatal PMMA intoxications in our study. Two manifestation forms could, however, be identified; about one third of the subjects were observed with signs and symptoms of severe intoxication, whereas two thirds were found already dead.

In the first group, progressive signs and symptoms were witnessed prior to death, including changes in mental status, autonomic excitation, and neuromuscular excitation. The reported signs and symptoms represented auditory hallucinations, severe hyperthermia, shivering, convulsions, acute respiratory distress, cardiac arrest, and sudden collapse. Most of these are typical clinical features of severe serotonin syndrome and increased noradrenergic neurotransmission. Agitation was not reported in any of our cases, which is consistent with the weak effects of PMMA on dopaminergic neurotransmission (Liechti, 2015; Simmler et al., 2014). As presented in the Additional results, only one individual out of the 27 fatal PMMA intoxications in our study was still alive at first contact with health care professionals, and this subject presented with coma and severe hyperthermia and developed cardiac arrest soon after admission to hospital. This was followed by multi-organ failure, and death was pronounced on the third day after admission (paper I). For comparison, Nicol et al. recently reported that 16 of the 17 fatal PMMA-related intoxications that were hospitalized had presented with serotonin syndrome. Body temperatures as high as 43.8°C were measured, and many of the deaths followed multi-organ failure (Nicol et al., 2015).

In the other and largest group, the subjects were found dead at the scene and it was unknown whether death was preceded by alarming intoxication symptoms (paper I). In several of these cases, the circumstances indicated that death might have occurred during sleep. The PMMA concentrations were generally high and at a level where severe serotonergic symptoms are expected. It is well known that serotonin syndrome in its most
pronounced stage might lead to unconsciousness, coma, and cardiovascular collapse (Fig. 3, Section 1.2.1). Other factors that might have contributed to reduced consciousness, coma, and death are hypoglycemia and hyperkalemia, which might follow the highly increased energy demands and muscular overstimulation that occur in serious PMMA poisoning. According to previous reports, hypoglycemia and hyperkalemia were present in most fatal PMMA intoxications (Nicol et al., 2015) and were also frequent features of PMA intoxications (Ling et al., 2001; Refstad, 2003). When severe and left untreated, hypoglycemia and hyperkalemia lead to reduced consciousness, muscular weakness, arrhythmia, and eventual collapse (Nicol et al., 2015). Another possible mechanism behind the development of hypoglycemia is related to insulin. PMMA has proven to be a potent MAO-A enzyme inhibitor, and previous reports have demonstrated that other MAO inhibiting drugs potentiate insulin release depending on the drug concentrations (Aleyassine and Gardiner, 1975). It is now known whether PMMA is capable of potentiating insulin release.

As mentioned, in as much as 26 of the 27 fatal PMMA intoxications in our study, the deceased were pronounced dead already at the first contact with healthcare professionals at the scene. This is in contrast with the findings in the Canadian outbreak, where more than half of the 27 fatal PMMA-related intoxications survived until hospitalization (Nicol et al., 2015). Possible explanations that so few subjects in our study survived until hospitalization might be that the victims and the witnesses underestimated the danger of the emerging symptoms, or that serious poisoning developed so fast that one did not have time to call an ambulance.

The two clinical manifestation forms found in our study, and the dominance of cases that were found already dead, are consistent with the findings described in fatal PMMA intoxications in Sweden, the UK, Canada, and Taiwan (Chen et al., 2012; Elliott and Evans, 2014; Kronstrand et al., 2015; Nicol et al., 2015; WHO, 2015). In the Taiwan outbreak of fatal PMMA intoxications, there was available information about the antemortem behavior for eight patients. PMMA was found to exert a dual effect, which was concentration-related (Chen et al., 2012):
a) **Suppression of vital functions associated with very high PMMA concentrations:** There was no cognitive impairment, and the users gradually fell asleep 2–5 hours after PMMA ingestion and were later found dead with very high PMMA concentrations of 12 μM–26 μM.

b) **Cognitive impairment associated with high PMMA concentrations:** Progressive cognitive impairment starting 0.5–6.5 hours after PMMA ingestion, including incoherent speech, hypertalkativeness, disinhibition, and irritability as if in a delirious state. This culminated in convulsions, collapse, and death with high PMMA concentrations of 7 μM–17 μM.

A dual and concentration-related effect of PMMA on behavior was also previously reported in rodents, where low doses induced neurocognitive impairment and high doses induced toxicity and death (Palenicek et al., 2011). We could not identify any systematic difference in the PMMA concentrations between the two manifestation forms in our study, but the available information about the antemortem events was fragmentary in most cases and this will blur the picture.

The findings of our study illustrate the very high risk of overdosing on the serotonergic ecstasy-like drug PMMA, and the importance of rapidly calling an ambulance at the onset of hyperthermia, shivering, reduced consciousness, or respiratory distress in order to increase the chances of survival of PMMA intoxication.

### 5.2. Risk factors for fatal PMMA intoxication

#### 5.2.1. **PMMA dose and repeated intakes**

As presented in papers I and II and the Additional results, the PMMA blood concentrations in the 27 fatal PMMA-related intoxications in Norway ranged from 0.1 μM to 27 μM with a median of 9.0 μM. These are generally very high and fatal PMMA concentrations even after adjusting for an expected postmortem increase of up to 2–3 times (Vevelstad et al., 2012). For comparison, it has been suggested that PMMA blood concentrations greater than 2.8 μM are toxic and possibly lethal (Chen et al., 2012; EMCDDA, 2003). The high PMMA
concentrations in our study strongly indicated that high or repeated doses of PMMA had been ingested. This was consistent with the available case information stating that many of the deceased had repeatedly administered stimulant drugs prior to death. Moreover, according to witnesses at least one half of the deceased presumed they were taking “amphetamine” or “ecstasy”. This disguise of the toxic and very unpopular drug PMMA as amphetamine or ecstasy seems to represent a decisive and major cause of its use. Further, the weak and delayed euphoria induced by PMMA compared to MDMA can easily prompt the users to repeat their PMMA intake and to increase the dose (Glennon et al., 1988; Palenicek et al., 2011). According to previous studies, PMMA users frequently believed they had taken a weak ecstasy pill and re-dosed within a short time period (Chen et al., 2012; Lin et al., 2007; Nicol et al., 2015; Westin and Brede, 2011; WHO, 2015). For comparison, MDMA is usually effective after a single tablet, and a total of one or two tablets is often used per occasion (Parrott, 2005). Because the margin of toxicity is narrower for PMMA compared to MDMA, re-dosing or high doses is more dangerous for PMMA (Steele et al., 1992). Our study leaves no doubt that the most important risk factor for a fatal outcome of PMMA poisoning is a high PMMA dose or repeated intakes.

Some fatal cases in our study, however, involved PMMA blood concentrations in the lower toxic range. In paper II, the lowest registered PMMA concentration was 4.6 μM in fatalities not involving opioids, underlying disease, or unfortunate circumstances. This PMMA concentration is similar to the highest concentration registered in the non-fatal cases, which was 4.8 μM. This overlap indicates that there are yet unknown inter-individual differences in sensitivity to the toxic effects of PMMA. This is supported by the Canadian report where a PMMA blood concentration as low as 0.6 μM was measured in the twenty PMMA-related deaths not involving opioids or life-prolonging care (Nicol et al., 2015).

5.2.2. Other risk factors

As presented in papers I and II, the PMMA concentrations tended to be lower in poly-drug fatalities involving both PMMA and amphetamine/methamphetamine compared to the PMMA-only fatalities. Moreover, the amphetamine/methamphetamine concentrations were very high in several of the fatalities. This indicates that not only the PMMA dose, but also the
total dose of psychostimulants is of importance for PMMA toxicity. This is as expected because all psychostimulant drugs will contribute to increasing catecholaminergic overstimulation and because the specificity of the drug actions on different monoamine neurotransmitters becomes weaker at higher drug doses (Parrott, 2002; Parrott et al., 2001).

As presented in papers I and II and the Additional results, about a quarter of the fatalities exhibited PMMA concentrations in the sub-toxic range below 2.8 μM. A majority of these deaths could be attributed mainly to the presence of morphine/heroin in the blood, which are potent respiratory depressants. There were, however, two PMMA-related fatalities not involving opioids, where the PMMA concentrations were as low as 0.1 μM and 0.2 μM. No other obvious toxicological contributor to death was identified, and this indicated that other underlying risk factors might have been involved. One of these subjects had reported chest pain prior to death, whereas an unfortunate body position might have contributed to suffocation in the other subject. A possible underlying heart disease would obviously be a risk factor for fatal PMMA toxicity, as would an unfortunate body position.

According to our studies, being asleep or being left unattended while intoxicated with PMMA was associated with a fatal outcome. As mentioned above, high doses of PMMA induce a life-threatening serotonergic syndrome with reduced consciousness as one of the expected symptoms. This argues that drug users who have recently taken large or repeated doses of serotonergic ecstasy-like drugs should not stay or be left alone, and especially if they have developed increased body temperature or twitching, which should prompt an emergency call. Moreover, food intake prevents hypoglycemia, and this is particularly important before going to sleep.

Although not published, we found no association between fatal PMMA intoxication and the presence of caffeine or cotinine in the blood samples compared to the non-fatal PMMA abuse cases. This speaks against an involvement of caffeinated foodstuffs like colas or coffee or tobacco smoking as risk factors for fatal PMMA intoxication.
5.2.3.  **PMMA concentrations in non-fatal abuse cases**

We are the first to publish the PMMA concentrations in non-fatal PMMA abuse cases, mainly representing suspected drugged drivers or subjects suspected of illegal drug abuse. As presented in papers I and II, the PMMA blood concentrations ranged from 0.1 μM to 4.8 μM with a median of 1.4 μM. Although most concentrations were moderate, the suggested toxic level of 2.8 μM was exceeded in one quarter of the non-fatal cases. In two subjects the PMMA concentrations were as high as 4.8 μM and 3.9 μM. The latter concentration was measured in blood sampled four hours after this subject was apprehended by the police, and the subject had a pulse of 86 and was considered as not-impaired by a physician at the time of sampling. Reportedly, this subject had used the same white powder, which had been assumed to be MDMA, in similar doses of 0.5 grams four times over ten hours as a friend who had a fatal outcome. This suggests that there are yet unknown protective factors in regards of PMMA toxicity.

Altogether, we found that the main risk factors for a fatal outcome of PMMA intoxication was a high dose or repeated intakes of PMMA, the disguise of the drug as amphetamine or ecstasy, concomitant use of other psychostimulants or potent opioids, and being asleep or left unattended. The overlapping of PMMA concentrations between the fatal and non-fatal cases indicated that there are yet unknown individual protective or disposing factors for fatal toxicity of this drug.

5.3.  **Association between CYP genetics, PMMA metabolism, and fatal PMMA intoxication**

Several previous reports have suggested that the neurotoxicity of ring-substituted amphetamines like PMMA is related to drug metabolism and CYP enzyme genetics, particularly regarding the polymorphically expressed CYP2D6 enzyme (Esteban et al., 2001; Monks et al., 2004). Early studies indicated that CYP2D6 PM individuals were prone to toxicity (Colado et al., 1995; Kraner et al., 2001; Tucker et al., 1994), while later studies found no evidence for this (Gilhooly and Daly, 2002; O'Donohoe et al., 1998) or suggested that CYP2D6 UM individuals might be predisposed to intoxication owing to the formation of
toxic metabolites (Carmo et al., 2007; Cherner et al., 2010; Cho et al., 1977; Esteban et al., 2001; Schwab M, 1998; Yamada et al., 2005).

5.3.1. Importance of CYP genotype

In paper II we showed that fatal PMMA intoxication was not associated with any particular CYP2D6 genotype. The CYP2D6 genotype frequencies in the fatalities did not differ significantly from those in the non-fatal PMMA abuse cases or from the reference population of non-abusers. In particular, we found no overrepresentation of CYP2D6 PM or UM among the PMMA fatalities. Hence, we could not identify any genetic CYP2D6 predictive marker on fatal toxicity of PMMA. Accordingly, our study does not lend support to previous theories that the toxicity of drugs like PMMA is related to CYP2D6 enzyme genetics. Similar conclusions have previously been drawn by other authors like de la Torre in regards to an influence of CYP2D6 genotype on the toxicity of MDMA (de la Torre et al., 2012).

Nevertheless, our studies of PMMA metabolism in blood samples from fatal and non-fatal PMMA intoxications and in HLMs have demonstrated that the CYP2D6 enzyme and genotype are important in the metabolism of PMMA, as presented in papers II and III. The higher the number of functional CYP2D6 gene copies, the more efficient the metabolism of PMMA and formation of the studied metabolites, and particularly regarding the major metabolite OH-MA. This is consistent with the study of Staack et al. demonstrating an involvement of the enzyme CYP2D6 in the formation of OH-MA from PMMA in HLMs (Staack et al., 2004b). In paper II, however, we found low concentrations of OH-MA also in CYP2D6 PM subjects, both in fatal and non-fatal cases. This indicates that other enzymes than CYP2D6 are also involved in the metabolism of PMMA to OH-MA in humans. This is in line with previous reports suggesting that other CYP isoenzymes might be involved in the metabolism of amphetamine-type drugs, and particularly in CYP2D6 PM (Kreth et al., 2000; Yamada et al., 2005). In paper III we showed that the formation of the catechol di-OH-MA was also influenced by CYP2D6 genetics. This is in line with previous rat studies demonstrating that di-OH-MA or thioether conjugates of this metabolite were detected only in the brain of rat strains used as models of the human CYP2D6 EM genotype, and not in models of CYP2D6 PM (Chu et al., 1996; Jones et al., 2005). As presented in paper III, the weak influence of CYP2D6 genetics on the
formation of PMA in our HLM study is in keeping with the literature, concluding that N-dealkylation of amphetamines occurs mainly via other enzymes like CYP2B6 (Kreth et al., 2000; Maurer et al., 2000b).

Previous reports have suggested that CYP isoenzymes of the 2C and 3A subfamilies might also be involved in the metabolism of ring-substituted amphetamines like MDMA and PMMA (Kreth et al., 2000; Yamada et al., 2005). In paper II we demonstrated that the genotype frequencies of clinically relevant gene variants of CYP2C9, CYP2C19, and CYP3A5 were similar in the fatal PMMA intoxications, the non-fatal PMMA abuse controls, and the reference group of non-abusers. Accordingly, we found that neither CYP2C nor CYP3A genetics were associated with fatal PMMA intoxication.

5.3.2. CYP2D6 phenotype

Although fatal PMMA intoxication was not associated with CYP2D6 genotype, paper II showed that it was associated with the CYP2D6 phenotype, estimated as the OH-MA/PMMA concentration ratio. The relative formation of OH-MA from PMMA was significantly lower in the PMMA fatalities compared to the non-fatal PMMA abuse cases. In 12% of the fatalities, the major metabolite OH-MA was not detected at all, although these individuals were genetically CYP2D6 EM. Moreover, there was no significant correlation between the CYP2D6 genotype and the estimated CYP2D6 phenotype in the fatalities, as opposed to the non-fatal cases. Accordingly, in the fatalities the relative metabolism of PMMA was low independently of the number of functional CYP2D6 alleles. This might indicate that the fatalities in general represented an earlier stage of PMMA metabolism because of death occurring rapidly after PMMA intake, in comparison with the non-fatal cases of mainly car drivers where the blood sampling might have taken place at a later metabolic stage. The scarcity of case information that was available indicated that the time interval between PMMA intake and death was rather short, from about 30 minutes to a few hours. For comparison, in the Canadian outbreak of 27 fatal PMMA intoxications the median time from PMMA exposure to hospital presentation was six hours (Nicol et al., 2015).
Another possible explanation contributing to the lower formation of OH-MA in the fatalities could be a higher degree of CYP2D6 enzyme inhibition, mediated by PMMA and amphetamine/methamphetamine, compared to the non-fatal cases. CYP2D6 is a low-capacity enzyme, and PMMA and co-administered amphetamine/methamphetamine are not only substrates, but also weak competitive inhibitors of this enzyme with an inhibitory constant (Ki) of 20–24 μM (de la Torre et al., 2012; Wu et al., 1997). The high Ki means that a PMMA or amphetamine concentration of 20–24 μM is required to give half-maximal CYP2D6 enzyme inhibition. Because the degree of enzyme inhibition is dose dependent, the drugs PMMA and amphetamine/methamphetamine can hardly induce any CYP2D6 enzyme inhibition at the recreational drug concentrations found in most of the non-fatal cases. In the fatalities, on the other hand, there should be a pronounced enzyme inhibition owing to the high concentrations of PMMA and amphetamine/methamphetamine. When there is a pronounced inhibition of the CYP2D6 enzyme, genetic CYP2D6 EM might be phenocopied to PM shortly after drug intake (de la Torre et al., 2012). This will result in a very low formation of OH-MA irrespective of CYP2D6 genotype. For comparison, MDMA is a very potent CYP2D6 enzyme inhibitor with a low Ki of 0.6 μM (de la Torre et al., 2012; Wu et al., 1997), and this drug was previously demonstrated to induce irreversible CYP2D6 enzyme inactivation within two hours after intake of a single recreational dose (O'Mathuna et al., 2008; Yang et al., 2006). Such a pronounced inhibition of the CYP2D6 enzyme by MDMA can also explain why the reference substance MDMA was hardly metabolized in our HLM studies in paper III, where the MDMA substrate concentration was 100 μM.

In summary, we cannot answer whether the low CYP2D6 metabolic ratio in the fatalities is due to death occurring shortly after intake of PMMA or to a more pronounced inhibition of the CYP2D6 enzyme, in comparison with the non-fatal cases. Irrespective of the cause, the concentrations of the fat-soluble drug PMMA in the fatalities were generally very high and the drug was only slightly metabolized to water-soluble metabolites, and this will result in very high concentrations of PMMA also in the brain where the neurotoxic serotonergic actions take place.
5.3.3. **Overrepresentation of CYP2D6 PM in non-fatal cases**

In paper II, we found that 30% of the non-fatal PMMA abuse controls were genetically CYP2D6 PM. This figure is significantly higher than the frequency of 6.2% in our reference group of non-abusers and the frequency of 3%–10% that was previously reported in the Caucasian population (Bernard et al., 2006). To our knowledge, such a high frequency of homozygous-deficient CYP2D6 metabolizers has not been previously reported in any Caucasian population. The frequency is reported to vary from 0% to 19% in different ethnic populations (Bernard et al., 2006). This overrepresentation of genetic CYP2D6 PM among the non-fatal cases in our study could theoretically indicate that this genetic trait is protective against fatal PMMA toxicity, and could thus add support for an involvement of CYP2D6-enzyme-mediated metabolism in PMMA toxicity. However, most of the findings in our studies speak against this. As previously mentioned, the relative formation of OH-MA from PMMA was significantly higher in the non-fatal cases than in the fatalities, and not lower as would have been expected if genetic CYP2D6 PM subjects had been protected against a fatal outcome. Actually, most of the fatal cases exhibited a CYP2D6 metabolic ratio corresponding to the ratio found in genetic CYP2D6 PM in the non-fatal cases. Second, there was a great predominance of partly deficient CYP2D6 metabolizers (IM) among the PMMA-only fatalities. Last but not least, we found no genetic CYP2D6 predictive marker for fatal PMMA toxicity. Taken together, our findings in the non-fatal PMMA abuse cases do not argue for any protection of deficient CYP2D6 metabolism in regards of fatal PMMA toxicity.

Another possible explanation for the overrepresentation of genetic CYP2D6 PM in the non-fatal PMMA abuse cases in paper II might be related to personality traits. The CYP2D6 enzyme is expressed at low levels in many parts of the human brain and is involved in the formation of the endogenous neurotransmitters serotonin and dopamine (Yu et al., 2003b). Interestingly, it has been suggested that the CYP2D6 PM trait is associated with a low serotonin and a high dopamine tone, as well as high impulsiveness and low harm avoidance (Bertilsson et al., 1989; Roberts et al., 2004). This is interesting because the serotonin system is the main venue for the effects of PMMA. It is reported that CYP2D6 PM individuals were more sensitive to the effects of methamphetamine on several objective and subjective measures of stimulation, euphoria, and good effects compared with CYP2D6 EM individuals,
despite similar plasma kinetics (Kaplan et al., 1996; Sellers and Tyndale, 2000). Sellers suggested that CYP2D6 PM individuals might achieve higher methamphetamine concentrations in the brain, or a steeper concentration-response relationship, than EM individuals. On the other hand, other studies argue against a predisposition for stimulant drug abuse in deficient CYP2D6 metabolizers. The frequency of genetic CYP2D6 PM was underrepresented in MDMA abusers compared with the general population (Kaplan et al., 1996), and the PM trait was associated with protection against methamphetamine dependence (Otani et al., 2008). Taken together, we have no good explanation for the overrepresentation of CYP2D6-deficient metabolizers in the non-fatal PMMA abuse controls, and it might simply represent random variation due to the limited sample size.

5.4. PMMA metabolite pattern

There are no previous reports addressing the PMMA metabolite pattern in humans. In paper III, the main metabolite formed from PMMA in HLMs was OH-MA (pholedrine), which constituted 87% of the metabolized PMMA, whereas the minor metabolites PMA, OH-A, and, di-OH-MA constituted only 1.0%–2.9% of the metabolized PMMA after four hours of incubation. To our knowledge, the present study is the first to demonstrate the formation of di-OH-MA, HM-MA, HM-A, and oxilofrine in humans or in incubations with HLMs after PMMA exposure. The PMMA metabolites detected in blood from the three fatal PMMA intoxications were in general consistent with the results in HLMs. The formation of the metabolite PMA was, however, more pronounced in vivo compared with the in vitro HLM experiments; PMA represented 30%–100% of the total metabolite concentration in blood in the fatalities, and up to 10% in HLMs. In the fatality with a very high PMMA concentration of 26.4 μM, we also detected low levels of HM-MA and HM-A in the blood. These non-toxic metabolites were not expected to be formed in the HLM study because the indispensable methyl donor S-adenosyl methionine was not added to the incubation solution (Helmlin et al., 1996; Kuwayama et al., 2009). Our findings of the major and minor metabolites from PMMA were consistent with previously published findings after administration of PMMA to rats (Rohanova and Balikova, 2009a; Staack et al., 2003) and with the results in a recent study of PMMA metabolism in HLMs (Lai et al., 2015).
5.4.1. **Catechol metabolites**

Previous reports have postulated that systemic metabolism is crucial for the serotonergic neurotoxicity of ring-substituted amphetamines like PMMA and MDMA, possibly by conjugation of the minor catechol metabolite di-OH-MA (Fig. 12) to form potent neurotoxic conjugates (Carmo et al., 2006; Perfetti et al., 2009). Although not neurotoxic in itself, di-OH-MA is reactive and can be rapidly oxidized and conjugated with glutathione/N-acetylcysteine to form neurotoxic conjugates than can penetrate or be formed in the brain (Carvalho et al., 2004b; Chu et al., 1996; de la Torre and Farre, 2004; Esteban et al., 2001; Hiramatsu et al., 1990; Jones et al., 2005; Lim and Foltz, 1988; McCann and Ricaurte, 1991; Miller et al., 1995; Monks et al., 2004). Rodent studies have shown that repeated doses of MDMA might lead to accumulation of neurotoxic di-OH-MA conjugates in the brain, which might induce the release of monoamines and a behavioral response comparable with serotonin syndrome in humans (Chu et al., 1996; Erives et al., 2008; Jones et al., 2005; Miller et al., 1996; Monks et al., 2004).

![Figure 12. The catechol dihydroxymethamphetamine (di-OH-MA).](image)

In paper III we have demonstrated that PMMA was metabolized to the catechol metabolite di-OH-MA in HLMs. To the best of our knowledge, this has not been shown before. The formation of di-OH-MA from PMMA was, however, two to seven times lower than from an equimolar dose of MDMA, which is a less toxic and more thoroughly studied drug. De la Torre et al. have previously demonstrated that di-OH-MA was actually the major intermediate metabolite of MDMA in HLMs when a methyl donor was added to the incubation medium (de la Torre et al., 2004). As soon as it is formed in the body, the reactive catecholamine di-OH-MA becomes rapidly inactivated either by methylation by the COMT enzyme to form the nontoxic metabolite HM-MA (Perfetti et al., 2009; Yokchue and Anderson, 2015) or by degradation by the MAO-A enzyme. Both enzymes are present in the brain and in liver cells. Consequently, the lower formation of di-OH-MA from PMMA in our
study, compared with the formation from MDMA, does not support the hypothesis of catechol metabolites as major determinants of PMMA toxicity in humans.

It could be speculated, however, as to whether the risk of achieving, and possibly accumulating, high levels of the reactive compound di-OH-MA in the brain of CYP2D6 EM individuals might still be higher with PMMA compared with MDMA, owing to the pattern of rapid re-dosing of PMMA combined with the 20 times more potent inhibition of the MAO-A enzyme exerted by PMMA (Matsumoto et al., 2014). Catechol compounds are metabolized by the MAO-A enzyme (Kopin, 1994), and when this enzyme is severely inhibited, the degradation of di-OH-MA might be reduced. Previous rat studies have demonstrated that multiple-dose administration of ring-substituted amphetamines more reliably produces neurotoxicity than single doses (Erives et al., 2008; Jones et al., 2005). However, further studies are needed to investigate whether di-OH-MA and its thioethers might be involved in PMMA toxicity.

In summary, our findings do not support the former hypothesis of catechol metabolites as major determinants of fatal PMMA toxicity in humans. Our conclusion is further supported by a recent study that has also challenged the former postulations about systemic metabolism being crucial for the serotonergic neurotoxicity of ring-substituted amphetamine drugs (Shokry et al., 2016). Shokry et al. showed that local administration of MDMA in the brain resulted in an uneven distribution of the drug, and that former postulations based on such research models might not be valid.

In the present work, we also studied the metabolism of methamphetamine in HLMs using pHLMs, CYP2D6 PM, and CYP2D6 UM (data not published). Trace amounts of di-OH-MA were formed from methamphetamine, but only in CYP2D6 UM where the maximum level constituted 0.07% of the initial methamphetamine concentration. This demonstrated that methamphetamine can also be metabolized to di-OH-MA in HLMs, but that the expected levels are very low.
6 Implications and future perspectives

As a part of the present work, we have developed and validated two analytical methods. The first method was developed for analysis of PMMA and PMA in whole blood, and since 2010 these drugs have been included in the routine toxicological screening analysis for narcotics, psychoactive drugs, and metabolites in our laboratory. The second method was developed for analysis of PMMA, PMA, and the metabolites OH-MA, OH-A, di-OH-MA, HM-MA, HM-A, and oxilofrine in whole blood and in HLMs.

The Norwegian outbreak of fatal and non-fatal PMMA intoxications and the results of the present work contributed to a national and global alert about the high toxicity of PMMA (WHO, 2015). The drugs PMMA and PMA were included in the Norwegian list of regulated narcotic drugs in 2010, and since that time, the emergence, use, and number of life-threatening intoxications with novel psychoactive drugs other than PMMA have increased dramatically in Norway, Europe, and the US.

At the planning stage of the present work, we also considered investigating whether PMMA toxicity might be related to inhibition of the MAO-A enzyme. This was, however, out of reach owing to limited time and resources. The toxicity of amphetamines was previously demonstrated to be severely increased if the MAO-A enzyme is inhibited, which results in intracellular flooding of monoamine neurotransmitters (Clark et al., 1964; Freezer et al., 2005; Mantle et al., 1976; Matsumoto et al., 2014; Scorza et al., 1997; Smythies et al., 1967; Vuori et al., 2003). While the present work was ongoing, Matsumoto et al. demonstrated that PMMA is a potent inhibitor of the MAO-A enzyme (Matsumoto et al., 2014). Using recombinant human MAO-A enzyme, PMMA was shown to be 20–24 times more potent than MDMA and methamphetamine, and 3 times less potent than PMA, at inhibiting this enzyme. This might contribute to explaining the high toxicity of PMMA and PMA compared to MDMA and methamphetamine. Other authors have suggested that the toxicity of ring-substituted amphetamines might be related to inhibition of the COMT enzyme and genetic COMT polymorphisms because this enzyme is involved in the rapid detoxification of
hydroxylated reactive metabolites that are formed in the body (Perfetti et al., 2009). Future studies are necessary to determine whether COMT is involved in PMMA toxicity.
7 Conclusions

This thesis has studied the risk factors for a fatal outcome of PMMA intoxication, with particular emphasis on metabolite pattern and CYP genetics.

During 2010–2012, a total of 27 fatal PMMA-related intoxications were registered at DFS in Norway. The PMMA blood concentrations were high and generally in the lethal range, with a median of 9.0 μM, ranging from 0.1 μM to 27 μM. In the non-fatal PMMA abuse cases the median PMMA blood concentration was 1.4 μM, ranging from 0.1 μM to 4.8 μM. Our findings demonstrate that high and toxic PMMA doses had been ingested in most of the fatalities. There was, however, some overlap between fatal and non-fatal PMMA concentrations, which indicates that there are currently unknown protective or predisposing factors in regards of fatal PMMA toxicity.

Two clinical manifestation forms of fatal PMMA intoxication were identified (n=12). In one third of the fatalities, progressive signs and symptoms were witnessed prior to death, mainly severe hyperthermia, shivering, convulsions, acute respiratory distress, cardiac arrest, and sudden collapse. These are typical clinical features of severe serotonin syndrome and increased noradrenergic neurotransmission. Two thirds of the fatalities were found dead at the scene, and many presumably died in their sleep. We could not identify any systematic difference in the PMMA concentrations between the two manifestation forms, but it is well known that serotonin syndrome in its most pronounced stage might lead to unconsciousness, coma, and cardiovascular collapse.

The major risk factors for a fatal outcome of PMMA poisoning were high doses or re-dosing of PMMA, the disguise of PMMA as “amphetamine” or “ecstasy”, concomitant use of amphetamine/methamphetamine or morphine/heroin, a high total concentration of psychostimulant drugs, and being asleep and left unattended. Only one of the 27 fatally intoxicated subjects was alive at first contact with health care professionals. Our findings indicate that the victims and witnesses underestimated the danger of the emerging symptoms, or that serious PMMA poisoning developed so fast that one did not have time to
call an ambulance. Drug users taking serotonergic ecstasy-like drugs should be advised not to be alone while under the influence, and to call the emergency services without delay at the onset of increased body temperature, shivering, reduced consciousness, or respiratory distress, in order to increase the chances of survival.

This work has generated new insights into the importance of genetic variation in CYP enzymes for the toxicity of amphetamine-like drugs such as PMMA. We found that the CYP2D6 enzyme was important for the metabolism of PMMA to OH-MA, and for the formation of most of the studied metabolites of PMMA. PMMA was mainly metabolized to OH-MA, whereas low levels of PMA, OH-A, di-OH-MA, oxilofrine, HM-MA, and HM-A were also formed. Our study showed that other enzymes than CYP2D6 are also involved in the metabolism of PMMA to OH-MA in humans. Contrary to what previous studies have suggested, we could not identify any genetic CYP2D6 predictive marker on fatal toxicity of PMMA. Accordingly, our study does not lend support to previous theories that the toxicity of ecstasy-like drugs like PMMA is related to CYP2D6 enzyme genetics. We also found no association between fatal toxicity of PMMA and CYP2C9, CYP2C19, or CYP3A5 genetics.

In the fatal PMMA intoxications, the relative formation of OH-MA from PMMA was significantly lower, and not correlated with the CYP2D6 genotype, compared with the nonfatal PMMA abuse cases where the relative metabolite formation was high and correlated with the CYP2D6 genotype. Our findings indicate that most PMMA deaths occurred rapidly and at an early stage of PMMA metabolism, or that there was a more pronounced inhibition of the CYP2D6 enzyme in the fatalities imposed by the high blood concentrations of the weak and dose-dependent CYP2D6 inhibitors PMMA and co-administered methamphetamine/amphetamine.

Our studies demonstrated, for the first time, that PMMA was metabolized to the catechol di-OH-MA in HLMs. The formation of di-OH-MA from PMMA was, however, two to seven times lower than from an equimolar dose of the less toxic drug MDMA. Our findings do not support the hypothesis of catechol metabolites as major determinants of fatal PMMA toxicity in humans.
There was an overrepresentation of the CYP2D6 PM genotype in non-fatal PMMA abuse cases, compared to our reference groups of non-abusers and the background Caucasian population. This could be related to personality traits or might simply represent random variation due to the limited sample size.
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Original papers I – III
The PMMA epidemic in Norway: Comparison of fatal and non-fatal intoxications

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A B S T R A C T
During a 6 month period (July 2010–January 2011) we observed 12 fatal intoxications and 22 non-fatal cases related to the drug paramethoxymethamphetamine (PMMA) in Norway (4.8 mill inhabitants). This toxic designer drug, also known as “Death”, is occasionally found in street drugs offered as “ecstasy” or “amphetamine”. The present study aimed to evaluate the cause of death, and to compare the PMMA blood concentrations in fatal and non-fatal cases. Methods for identification and quantification of PMMA are presented. The median age of fatalities was 30 years (range 15–50) with 67% males; in non-fatal cases 27 years (20–47) with 86% males. In the 12 fatalities, the median PMMA blood concentration was 1.92 mg/L (range 0.17–3.30), which is in the reported lethal range of 0.6–3.1 mg/L in peripheral blood and 1.2–15.8 mg/L in heart blood. In the 22 non-fatal cases, the median PMMA concentration was 0.07 mg/L (range 0.01–0.65). Poly-drug use was frequent both in fatal and non-fatal cases. The PMMA concentrations ranging from 0.00 to 0.26 mg/L in both groups likely represented a PMMA metabolite. Three fatalities were attributed to PMMA only, six to PMMA and other psychostimulants drugs, and three to PMMA and CNS depressant drugs, with median PMMA concentrations of 3.05 mg/L (range 1.58–3.30), 2.56 (1.52–3.23) and 0.52 mg/L (0.17–1.24), respectively. Eight victims were found dead, while death was witnessed in four cases, with symptoms of acute respiratory distress, hyperthermia, cardiac arrest, convulsions, sudden collapse and/or multiple organ failure. In summary, all fatalities attributed to PMMA had high PMMA blood concentrations compared to non-fatal cases. Our sample size was too small to evaluate a possible impact of poly-drug use. A public warning is warranted against use and overdose with illegal “ecstasy” or “speed” drugs.

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1. Introduction
The toxic designer drug paramethoxymethamphetamine (PMMA) is the 4-methoxy analogue of methamphetamine (MA). It is partly metabolized to the more well-known toxic drug paramethoxymethamphetamine (PMA), which is the 4-methoxylated analogue of amphetamine (A) (Fig. 1). PMMA appeared later on the illegal drug scene than PMA, in the 1990s and 1970s, respectively. PMMA and PMA are sometimes offered on the clandestine market as a MDMMA (methyleneoxymethamphetamine, “ecstasy”) substitute, often mixed with A, MA and/or MDMMA. Both PMMA and PMA have abuse potential and are listed in many countries as substances controlled by legislation [1]. The effects mimic some of the psychological effects of ecstasy and “speed” (methamphetamine, amphetamine). However, the toxicity of PMMA and PMA, earning the street name “Death”, is reported to be substantially higher than with ecstasy and amphetamines, as documented by more than 90 fatal and many severe poisonings attributed to the ingestion of PMMA/PMA in Canada, USA, Australia and Europe [1]. As PMA and PMMA presumably account for only a small part of the apparent ecstasy ingestion, the relative number of fatalities is high [1,2].

During a 6 month period (July 2010–January 2011), we observed the uncommon appearance in Norway (4.8 mill inhabitants) of several unnatural deaths in young adults, which were all apparently related to recent intake of A, MA or “ecstasy”. The fatalities were subjected to forensic autopsy and standard toxicological blood drug analysis, but no obvious cause of death was found. As there is little mortality attributable to A/MA when used alone, even in overdose [3], we performed supplementary analyses to search for other toxic agents, possibly related to
amphetamines. This brought to light a Norwegian epidemic of fatal and non-fatal PMMA intoxications, which at the time of writing is still ongoing.

The present study aimed to evaluate the cause of death and to compare the PMMA blood concentrations in fatal and non-fatal cases. Methods for identification and quantification of PMMA will be presented.

2. Materials and methods

2.1. Selection of cases

The Division of Forensic Medicine and Drug Abuse Research (DFMDA) at the Norwegian Institute of Public Health (NIPH) has the national responsibility for forensic toxicology analysis in biological specimens in all cases of suspected driving under the influence of drugs/alcohol (DUI), and in most cases of suspected unnatural death where the division serves 93% of the Norwegian population (except Mid-Norway). DFMDA receives blood specimens from about 9000 suspected criminal offences and 1600 postmortem cases annually.

In the unnatural deaths apparently related to the use of classical amphetamines or "ecstasy" emerging in the early autumn of 2010, we performed supplementary screening analyses in blood to search for more toxic amphetamine derivatives. This resulted in the identification of PMMA, in toxic and lethal concentrations. Simultaneously, a warning was issued from the National Criminal Investigation Service (NCIS/Kripos) that PMMA was recently detected in multiple Norwegian drug seizures of powder sold as "ecstasy" or "speed". The first positive case was seized on July 9th 2010. Consequently, we performed retrospective PMMA/PMA analysis in all blood samples received in our laboratory after May 31st 2010, from cases of unnatural death in which (a) there was any indication of recent A, MA or MDMA use, and/or (b) A, MA and/or MDMA was identified in blood or urine samples, above or below the analytical cut-off. Routine screening/confirmation of blood samples for PMMA/PMA in cases of unnatural death and suspected criminal offences was implemented in September 27th. Hence, the PMMA fatalities included in this study were diagnosed in July 2010-January 2011, while the included non-fatal cases represented suspected DUI or drug abuse during the same time period.

2.2. Sample collection

All of the fatal PMMA intoxications were subjected to a forensic autopsy, performed at the DFMDA, Gades Institute (University of Bergen) or the Department of Pathology at the University Hospital of North-Norway. Postmortem whole blood specimens of peripheral blood and urine or vitreous humour were collected in 25 ml Sterilin tubes (Sterilin Limited, Caerphilly, UK) containing 200 mg potassium fluoride as preservative. Specimens were brought to the DFMDA for analysis.

Whole blood samples from non-fatal cases of DUI or drug abuse were received in 4 mL BD Vacutainer® Plus Plastic Blood Collection Tubes (BD Vacutainer Systems, Franklin Lake, NJ, USA) containing 10 mg sodium fluoride and 8 mg potassium oxalate.

The collected samples were stored at 4 °C prior to processing.

2.3. Drug screening

All blood samples received by the DFMDA were screened for alcohols, ketone bodies and 31 legal and illegal psychoactive drugs and narcotics using Head-Space Gas Chromatography (HS-GC) and Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS/MS) [4,5]. PMMA and PMA were included in the UPLC-MS/MS screening. Additionally, all postmortem blood samples were routinely screened for 47 psychoactive drugs and metabolites using a previously described LC–MS–method [6], but with a slightly modified repertoire because some compounds are now analysed by UPLC-MS/MS [5].

2.4. Confirmation method for PMMA/PMA

PMMA and PMA were quantitated with a dedicated UPLC-MS/MS method, as described in the following. PMMA and PMMA and the internal standard amphetamine d11 were purchased from Cerilliant Corporation (Round Rock, TX, USA). Standard compounds were stored according to supplier recommendations (solid substances at room temperature, ampoules at 4 °C). HPCL-grade methanol and acetonitrile were purchased from Lab-scan Ltd. (Dublin, Ireland) and ammonium bicarbonate from Sigma–Aldrich (St. Louis, MO, USA). All water used was provided from our MilliQ-A10 purification system (Millipore, Billerica, MA, USA). 5 mL glass tubes were purchased from Apodan (Copenhagen, Denmark) and 300 μL auto sampler PP-vial from Waters (Milford, MA, USA).

We prepared stock solutions in methanol, and calibrator and quality control (QC) solutions were prepared by appropriate dilution of stock solutions with water. The stock and aqueous solutions were stored at −20 °C and 4 °C, respectively. The internal standard solution was diluted with water and contained 270 ng/mL amphetamine-d11. We prepared a 5 mmol/L solution of ammonium bicarbonate by dissolving 400 mg ammonium bicarbonate in 1 L water, which gives a pH of 7.9.

2.4.1. Sample preparation

0.5 mL aliquots of whole blood were transferred to separate 5 mL glass tubes, and stored at 4 °C until analysis. We added 50 μL of internal standard (amphetamine d11) to 0.5 mL of whole blood and performed protein precipitation with 0.5 mL ice cold acetonitrile. 100 μL of supernatant was transferred to vials after centrifugation and diluted by adding 100 μL water.

2.4.2. Instrumental set-up

A Waters Aquity UPLC-system was used for separation, applying an Aquity UPLC® BEH phenyl (1.7 μm 2.1 mm × 100 mm) column, with gradient elution at a flow rate of 0.5 mL/min with 100% methanol (mobile phase A) and 5 mmol/L aqueous ammonium bicarbonate, pH 7.9 (mobile phase B). The column temperature was held at 65 °C, and the injection volume was 5 μL using partial loop injection with a needle overfill flush of 3 μL. Weak wash was performed with 600 μL methanol:water (10:90), and strong wash with 200 μL methanol:water (90:10), for each sample. Linear change was used for all steps in the gradient. The gradient started at 2.5% A increasing to 5% after 0.5 min, 30% after 2 min, 50% after 3.6 min, held at 50% until 4 min, then increased to 80% at 4.3 min, kept at 80% until 4.75 min, and returned to 2.5% at 4.85 min. The total cycle time was 5.5 min.

A Waters Quattro Premier XE tandem mass spectrometer, equipped with a Z-spray electrospray interface, was used for all analyses. Positive ionization was performed in the multiple reaction monitoring (MRM) mode, with two transitions for PMMA and PMMA and one transition for amphetamine d11. The capillary voltage was set to 1.0 kV, the source block temperature was 120 °C, and the desolvation gas (nitrogen) was heated to 500 °C and delivered at a flow rate of 900 L/h. The cone gas (nitrogen) was set to 60 L/h, and the collision gas (argon) pressure was maintained at 0.005 mbar in the collision cell. The appropriate MRM transitions, cone voltages, and collision energy for the individual analytes were determined by direct infusion into the mass spectrometer. The MRM transitions, cone voltage (CV), and collision energy (CE) for the measurement of the analytes and the internal standard
were amphetamine d11, 147.1 > 98.0, CV = 15, CE = 18; PMMA 180.2 > 121.1 CV = 15, CE = 18, 180.2 > 149.1 CV = 15, CE = 12; PMMA 166.1 > 121.1 CV = 15, CE = 18, 166.1 > 149.1 CV = 15, CE = 12. System operation and data acquisition were controlled using Mass Lynx 4.1 software. Analysts were identified by comparing the retention times and ion ratios of the respective MRM transitions with the corresponding calibrators and QC samples. Data were processed with the QuanLynx program, using peak height for quantification.

2.4.3. Validation results

The validation was performed with 7 calibrators ranging from 0.001 to 3.0 mg/L for PMMA and 0.01 to 3.6 mg/L for PMA and fitted using a quadratic curve with 1/x weighing excluding the orio. The lower limit of quantitation (LOQ) was set as the lowest calibration standard for both PMMA and PMA, evaluated by performance of calibrators (RSD and bias within ± 20%). The interassay precisions for low and high QC samples run at 0.02 and 0.2 mg/L for PMMA and 0.2 and 2 mg/L for PMA with two replicates on nine different days were 9% and 6% for PMMA and 9% and 7% for PMMA (n = 18), and the accuracy expressed as bias was –4% and –2% for PMMA and –2% and –3% for PMA. The within assay precision for low and high QC samples were 3% and 4% for PMMA and 4% and 6% for PMMA (n = 10), and the accuracy expressed as bias was –1% and –3% for PMMA and –4% and –8% for PMA.

Matrix effects were evaluated at 0.002 mg/L by the method proposed by Matuszewski et al. [7]. A value below 100 indicates ion suppression, while a value above 100 indicates ion enhancement. A total of eight different sources of whole blood were tested, three from living persons and five autopsy blood samples. The samples were determined to be negative for PMMA/PMA by screening [5] before spiking. Six replicates of mobile phase were included in the test. A matrix effect of 82% for PMA and 81% for PMMA was found, indicating some ion suppression. If correction by internal standard is included in the calculation, the suppression is negligible, with matrix effects calculated to be 100% and 99%, respectively.

2.5. Statistics

Statistic parameters were calculated using the Statistical Package for Social Sciences (SPSS) version 17.0. Differences between groups were examined using the non-parametric Mann–Whitney U-test. Statistical comparisons were considered significantly different if p < 0.05 (two-tailed).

3. Results

The median age of the 12 PMMA-related fatalities was 30 years (range 15–50) with 67% males, in the 22 non-fatal cases the median age was 27 years (20–47) with 86% males. Different geographical regions of Norway were represented both in fatal and non-fatal cases.

The postmortem specimens were collected median 2, range 1–6 days after death. The specimens were sent to FDMA within median 5, range 2–9 days after autopsy.

The blood concentrations of PMMA, PMA, MA and A in fatal and non-fatal cases are presented in Table 1. The PMMA concentrations, ranging from not detectable to 0.26 mg/L in both groups, likely represented a PMMA metabolite.

The median concentration ratio of amphetamines relative to PMMA, calculated as [A + MA]/PMMA, was 0.2 (range 0.0–22.6) in fatal and 7.9 (0.0–107.3) in non-fatal intoxications, a difference which is significant (p < 0.01).

Concomitant use of other psychoactive drugs was frequent both in fatal and non-fatal cases, mainly A/MA, cannabis, benzodiazepines and/or ethanol. Two of the deceased had recently used heroin, according to the witness histories, the detection of 6-monoacetylmorphine in the urine samples and morphine in the blood. Use of heroin/morphine was not involved in any non-fatal cases, whereas a therapeutic blood concentration of tramadol was found in one case and a recreational concentration of gammahydroxybutyrate (GHB) in three cases.

The individual circumstances of death and results of the forensic autopsy and toxicological analyses of the 12 fatalities are presented in Table 2. Death was witnessed in four subjects, while the eight other victims were found dead, mainly after presumed sleep.

Based on the available information, the fatalities were categorized according to the main cause of death (Fig. 2 and Table 3). All fatalities were attributed to drug intoxication, and the manner was classified as accidental. Three fatalities were attributed to PMMA only, six to PMMA and other psychostimulant drugs, and three to PMMA and CNS depressant drugs, mainly morphine/heroin1 or ethanol, with median PMMA concentrations of 3.05 mg/L (range 1.58–3.30), 2.56 (range 1.52–3.23) and 0.52 mg/L (range 0.17–1.24), respectively.

In fatalities attributed to PMMA and CNS depressants, the PMMA concentrations tended to be lower than in fatalities attributed to PMMA only (Fig. 2).

In one fatal developing multi-organ failure, both antemortem (AM) serum and postmortem (PM) femoral blood samples were available for analysis, sampled 52 h before and 48 h after death, respectively. The AM–PM concentrations of PMMA were 0.97–2.22 mg/L, and the corresponding concentrations of PMMA, MA and A were 0.08–0.26 mg/L, 0.03–0.09 mg/L and 1.07–3.11 mg/L, respectively.

4. Discussion

4.1. Comparison of blood drug concentrations in fatal and non-fatal cases

In the 12 fatal intoxications related to PMMA, the mean PMMA concentration in peripheral blood was 2.02 mg/L. In 11 of these fatalities, the PMMA concentrations were above 0.5 mg/L, and in most cases greater than 1.24 mg/L. These are high PMMA concentrations within the fatal range reported by other authors [1.8–12]. For comparison, PMMA or PMMA blood concentrations above 0.5 mg/L are reported to be associated with toxic and possibly lethal effects [13,14]. In heart blood, a mean lethal PMMA concentration of 4.3 ± 4.8 mg/L was reported in Taiwan in 2006 (n = 8) [10]. In femoral blood, a concentration of 0.85 mg/L was reported in Germany 2002 (n = 1) and 0.6–3.1 mg/L in Denmark 2000 (n = 2) [8.9]. For PMMA fatalities, postmortem blood concentrations ranging from 0.24 to 5.7 mg/L are reported [1,2,8,9,11–21].

1 A high MA concentration was also found in ID9.
Table 2
Summary of the 12 fatal PMMA intoxications, with drug concentrations detected in postmortem peripheral blood.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMMA</th>
<th>PMA</th>
<th>MA</th>
<th>A</th>
<th>MDMA</th>
<th>Alcohol (%)</th>
<th>Other drugs(^b)</th>
<th>Circumstances of death</th>
<th>Forensic autopsy findings</th>
<th>Cause of death</th>
<th>Manner of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>20–29</td>
<td>1.58</td>
<td>0.10</td>
<td>0.36</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Found dead in the early morning, many hours after use of amphetamine, ecstasy, alcohol.</td>
<td>Lung edema.</td>
<td>I</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>40–49</td>
<td>3.23</td>
<td>0.25</td>
<td>0.07</td>
<td>0.57</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Found dead at mid-day, with hyperthermia and rigor mortis.</td>
<td>Moderate aortic valve stenosis, cardiac hypertrophy, lung edema.</td>
<td>II</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>15–19</td>
<td>0.52</td>
<td>0.08</td>
<td>0.09</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Morphine (0.04)</td>
<td>Severe auditory hallucinations after intake of &quot;speed&quot;, ecstasy and other drugs. Some hours later observed deeply sedated. Found dead many hours later.</td>
<td>Pulmonary congestion, sparse bleeding in subcutaneous tissues, sparse aspirated food in lower airways.</td>
<td>III</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>20–29</td>
<td>3.05</td>
<td>0.21</td>
<td>0.15</td>
<td>n.d.</td>
<td>n.d.</td>
<td>THC (6.29 μg/L)</td>
<td>At noon witnessed seizures and immediate cardiac arrest. Intake of ecstasy, cannabis and alcohol the previous evening, subsequent sleep. Known to have epilepsy.</td>
<td>Lung edema and dilatation of right cardiac ventricle. Possibly acute toxic glomerulosclerosis.</td>
<td>I</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>40–49</td>
<td>2.92</td>
<td>0.05</td>
<td>1.64</td>
<td>0.92</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Respiratory distress 1.5 h after injection of &quot;amphetamine&quot;. Known amphetamine abuse.</td>
<td>Pulmonary congestion.</td>
<td>II</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>20–29</td>
<td>2.22</td>
<td>0.26</td>
<td>0.09</td>
<td>3.11</td>
<td>n.d.</td>
<td>THC (0.94 μg/L)</td>
<td>Severe hyperthermia, shakiness and dehydration 2 h after the intake of ca 0.2 g &quot;amphetamine&quot;. Cardiac arrest at admission to hospital. Goma, multi organ failure, hyperkalemia, disseminated intravascular coagulation, hypoglycemia. Death 3 days after admission. Pulmonary congestion, widespread petechial bleeding in airways and organs. Brain and kidneys swollen.</td>
<td>During 10 h repeated ingestion of 0.5 g white powder thought to represent MDMA, totally 2 g. Friends with simultaneous intake of the same drug in similar doses survived, unknown whether they developed symptoms. Sparse food aspirated in airways. Slight superficial bleeding in gastric and duodenal mucosa.</td>
<td>I</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>20–29</td>
<td>3.30</td>
<td>0.23</td>
<td>0.10</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Observed surprisingly awake at night in spite of one day partying with alcohol and repeated &quot;amphetamine&quot; use. Found dead the next day with rigor mortis and rectal temperature 41°C.</td>
<td>Pulmonary congestion, severe coronary atherosclerosis in LAD.</td>
<td>II</td>
<td>Acc</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>30–39</td>
<td>1.61</td>
<td>0.07</td>
<td>3.43</td>
<td>0.18</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In the majority of the 22 non-fatal intoxications, the whole blood PMMA concentration at the time of sampling was low or moderate, and within the range associated with recreational use of PMMA and PMA [1]. However, one subject had a high PMMA concentration of 0.65 mg/L, which is above the reported toxic range. At the time of apprehension, 4 h before sampling, the concentration was probably even higher. However, this individual was considered to be not-impaired in the Clinical Test of Impairment performed by a physician at the time of blood sampling. Reportedly, this subject had ingested the same white powder drug and in similar doses as one of the fatalities, about 2 g per subject. As one subject became lethally intoxicated while the other presumably suffered no severe symptoms after similar PMMA intakes, this might indicate yet unrevealed mechanisms for interindividual differences in the sensitivity to the fatal PMMA effects.

The fatalities demonstrated PMMA blood concentrations which were many times higher than in non-fatal cases. Moreover, the level of PMMA relative to A/MA was significantly higher in the fatalities than in non-fatal cases. However, at least some of the first mentioned difference can probably be attributed to postmortem drug redistribution (PMR) from drug-rich tissues to blood, resulting in gradually increased drug concentrations some time after death. Our samples were collected 1–6 days after death. PMR is particularly significant for drugs with a volume of distribution (Vd) above 3–4 L/kg [22]. Although the human Vd is not known for PMMA, 6.4 L/kg is reported in rats [23], and structurally related amphetamines have Vd’s in the range of 3–6 L/kg. A PMMA heart/peripheral blood ratio of 2.5 is reported (n = 7) [8]. For PMA, a postmortem/antemortem (PM/AM) concentration ratio of 1.04 was reported in one former Norwegian PMMA fatality [12]. In ID6 in our study, both AM serum and PM peripheral blood samples were available for analysis. In spite of the long time period between sampling and death, and the unknown blood–plasma ratio of PMMA and PMA, the detected drug concentrations might indicate a PM/AM concentration ratio of about 2–3 for PMMA and PMA, and probably more.

The moderate PMA blood concentrations in fatal and non-fatal cases, probably representing a PMMA metabolite, are in good agreement with the fact that PMA has not been detected in any

---

**Table 1.** Intoxication patterns of the 22 PMMA fatalities.

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age</th>
<th>Cause of death</th>
<th>Drugs detected</th>
<th>Concentrations (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 F</td>
<td>30–39</td>
<td>0.17</td>
<td>Intoxication</td>
<td>Morphine (0.28), codeine (0.03), promethazine (0.05), quetiapine (1.00), THC (3.46)</td>
<td>n.d.</td>
</tr>
<tr>
<td>10 F</td>
<td>20–29</td>
<td>2.89</td>
<td>Intoxication</td>
<td>Erythromycin, promethazine (0.03), percocet (0.11), THC (7.46)</td>
<td>n.d.</td>
</tr>
<tr>
<td>11 M</td>
<td>50–59</td>
<td>1.24</td>
<td>Intoxication</td>
<td>Morphine (0.39), N-demethyl diazepam (0.08), THC (3.46)</td>
<td>0.02</td>
</tr>
<tr>
<td>12 M</td>
<td>30–39</td>
<td>1.52</td>
<td>Death</td>
<td>MDA (0.05), cocaine (0.01), THC (0.58)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**Fig. 2.** Boxplot of the PMMA concentrations in peripheral blood of the 12 fatalities categorized according to the main cause of death, in comparison with the blood concentrations in the 22 non-fatal cases related to PMMA. The middle line is the median, the box represents the 25–75 percentile and the whiskers represent the minimum and maximum observation. Outliers are marked with a circle, and extreme values with an asterisk. The number of observations are in brackets.
Norwegian PMMA seizure since the drug reappeared in Norway on July 9th 2010.

4.2. Impact of other psychoactive drugs on PMMA toxicity

It is reported that the combination of PMMA with other stimulants, alcohol or cannabis, or with drugs affecting serotonin receptors, may increase the PMMA toxicity because of strong catecholaminergic stimulation [24–26]. We found the highest PMMA concentrations in fatalities attributed to PMMA alone, while the concentrations tended to be lower in fatalities attributed to PMMA and other psychoactive drugs. However, the sample size in this study is too small and the power too low to find a possible significant impact of other psychoactive drugs on the PMMA toxicity.

In the two PMMA fatalities related to heroin, the PMMA concentrations tended to be lower than in the other fatalities. For ID3, the PMMA concentration of 0.52 mg/L was within the lower toxic range, combined with a morphine concentration of 0.04 mg/L, which is not high but still within a range associated with heroin-related fatalities. This subject suffered severe hallucinations prior to death, a characteristic symptom of PMMA toxicity [1]. This indicates that PMMA concentrations even in the lower toxic range might be lethal, possibly in combination with a potent opiate. With a PMMA blood concentration of 0.17 mg/L, ID9 exhibited the lowest PMMA concentration of all the twelve fatalities. This level is within the reported range of recreational use, and to our knowledge, fatalities are not formerly reported at such low concentrations of PMMA or PMA. In this case, the forensic pathologist considered the high morphine concentration of 0.20 mg/L to represent the major cause of death. Moreover, this subject was cold when found dead a few hours after the last vital observation. This could also speak against severe PMMA toxicity in this individual.

4.3. Cause of death

The autonomic, somatic and cognitive signs and symptoms witnessed before death, e.g. hyperthermia, shivering, hyperactivity, muscle spasms, convulsions, hallucinations, sudden collapse, cardiac arrest, coma and multiple organ failure, represent common symptoms of PMMA and PMA intoxication [1]. Together with tachycardia and hypertension, such symptoms also represent the major features of serotonin syndrome and of excessive catecholamine activity [27]. In fact, PMMA is more selective for serotonergic neurons than MDMA, enhancing serotonin levels in the CNS by inhibiting reuptake. It also inhibits central and peripheral monoamine oxidase A (MAO-A), which prevents the breakdown of serotonin and other monoamines [1]. With repeated PMMA use or overdosing, the MAO inhibition leads to greatly increased discharge of catecholamine in response to whatever dose of stimulants or stimuli [3]. The severely increased extraneuronal and peripheral serotonin levels result in acute serotonin syndrome [23], while the increased levels of other monoamines are responsible for most of the serious effects on the cardiovascular system [3].

As opposed to MDMA and amphetamine, the transport of PMMA over the blood–brain-barrier is poor [28]. A consequence is the well-known slow onset of stimulant action. Moreover, PMMA is reported to have a weak euphoriant effect that is probably mediated by noradrenaline, while the more attractive euphoriant actions on dopaminergic neurons, which are typical of A and MDMA euphoria, are faulty [1]. At the same time, peripheral sympathomimetic effects as tachycardia and increased blood pressure clearly demonstrate that the ingested drug is “active”. Altogether, these drug properties may easily encourage users to repeat their intake (“stacking”) to achieve the desired but never achieved response, resulting in PMMA overdoses. Besides, repeated intake of stimulants is commonplace, to keep up the desired effects until the dopaminergic pathways are exhausted.

At least three of the fatalities might have been directly preceded by arrhythmia and acute cardiac failure, and one fatality was preceded by convulsions in an epileptic subject. One of the known effects of catecholamines is sensitization of the myocardium to ectopic stimuli, increasing the risk of ventricular arrhythmias [3]. QRS interval prolongation and hyperkalemia are reported to be unique and frequent features of PMA poisoning [2,12]. It is suggested that PMA might have sodium-channel-blocking properties, which could explain the tendency of QRS prolongation and convulsions.

At autopsy, pulmonary and other organ congestion was a common finding. In some subjects, mucosal haemorrhage in the nose, gastric mucosa or in organ membranes was noted. Petechial haemorrhages in various organs are also incidentally described in “ecstasy” fatalities [3]. Two victims had findings consistent with acute renal damage, which is also an expected complication of severe PMMA intoxication [1].

Two victims exhibited severe hyperthermia and rigor mortis when they were found dead. Both had a high blood PMMA concentration and a moderate A or high MA concentration, with no other drugs detected. It is well known that rigor mortis may start at the time of death, e.g. in severely hyperthermic victims of PMA/A/
MA intoxication after repeated use of “ecstasy” tablets containing 50 mg PMA [29]. In a study where rats had been administered a neurotoxic dose of the structurally similar drug MDMA five weeks previously, the hyperthermic response to any dose of MDMA was enhanced, particularly after repeated administration, compared to non-pre-treated rats. The increase was further marked at high ambient temperature [30]. For the hyperthermic victims in our study, it is not known whether they had been exposed to repeated doses or only one high dose of PMMA.

Other environmental influences are also reported to strengthen the individual response to stimulant drugs in rapid and unpredictable ways, e.g. overcrowding, loud music and prolonged dancing [24]. Such factors might have been of importance in at least one of our fatalities.

Nine of twelve deaths apparently occurred closely related to presumed sleep. Eight of them were found dead many hours after the last vital observation. Acute death during sleep could possibly be explained by severe hypoglycemia and/or hyperkalemia, which are reported to be unique and frequent features of PMA poisoning [2, 12]. Further, PMMA/PMA may have MAO-inhibiting properties, and other MAO inhibitors have been reported to stimulate insulin release [2].

Regarding possible mechanisms for individual differences in the sensitivity to the fatal PMMA effects, genotypic and phenotypic variability in metabolic clearance of PMMA might be involved. PMMA is extensively metabolized by the polymorphic CYP2D6 enzyme to the sympathomimetic 4-hydroxymethamphetamine (phendrine) [31]. This warrants further investigations.

5. Conclusion

The investigation of this large PMMA epidemic demonstrated that all fatalities attributed to the ecstasy-substitute PMMA had high PMMA blood concentrations, compared to non-fatal cases. Our sample size was too small to evaluate a possible impact of poly-drug use. A public warning is warranted against use and overdose with illegal “ecstasy” or “speed” drugs.

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References

Is toxicity of PMMA (paramethoxymethamphetamine) associated with cytochrome P450 pharmacogenetics?

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A B S T R A C T

In 2010–2013, 29 fatal intoxications related to the designer drug paramethoxymethamphetamine (PMMA, 4-methoxymethamphetamine) occurred in Norway. The current knowledge about metabolism and toxicity of PMMA in humans is limited. Metabolism by the polymorphic cytochrome P450 (CYP)2D6 enzyme to the psychoactive metabolite 4-hydroxymethamphetamine (OH-MA), and possibly by additional enzymes, is suggested to be involved in its toxicity. The aim of this work was to study the association between CYP genetics, PMMA metabolism and risk of fatal PMMA toxicity in humans. The frequency distribution of clinically relevant gene variants of CYP2D6, CYP2C9, CYP2C19 and CYP3A45, and the phenotypic blood CYP2D6 metabolic ratio (OH-MA/PMMA) in particular, were compared in fatal PMMA intoxications (n = 17) and nonfatal PMMA abuse controls (n = 30), using non-abusers (n = 305) as references for the expected genotype frequencies in the Norwegian population. Our study demonstrated that the CYP2D6 enzyme and genotype are important in the metabolism of PMMA to OH-MA in humans, but that other enzymes are also involved in this biotransformation. In the fatal PMMA intoxications, the blood concentrations of PMMA were higher and the CYP2D6 metabolic ratios were lower, than in the nonfatal PMMA abuse controls (median (range) 2.1 (0.03–5.0) vs 0.3 (0.1–0.9) mg/L, and ratio 0.6 (0.0–4.6) vs 2.1 (0.2–7.4) p = 0.021, respectively). Overall, our findings indicated that, in most cases, PMMA death occurred rapidly and at an early stage of PMMA metabolism, following the ingestion of large and toxic PMMA doses. We could not identify any genetic CYP2D6, CYP2C9, CYP2C19 or CYP3A45 predictive marker on fatal toxicity of PMMA in humans. The overrepresentation of the CYP2D6 poor metabolizer (PM) genotype found in the nonfatal PMMA abuse controls warrants further investigations.

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1. Introduction

In 2010–2013, a cluster of 29 fatal poisonings related to the toxic designer drug paramethoxymethamphetamine (PMMA, 4-methoxymethamphetamine) was revealed in Norway [1]. PMMA is a ring-substituted monomethoxy methamphetamine (MA) derivative, and an N-methylated derivative of the pharmacologically similar designer drug paramethoxymethamphetamine (PMA, 4-methoxymethamphetamine) (Fig. 1). The ring-substitution adds potent serotonergic, hallucinogenic and MDMA-like (ecstasy, 3,4-methylenedioxy-methamphetamine) properties to methamphetamine/amphetamine drugs [2]. PMMA and PMA are substitute drugs occasionally found in powder or tablets illegally sold as “amphetamine” or “ecstasy”. These drugs act by increasing the release and inhibiting the reuptake of serotonin and by reversibly inhibiting the enzyme monoamine oxidase A (MAO-A) [3,4]. The toxicity of PMMA is regarded as substantially higher than for amphetamine, methamphetamine and MDMA [5], as indicated by 131 fatal and 31 nonfatal poisonings associated with the abuse of PMMA worldwide [1,6–10]. The toxicity of PMMA is positively correlated with the PMMA dose and the blood drug level, but the existing literature indicates that certain human subjects may have an increased risk of PMMA toxicity [1]. This has also been suggested for other designer amphetamines like 4-methylthioamphetamine (4-MTA) [11].
Fig. 1. Chemical structure and major metabolic pathways of PMMA, PMA, methamphetamine, amphetamine and MDMA; PMMA, paramethoxymethamphetamine, PMA, paramethoxyamphetamine, MDMA, 3,4-methylenedioxyamphetamine, CYP2D6, cytochrome P450 2D6.

Genetic and phenotypic variability in the PMMA metabolism might be relevant vulnerability traits.

The current knowledge about the PMMA metabolism and toxicity in humans is limited. The available information about metabolism is based solely on two rat studies [12,13], one study using human liver microsomes [14] and one urine sample from a single individual [12]. As is the case with many synthetic amphetamine analogues, PMMA metabolism probably occurs mainly through O-demethylation via the polymorphic cytochrome P450 (CYP) 2D6 enzyme to the psychoactive and potentially toxic metabolite 4-hydroxymethamphetamine (OH-MA, pholedrine) followed by conjugation with glucuronide or sulphate [14,15]. N-demethylation to PMA is a minor pathway, mainly by other CYP enzymes and MAO-A [16]. PMA is the only metabolite unique for PMMA, as the other PMMA metabolites are also formed from MDMA [17] and methamphetamine [18] by the same enzymes (Fig. 1).

Due to genetic variation and pharmacological interactions, CYP2D6 catalytic activity varies considerably in all populations examined. Based on CYP2D6 genotype, subjects can be classified into four CYP2D6 phenotype categories: poor (PM), intermediate (IM), extensive (EM) and ultrarapid metabolizers (UM) [19]. It has been suggested that CYP2C isozymes might be involved in the deamination of amphetamines and that CYP2C gene variants might contribute to interindividual differences in drug action [16]. Also CYP isoenzymes of the 1A, 2B and 3A subfamilies have the capacity to metabolize ring-substituted amphetamines, possibly of particular importance in CYP2D6 poor metabolizers [20].

Previous reports on the association between CYP2D6 genotypes, drug metabolism and the risk of stimulant drug toxicity have been conflicting. Early studies on PMA, MDMA and other ring-substituted amphetamines suggested that the CYP2D6 PM phenotype could be a predisposing factor for acute drug toxicity, due to the high toxicity of these parent drugs in high doses [21–23]. However, later studies found no evidence for this [24–27]. Instead, it was suggested that the CYP2D6 UM phenotype might predispose users to toxic effects, due to toxic metabolites [11,16,28–32]. Active metabolites were proposed to be involved in the toxicological effects of some amphetamines, particularly hydroxy- and dihydroxy- (catechol) metabolites or their reactive conversion products [33–35]. It has been questioned whether systemic metabolism is important for PMMA toxicity, as has been reported for MDMA where this is proposed to be a crucial step for toxicity [32].

The aim of the present work was to study the association between CYP genetics, PMMA metabolism and the risk of fatal PMMA toxicity in humans, by comparing the frequency distribution of clinically relevant gene variants of CYP2D6, CYP2C9, CYP2C19 and CYP3A5 enzymes, and the phenotypic blood CYP2D6 metabolic ratio (OH-MA/PMMA) in particular, in fatal PMMA intoxications and nonfatal PMMA abuse controls, using non-abusers as references for the expected genotype frequencies in the Norwegian population.

2. Material and methods

Our cases included all of the fatal PMMA intoxications recorded in Norway during the study period from June 2010 to February 2013. Our control group included nonfatal PMMA-related drug abuse cases recorded in the Forensic Toxicology Database at the Norwegian Institute of Public Health (NIPH) during the same time period. As references for CYP genotype frequencies in the Norwegian population with no known drug abuse, we included healthy living blood donors and natural death cases not related to drug use, reference groups C2 and C3, respectively (Table 1). The exclusion and inclusion criteria for these four groups are described below. The study was approved by the Regional Committee for Medical and Health Research Ethics, and by the Higher Prosecution Authority as the owner of the forensic samples.

2.1. Inclusion in the study

2.1.1. Fatal PMMA intoxications

During the study period of almost three years, PMMA was detected in post-mortem blood samples from 27 forensic autopsy cases at NIPH and in 2 cases from Mid-Norway (personal communication with Lars Sørdal, St. Olavs Hospital, Trondheim). Based on our study's inclusion and exclusion criteria, the forensic toxicology results and the forensic pathologist's classification of cause of death, 12 of these 29 cases were not found to be eligible for inclusion in our study, due to violent cause of death ($n = 2$), no remaining blood ($n = 2$), genotyping failed ($n = 2$) or heroin/morphine being judged as the major cause of death ($n = 6$). These 17 fatal PMMA intoxications constituted our cases. The influence of other drugs as a cause of death is illustrated in Table 2. Based on the available information including the pathologist's conclusion, the fatal PMMA intoxications were categorized as “Fatal PMMA only” with no major contribution of other drugs, and as “Fatal PMMA polydrug” intoxications when a significant concentration of other psychostimulant or sedative drugs was detected in blood. According to the existing literature, we defined a blood amphetamine or methamphetamine concentration above 0.2 mg/L (i.e. lower recreational level) to be “significant” [36] (analytical cut-off 0.03 mg/L), or any concentration of the more toxic substances MDMA, cocaine or ethanol above the analytical cut-off applied in
Table 1
Involvement of drugs in the fatal PMMA intoxications, the nonfatal PMMA abuse controls and in the reference groups C2-C3, and major cause of death.

<table>
<thead>
<tr>
<th>Group</th>
<th>Classification</th>
<th>Drug involvement (and major cause of death for the cases)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>Fatal PMMA intoxications</td>
<td>PMMA+/ other drugs of abuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>‘PMMA only’</td>
<td>No major influence of other drugs of abuse/medicinal drugs</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>‘PMMA polydrug’</td>
<td>PMMA in combination with significant concentration of other psychostimulant drug(s)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PMMA and ethanol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(1)</td>
</tr>
<tr>
<td>Controls</td>
<td>Nonfatal PMMA abuse controls</td>
<td>PMMA+/ other drugs of abuse/medicinal drugs</td>
<td>30</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Healthy blood donors</td>
<td>No reported drugs of abuse</td>
<td>100</td>
</tr>
<tr>
<td>C3</td>
<td>Natural deaths</td>
<td>Death not related to drugs of abuse/medicinal drugs</td>
<td>205/211&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fatal heroin/morphine/opiate intoxications were excluded.
<sup>b</sup> Methamphetamine and/or amphetamine (n=9), MDMA (n=1) and/or cocaine (n=1).
<sup>c</sup> Blood alcohol concentration 2.5 g/L.
<sup>d</sup> CYP2D6 genotype data were available for n=205, CYP2C19 genotype available for n=211.

Table 2
Influence of other drugs in the fatal PMMA intoxications.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Major cause of death</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatal ‘PMMA only’</td>
<td>PMMA. No major influence of other drugs of abuse/medicinal drugs</td>
<td>7</td>
</tr>
<tr>
<td>Fatal PMMA polydrug</td>
<td>PMMA in combination with significant concentration of other psychostimulant drug(s)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>PMMA and ethanol (blood ethanol concentration 2.5 g/L)</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Methamphetamine and/or amphetamine (n=9), MDMA and cocaine (n=1). Fatal heroin/morphine intoxications were excluded.

Routine cases at NIPH. The cut-offs were 0.039 mg/L, 0.015 mg/L and 0.1%<sub>0</sub>, respectively.

2.1.2. Nonfatal PMMA abuse controls

The controls included all individuals apprehended by the Norwegian police during the study period, due to suspected driving under the influence of drugs/alcohol (DUI) or drug abuse, and who presented PMMA whole blood concentrations above 0.09 mg/L. This concentration limit was defined based on previous reports stating that significant pharmacological effects might be expected at PMMA concentrations above this level. PMMA blood concentrations of 0.09–0.6 mg/L are referred to as typical recreational concentrations [6]. In total, PMMA was detected above the analytical cut-off (0.009 mg/L) in the blood specimens of 141 individuals. Of these, 30 individuals had PMMA blood concentrations above 0.09 mg/L and blood samples available for analysis. These 30 subjects represented our controls.

2.1.3. Healthy blood donors (reference group C2)

The reference group C2 included 100 healthy Norwegian blood donors with whole blood samples drawn in 2002. All donors had to fulfill the standard eligibility criteria for blood donation at Oslo University Hospital including health criteria and no use of illicit drugs.

2.1.4. Natural deaths (reference group C3)

The reference group C3 included previously published natural death cases from a Swedish population, these data were kindly provided by the authors [37]. CYP2D6 genotype data were available from 205 of these deaths, and CYP2C19 genotype results were available for 211 of these deaths. These were consecutive cases occurring in Sweden in 2002, the great majority being middle-aged males, in whom the cause of death was judged by the forensic pathologist as natural death, and not intoxication or suicide. The great majority of the Swedish population is Caucasian (similar to Norway).

2.2. Analysis

2.2.1. Specimens used for analysis

In all of the post-mortem cases (Cases and reference group C3), femoral whole blood samples were collected during autopsy with 1% potassium fluoride added as preservative. In the nonfatal PMMA abuser controls, we used venous whole blood samples drawn shortly after the time of apprehension by the police, with sodium fluoride added as preservative and sodium heparin as anticoagulant. In the healthy blood donor references (C2), we used whole blood samples collected in K<sub>2</sub>EDTA tubes at Oslo University Hospital.

2.2.2. Toxicological analysis

Before the time of inclusion in our study, a broad analysis of psychoactive/toxic drugs and metabolites had been performed in the blood samples of the fatal PMMA intoxications and the controls, using ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) screening methods for common drugs of abuse (amphetamine, opiates, benzodiazepines, cocaine, cannabis and other relevant substances) [38] and GHB [39]. The fatal PMMA cases were also screened for a range of prescription drugs and metabolites [40].
PMMA and PMA were either included in the drugs of abuse screening method or screened specifically by UHPLC-MS/MS. For the controls, ethanol was screened by an enzymatic alcohol dehydrogenase method [41], while for the fatal PMMA cases ethanol was screened by a chromatographic method [42], according to our routine. Confirmation analysis was performed for illicit and medicinal drugs whenever detected by the screening analysis, or on suspicion.

In the fatal PMMA intoxications and in controls, the following PMMA metabolites were determined using UHPLC-MS/MS following enzymatic hydrolysis (deconjugation) with β-glucuronidase and sulfatase: 4-hydroxymethamphetamine (OH-MA), 4-hydroxyamphetamine (OH-A), paramethoxyamphetamine (PMA), 4-hydroxy-3-methoxyamphetamine (HM-A), 4-hydroxy-3-methoxyamphetamine (HM-MA) and oxilofrine (4-hydroxyephedrine). Analysis of the unstable catechol PMMA-metabolite dihydroxymethamphetamine (di-OH-MA) was repeatedly attempted by various approaches, but was not possible in the post-mortem blood sample material.

In the reference group C3 samples, analyzed at the National Board of Forensic Medicine (NBFM) in Sweden, a wide range of basic and acidic prescription drugs were determined in femoral blood using capillary gas chromatography (GC) fitted with a nitrogen-phosphorus detector [43]. Ethanol was determined in blood by head-space GC according to a previously described method [44]. Urine and blood samples were also analyzed for different classes of illicit drugs (opiates, cannabis, amphetamines, cocaine and gamma-hydroxybutyrate) after a direct request by the forensic pathologist responsible.

2.2.3. Specific method for PMMA and metabolites

The enzymes β-glucuronidase (type II, from Patella vulgata 1,000,000–3,000,000 units/g solid) and sulfatase (type H-I from Helix Pomatia, ≥10,000 units/g solid) were obtained from Sigma–Aldrich (St. Louis, MO). Amphetamine and OH-MA were purchased from Sigma–Aldrich, PMMA and PMA from Cerillant (Round Rock, TX, USA) and methamphetamine, HM-A and HM-MA from Lipomed (Arlesheim, Switzerland). Oxilofrine and OH-A were obtained from National Measurement Institute (Sydney, NSW, Australia) and di-OH-MA from Cayman Chemicals (Ann Arbor, MI, USA). All the references were of >98% purity. Standard compounds were stored according to supplier recommendations (solid substances mainly at room temperature, solutions at 4 °C). Internal standards 13C6 amphetamine, 13C6 methamphetamine, 13C6 MDMA, 13C6 PMMA and 13C6 PMA were purchased from Chiron (Trondheim, Norway).

The samples were added ascorbic acid as stabilizer and internal standard, and were hydrolysed for 2 h at 60 °C with β-glucuronidase (1800 u) and sulfatase (166 u) as deconjugation agents. Protein precipitation was performed with 0.5 ml acetonitrile:-methanol 85:15 (v/v), the supernatant was evaporated and the residue dissolved in 0.2 ml MeOH:water (20:80).

Analysis was performed with a Waters UPLC-system (Waters, Milford, MA, USA). Separation was performed on an Acquity HSS T3 (1.8 μm 2.1 × 100 mm) column from Waters. The column temperature was held at 65 °C. The system used gradient elution at a flow rate of 0.5 ml/min with 100% methanol (mobile phase A) and 10 mmol/L aqueous ammonium formate, pH 3.1 (mobile phase B). The gradient had an initial composition of 2.5% B for 0.3 min, increased to 25% B in 5 min, switched to 100% B at 5.01 min and held for 1 min. At 6.01 min the gradient was returned to initial conditions at 2.5% B. The total cycle time was 8 min. An injection volume of 5 μL was used, and 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 performed with a Waters Quattro Premier XE tandem mass spectrometer. Additional data regarding the MRM transitions, cone voltage (CV) and collision energy (CE) for the measurement of the analytes and the internal standards together with retention time and calibration range are given in Table 3.

Table 3 Instrumental parameters and calibration range (ng/mL).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Target ion/qualifier ion transition</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
<th>Retention time (min)</th>
<th>Calibration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>136.1 &gt; 91.1</td>
<td>14</td>
<td>20</td>
<td>4.03</td>
<td>0.007–3.4</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>150.1 &gt; 91.1</td>
<td>15</td>
<td>20</td>
<td>4.30</td>
<td>0.015–7.5</td>
</tr>
<tr>
<td>PMA</td>
<td>166.1 &gt; 121.1</td>
<td>15</td>
<td>18</td>
<td>4.61</td>
<td>0.003–1.7</td>
</tr>
<tr>
<td>PMMA</td>
<td>180.2 &gt; 121.1</td>
<td>15</td>
<td>18</td>
<td>4.78</td>
<td>0.009–4.5</td>
</tr>
<tr>
<td>OH-A</td>
<td>152.1 &gt; 107.1</td>
<td>15</td>
<td>18</td>
<td>2.21</td>
<td>0.002–0.9</td>
</tr>
<tr>
<td>OH-MA</td>
<td>166.1 &gt; 107.0</td>
<td>18</td>
<td>20</td>
<td>2.35</td>
<td>0.017–8.3</td>
</tr>
<tr>
<td>HMA</td>
<td>182.1 &gt; 137.1</td>
<td>22</td>
<td>26</td>
<td>2.87</td>
<td>0.001–0.5</td>
</tr>
<tr>
<td>HMMA</td>
<td>196.1 &gt; 137.1</td>
<td>22</td>
<td>26</td>
<td>2.95</td>
<td>0.002–1.0</td>
</tr>
<tr>
<td>Oxilofrine</td>
<td>182.0 &gt; 149.0</td>
<td>20</td>
<td>30</td>
<td>1.31</td>
<td>0.002–0.9</td>
</tr>
<tr>
<td>13C6-amphetamine</td>
<td>142.1 &gt; 97.10</td>
<td>14</td>
<td>20</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td>13C6-methamphetamine</td>
<td>156.1 &gt; 97.1</td>
<td>15</td>
<td>20</td>
<td>4.30</td>
<td></td>
</tr>
<tr>
<td>13C6-PMA</td>
<td>172.1 &gt; 127.1</td>
<td>15</td>
<td>18</td>
<td>4.61</td>
<td></td>
</tr>
<tr>
<td>13C6-PMMA</td>
<td>186.2 &gt; 127.1</td>
<td>15</td>
<td>18</td>
<td>4.78</td>
<td></td>
</tr>
<tr>
<td>13C6-MDMA</td>
<td>200.1 &gt; 139.1</td>
<td>20</td>
<td>14</td>
<td>4.39</td>
<td></td>
</tr>
<tr>
<td>200.1 &gt; 169.1</td>
<td>20</td>
<td>14</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PMA, parahydroxymethamphetamine, PMMA, parahydroxymethamphetamine, OH-A, 4-hydroxyamphetamine, OH-MA, 4-hydroxyamphetamine, HM-A, 4-hydroxy-3-methoxymethamphetamine, HM-MA, 4-hydroxy-3-methoxymethamphetamine, oxilofrine, 4-hydroxyephedrine.
The respective 13C6-analogue was used as internal standard for amphetamine, methamphetamine, PMA and PMMA. For the rest of the compounds 13C6-MDMA was used as internal standard, because this internal standard would not be influenced by high concentrations of any compounds in the samples.

2.2.4. Genotyping

The most common clinically relevant sequence variants of CYP2D6, CYP2C9, CYP2C19 and CYP3A5 as well as CYP2D6 gene copy numbers were analyzed at Oslo University Hospital in the blood samples from the cases, the controls and the reference groups, except for the natural death references (C3) where sequence variants of CYP2C9 and CYP3A5 were not analyzed because these cases represented a part of previously published work[45]. For analysis of sequence variants, real-time PCR with melt curve analysis was used (except for the previously published natural death cases, where pyrosequencing was already performed at the National Board of Forensic Medicine (NBFM) in Sweden). For analysis of CYP2D6 gene copy numbers, quantitative real-time PCR was used (except for the post-mortem samples of fatal PMMA intoxications where this method proved unsuccessful, therefore pyrosequencing was performed at the NBFM). DNA was extracted from whole blood using the MagNA Pure LC instrument (Roche Applied Science, Penzberg, Germany). The analysis of single nucleotide variants and small deletions was performed by real-time PCR and melt curve analysis with hybridization probes (qualitative assays) on the LightCycler® 480 instrument (Roche Applied Science, Penzberg, Germany). The CYP2D6 gene copy numbers were determined by quantitative real-time PCR and normalization to ALB and RPPH1 reference genes [46,47]. Regarding the pyrosequencing, this method is validated and has proved to be a robust and safe method for genotyping [48] and for analysis of gene copy numbers [49] in post-mortem blood samples (with EDTA or fluoride).

2.2.5. Definitions and calculation of parameters

The frequency of concurrent use of other stimulant drugs (methamphetamine, amphetamine, MDMA and/or cocaine) was estimated based on the detection of these drugs or their metabolites in the blood samples above the analytical cut-off routinely applied at NIPH (see Section 2.1.1).

In order to compare the metabolism in post- and ante-mortem blood samples, which are not directly comparable due to drug redistribution after death, molar-drug-metabolite concentration ratios were calculated. Molar units were used instead of weight units, to avoid detecting concentration ratio differences caused by different molecular weights of the substances. Molecular weights used were PMMA 179.3 g/mol and OH-MA 165.2 g/mol.

The individual concentration ratios of PMMA/other CYP2D6-metabolized stimulant drugs were estimated by dividing the molar blood concentration of PMMA by the sum of the molar concentrations of such stimulant drugs:

\[
\text{Concentration ratio of PMMA/other 2D6–stimulants} = \frac{[\text{PMMA}]}{[\text{methamphetamine + amphetamine + MDMA}]}
\]

The individual CYP2D6 metabolic ratio was estimated by dividing the molar blood concentration of OH-MA (the major CYP2D6-mediated PMMA metabolite) by the molar concentration of the parent drug PMMA:

\[
\text{CYP2D6 metabolic ratio} = \frac{[\text{OH–MA}]}{[\text{PMMA}]}
\]

For OH-MA, the hydrolysed blood concentrations were used, i.e. the sum of free, glucuronidated and sulfated metabolite.

A high metabolic ratio would indicate efficient CYP2D6 metabolism, i.e. that a high percentage of the CYP2D6 substrate PMMA, and if present also methamphetamine and/or MDMA, in the blood had been metabolized to OH-MA. A low metabolic ratio would indicate deficient CYP2D6 metabolism, or a short time period between the PMMA intake and the blood sampling or death.

It was also attempted to calculate several other metabolic ratios, using variable combinations of the PMMA metabolites formed by the CYP2D6 enzyme, since the blood samples from the fatal and nonfatal PMMA cases were also analyzed for a number of other deconjugated PMMA metabolites than OH-MA, including PMMA. Many of these substances are also metabolites of methamphetamine, amphetamine and MDMA. The use of these alternative ratios, however, did not change any of the results or conclusions of our study. Altogether, the calculated CYP2D6 metabolic ratio turned out to be the most informative phenotypic parameter that could be calculated for our complex sample material.

2.3. Statistical analysis and power

Statistic parameters were calculated using the IBM Statistical Package for Social Sciences (SPSS) version 20.

Categorical data (genotype frequencies and sequence variant frequencies) were compared using Chi square test, or Fisher’s exact test for small counts.

For continuous data (drug concentration ratios), Student’s t-test was used for parametric data, and Mann–Whitney U-test for non-parametric data. Normality was tested by QQ-plots and Kolmogorov–Smirnov test.

Statistical comparisons were considered significantly different if \( p < 0.05 \) (two-tailed).

3. Results

3.1. Demographics, drug and metabolite concentrations

The median age of the 17 fatal PMMA intoxications was 31.7 years (range 25–51) and in the nonfatal PMMA abuse controls 30.9 years (range 20–46) (\( p = 0.30 \), t-test), with 88.2% (n = 15) vs 93.3% (n = 28) males, respectively (\( p = 0.61 \), Fisher’s test). The frequency of concurrent use of other stimulant drugs (methamphetamine, amphetamine, MDMA and/or cocaine) in the 17 fatal PMMA intoxications was 76.5% (n = 13) and in the nonfatal PMMA abuse controls 73.3% (n = 22) (\( p = 1.00 \), Fisher’s test).

The molar blood concentration ratio of PMMA relative to other CYP2D6-metabolized stimulants (PMMA/(methamphetamine + amphetamine + MDMA)), the blood concentrations of PMMA, OH-MA and PMA, and the CYP2D6 metabolic ratio (concentration ratio of OH-MA relative to PMMA) in the fatal PMMA intoxications and in the nonfatal PMMA abuse controls are shown in Table 4.

The molar blood concentration ratio of PMMA relative to other CYP2D6-metabolized stimulants was significantly higher in the fatal PMMA intoxications than in the nonfatal PMMA abuse controls (median (range) 3.4 (0.0–289.5) vs 0.8 (0.1–17.3), \( p = 0.039 \), Table 4).

As for the blood concentrations of PMMA, OH-MA and PMA, the fatal and nonfatal cases are not directly comparable because some of the difference between these groups is due to post-mortem redistribution of the drug and metabolite. Within the two subgroups of fatal PMMA intoxications, the blood concentration of OH-MA was significantly higher in the ‘PMMA only’ fatalities than in the ‘PMMA polydrug’ fatalities (median (range) 2.7 (0.4–6.5) mg/L vs 0.5 (0.0–2.0) mg/L, \( p = 0.016 \), Table 4). The blood concentration of PMMA tended to be higher in the ‘PMMA only’ fatalities than in the ‘PMMA polydrug’ fatalities (\( p = 0.085 \), Table 4), whereas the PMA concentrations were not different.
Table 4
Drug and metabolite concentrations and CYP2D6 metabolic ratio in the fatal PMMA intoxications and the nonfatal PMMA abuse controls.

<table>
<thead>
<tr>
<th></th>
<th>Fatal PMMA (n = 17)</th>
<th>Nonfatal PMMA (n = 30)</th>
<th>p-Value (test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median (range)</td>
<td>Mean</td>
</tr>
<tr>
<td>Concentration ratio of PMMA/other 2D6-stimulants&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.5</td>
<td>2.9</td>
<td>0.039 (Mann-Whitney)</td>
</tr>
<tr>
<td>PMMA blood concentration (mg/L)</td>
<td>3.4 (0.0–289.5)</td>
<td>0.8 (0.1–17.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.03–5.0)</td>
<td>(0.1–0.9)</td>
<td></td>
</tr>
<tr>
<td>µM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.7 µM</td>
<td>1.8 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.9 (0.2–27.9) µM</td>
<td>1.4 (0.5–4.8) µM</td>
<td></td>
</tr>
<tr>
<td>OH-MA blood concentration (mg/L)</td>
<td>1.7</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 (0.0–6.5)</td>
<td>0.5 (0.03–2.8)</td>
<td></td>
</tr>
<tr>
<td>µM</td>
<td>10.0 µM</td>
<td>4.2 µM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.2 (0.0–39.4) µM</td>
<td>2.9 (0.2–16.8) µM</td>
<td></td>
</tr>
<tr>
<td>CYP2D6 metabolic ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1</td>
<td>2.4</td>
<td>0.021 (Mann-Whitney)</td>
</tr>
<tr>
<td></td>
<td>0.6 (0.4–6.6)</td>
<td>2.1 (0.7–4.2)</td>
<td></td>
</tr>
<tr>
<td>PMA blood concentration (mg/L)</td>
<td>0.13</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.00–0.47)</td>
<td>(0.02–0.10)</td>
<td></td>
</tr>
<tr>
<td>Fatal PMMA only&lt;sup&gt;d&lt;/sup&gt; (n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMMA blood concentration (mg/L)</td>
<td>2.7</td>
<td>1.7</td>
<td>0.085 (t-test)</td>
</tr>
<tr>
<td></td>
<td>2.6 (1.3–5.0)</td>
<td>1.7 (0.03–2.9)</td>
<td></td>
</tr>
<tr>
<td>OH-MA blood concentration (mg/L)</td>
<td>3.1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.7 (0.4–6.5)</td>
<td>0.5 (0.0–2.0)</td>
<td></td>
</tr>
<tr>
<td>CYP2D6 metabolic ratio&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.4</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9 (0.4–4.0)</td>
<td>0.4 (0.0–4.6)</td>
<td></td>
</tr>
<tr>
<td>PMA blood concentration (mg/L)</td>
<td>0.18</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.13 (0.03–0.47)</td>
<td>0.06 (0.00–0.24)</td>
<td></td>
</tr>
</tbody>
</table>

PMMA, paramethoxymethamphetamine; OH-MA, 4-hydroxymethamphetamine.
<sup>a</sup> Molar blood concentration ratio of PMMA relative to other 2D6-stimulated stimulant drugs (sum of methamphetamine, amphetamine, and MDMA).
<sup>b</sup> Molar concentrations, for calculation of molar concentration ratios.
<sup>c</sup> Post-mortem blood concentrations are not directly comparable.
<sup>d</sup> Blood concentration ratio of OH-MA (4-hydroxymethamphetamine) relative to PMMA.
<sup>e</sup> PMMA in combination with a significant concentration of other psychostimulant drug(s) or ethanol.

In all of the nonfatal PMMA abuse controls (including the CYP2D6 PM), and in 15 of the 17 fatal PMMA intoxications, the metabolite OH-MA was detected above the level of quantification (0.017 mg/L). The two fatalities with no detectable OH-MA were both CYP2D6 EM, and represented 'PMMA polydrug' fatalities.

3.2. CYP genotypes and predicted phenotypes

The CYP2D6 genotype frequencies and the predicted CYP2D6 phenotypes in the fatal PMMA intoxications, the nonfatal PMMA abuse controls and the reference control groups are presented in Tables 5 and 6.

Table 5
Frequency of CYP2D6 genotypes in fatal PMMA intoxications, nonfatal PMMA abuse controls, and reference groups.

<table>
<thead>
<tr>
<th>CYP2D6 genotype</th>
<th>Fatal PMMA</th>
<th>Nonfatal PMMA</th>
<th>Healthy blood donors C2</th>
<th>Natural deaths C3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Freq</td>
<td>n</td>
<td>Freq</td>
</tr>
<tr>
<td>UM 1xN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0.059</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>EM 1/1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7</td>
<td>0.412</td>
<td>11</td>
<td>0.367</td>
</tr>
<tr>
<td>IM 1/1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>IM 1/4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7</td>
<td>0.412</td>
<td>9</td>
<td>0.300</td>
</tr>
<tr>
<td>IM 1/5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>1</td>
<td>0.033</td>
</tr>
<tr>
<td>IM 1/6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>IM 1/4xN&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>0.059</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>PM 4/4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>0.059</td>
<td>7</td>
<td>0.233</td>
</tr>
<tr>
<td>PM 4/5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>1</td>
<td>0.033</td>
</tr>
<tr>
<td>PM 4/6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>1</td>
<td>0.033</td>
</tr>
<tr>
<td>PM 3/4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>PM 5/5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0.000</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>Total, n</td>
<td>17</td>
<td>30</td>
<td>100</td>
<td>205</td>
</tr>
</tbody>
</table>

CYP2D6, cytochrome P450 2D6; Freq, relative frequencies; n, number of individual samples studied; UM CYP2D6, ultrarapid metabolizer; EM, CYP2D6 extensive metabolizer; IM, CYP2D6 intermediate metabolizer; PM, CYP2D6 poor metabolizer.
<sup>a</sup>In Natural deaths: 1xN<sup>2</sup>.
<sup>b</sup>In Natural deaths: 1/1 (n = 39), 1/2 (n = 45), 2/2 (n = 24) and 1xN<sup>4</sup> (n = 1).
<sup>c</sup>In Natural deaths: 1/1 (n = 100), 1/2 (n = 109), 2/2 (n = 52) and 1xN<sup>4</sup> (n = 1).
In the fatal PMMA intoxications (n = 17), the frequency distribution of the CYP2D6 genotypes and phenotypes did not differ significantly from the nonfatal PMMA abuse controls or from the reference groups.

In the subgroup of fatal ‘PMMA only’ intoxications (n = 7), the CYP2D6 IM phenotype was significantly overrepresented in comparison with the fatal ‘PMMA polydrug’ intoxications, the nonfatal PMMA abuse controls and the reference group of healthy blood donors/natural deaths (0.857 vs 0.200, 0.333 and 0.390, respectively, p < 0.05).

In the nonfatal PMMA abuse controls, the CYP2D6 PM genotype was significantly overrepresented compared to the reference group of non-abusers (healthy blood donors/natural deaths) (0.300 vs 0.062, p < 0.001).

The genotype frequencies for CYP2C9, CYP2C19 and CYP3A5 and the respective predicted CYP phenotypes were similar in the fatal PMMA intoxications, the nonfatal PMMA abuse controls and the reference groups (Online Resources 1–3).

3.3. CYP2D6 metabolic ratio (PMMA metabolism)

In the nonfatal PMMA abuse controls, there was a significant positive correlation between the CYP2D6 genotype (i.e. the number of functional CYP2D6 gene copies) and the CYP2D6 metabolic ratio (OH-MA/PMMA). The metabolic ratio was significantly lower in PM in comparison with IM (p = 0.001, t-test) and EM (p < 0.001, t-test, Fig. 2 top). Also in fatal and nonfatal PMMA intoxications altogether, there was a clear positive correlation between the CYP2D6 metabolic ratio and the CYP2D6 genotype (PM vs EM p = 0.000, PM vs IM p = 0.000).

In the fatal PMMA intoxications, there was no significant correlation between the CYP2D6 genotype and the CYP2D6 metabolic ratio (PM vs EM p = 0.47, t-test, Fig. 2 bottom). The blood CYP2D6 metabolic ratio was significantly lower in the fatal PMMA intoxications than in the nonfatal PMMA abuse controls (median (range) 0.6 (0.0–4.6) vs 2.1 (0.2–7.4) mg/L, respectively, p = 0.021, Mann–Whitney–U test, Table 4). The CYP2D6 metabolic ratio in the ‘PMMA only’ fatalities and the PMMA ‘polydrug’ fatalities were not significantly different (median (range) 0.9 (0.4–4.0) vs 0.4 (0.0–4.6) mg/L, respectively, p = 0.088, Mann–Whitney–U test, Table 4).

4. Discussion

This is one of the first studies examining the PMMA metabolism and impact of CYP genotype in humans, and the involvement of metabolism in PMMA toxicity. Several previous publications have recommended that such studies should be performed in humans regarding PMMA and similar stimulant drugs. Our study demonstrated that the CYP2D6 enzyme and genotype are important in the metabolism of PMMA to OH-MA in humans, but that other enzymes are also involved in this biotransformation. However, we
could not identify any genetic CYP2D6, CYP2C9, CYP2C19 or CYP3A5 predictive marker on fatal toxicity of PMMA in humans.

4.1. CYP2D6

The results of our study demonstrated a significant positive correlation between the CYP2D6 genotype and the CYP2D6 metabolic ratio in the nonfatal PMMA abuse controls. The higher the number of functional CYP2D6 gene copies, the higher the metabolic ratio, i.e., the more efficient metabolism of PMMA to OH-MA by CYP2D6. Also in the nonfatal and fatal PMMA intoxications altogether, there was a clear positive correlation. This indicates that the CYP2D6 enzyme, and the CYP2D6 genotype, is important in the metabolism of PMMA to the major metabolite OH-MA in humans, which is in line with what has been formerly reported in human liver microsomes [14]. However, it should be noted that detectable, but low, blood concentrations of OH-MA were also found in all subjects with CYP2D6 PM genotype, irrespective of whether these represented fatal PMMA intoxications or nonfatal PMMA abuse controls. This indicates that other enzymes than CYP2D6 are also involved in the metabolism of PMMA to OH-MA in humans.

In the fatal PMMA intoxications, however, there was no significant correlation between the CYP2D6 genotype and the CYP2D6 metabolic ratio. Moreover, the CYP2D6 metabolic ratio in the fatal PMMA intoxications was significantly lower than in the nonfatal PMMA abuse controls (median 0.6 vs 2.1, p = 0.021, Table 4). There are many possible explanations for this difference, involving sample size, time course, PMMA dose, post-mortem redistribution artefacts and CYP2D6 interactions.

First, our sample size is limited and the power may be too low to detect a CYP2D6 genotype-phenotype correlation using population data. The fatal PMMA intoxications counted only 17 cases, in comparison with the somewhat larger population of 30 nonfatal PMMA abuse controls. It must be kept in mind that the CYP2D6 metabolic ratio used in our study was influenced not only by the OH-MA formed from PMMA but also by OH-MA formed from methamphetamine or MDMA, which were frequently co-ingested in both groups.

Secondly, the lower CYP2D6 metabolic ratio in the fatal PMMA intoxications may indicate that, in the majority of these cases, death occurred rapidly after ingestion of PMMA, i.e., at an early stage of PMMA metabolism, while a later metabolic stage had been reached at the time of sampling in the nonfatal PMMA cases (mainly DUI cases). In 2 of the 17 PMMA fatalities, the OH-MA metabolite was not detected at all (above the limit of quantification), even though both of these individuals were CYP2D6 EM. (One had a blood alcohol concentration of 2.5 g/l, the other had a very high blood methamphetamine concentration of 3.4 mg/l). Rapid death was also supported by the scarce case information that was available in some of the fatalities, indicating that the time interval between PMMA intake and death had been short in these cases, from about 0.5 to a few hours.

Thirdly, the generally very high PMMA concentrations found in the PMMA fatalities, even after adjusting for an expected post-mortem increase of about 2–3 times or more [1], strongly indicates ingestion of high PMMA doses in the majority of the fatal cases. The PMMA concentrations in the nonfatal PMMA abuse controls were far lower and in most cases within the reported recreational range for PMMA. It has been reported that PMMA toxicity is strongly related to dose [16]. In support of the high-dose theory was the very high blood concentration ratio found in the fatal PMMA intoxications, of ‘PMMA vs other CYP2D6-metabolized stimulants’, compared to the nonfatal PMMA abuse controls.

Fourthly, the CYP2D6 metabolic ratio in the fatal PMMA intoxications might have been falsely lowered after death due to post-mortem redistribution artefacts. The numerator in this ratio was the polar metabolite OH-MA which might exhibit less post-mortem redistribution than the more lipid-soluble parent drug PMMA. In humans, a post-/ante-mortem concentration ratio of up to 2–3 or more is suggested for PMMA [1], while for OH-MA it is unknown. In rats, the distribution volume has been reported to be about 3–4 L/kg for OH-MA [50] and 6.4 L/kg for PMMA [13]. These sparse data may indicate a lower propensity for post-mortem redistribution of OH-MA than for PMMA. Fifthly, regarding drug interaction, our results argue against a more potent CYP2D6 enzyme autoinhibition or co-drug inhibition in the PMMA fatalities than in the nonfatal PMMA abuse controls. The high-affinity low-capacity CYP2D6 enzyme has been reported to be rapidly and completely inactivated even by one recreational drug dose, and the frequency of concomitant use of other CYP2D6 inhibitors (like methamphetamine and amphetamine) was similar in both of our groups [27,51–55]. Regarding concomitant use of antidepressants or antipsychotics, of which many are also CYP2D6 inhibitors, such drugs were not detected in any of the fatalities, while such substances were not analyzed routinely in the nonfatal PMMA abuse controls.

Overall, we suggest that the lack of significant correlation between CYP2D6 genotype and CYP2D6 metabolic ratio in the fatal PMMA intoxications compared to the nonfatal PMMA abuse controls was mainly attributable to rapid death following the ingestion of large PMMA doses, with some contribution of low sample size and post-mortem redistribution artefacts.

Regarding ‘extreme’ CYP2D6 genotypes, we found no overrepresentation of PM (homozygous deficient) or UM (with gene multiplication) in the fatal PMMA intoxications in our study. The CYP2D6 genotype frequencies in this group corresponded to what we found in our reference group of non-abusers (healthy blood donors/natural deaths), and also to what has formerly been reported in the Caucasian population [56]. Although the conclusions of former studies have been somewhat conflicting, our findings are in agreement with the majority of such studies that report no clear evidence for increased risk of acute stimulant drug toxicity or fatality in populations with ‘extreme’ CYP2D6 genotypes (PM or UM) [23–27,57]. Furthermore, in large fatal drug intoxications studies regardless of the type of drug, the frequency of CYP2D6 PM was not above what was expected in the reference population [37,58]. It should be noted, however, that in many former studies PM subjects have been excluded, so the evidence is not yet sufficient [59].

In the subgroup of fatal ‘PMMA only’ intoxications, the CYP2D6 IM genotype was significantly overrepresented (6 out of 7 individuals), in comparison with the fatal ‘PMMA polydrug’ intoxications and the two reference groups of non-abusers where the IM genotype frequencies were as expected in the Caucasian population (about 0.20–0.40) [56]. This shows that the majority of our ‘PMMA only’ fatalities were genetically CYP2D6 heterozygous extensive metabolizers (i.e. intermediate metabolizers), while the majority of the ‘PMMA polydrug’ fatalities were homozygous extensive metabolizers. We have no good explanation for this difference, since the CYP2D6 metabolic ratio, i.e. the CYP2D6 metabolism of PMMA, was similar in these two groups. The finding might be due to a limited sample size for a population genetic study.

Surprisingly, we found an unexpected high frequency of CYP2D6 PM in the nonfatal PMMA abuse controls (0.30). In our reference groups of non-abusers and in the fatal PMMA intoxications, these frequencies corresponded to what is reported in the Caucasian population (0.059–0.062 in our study, vs 0.030–0.100 formerly reported) [56]. This overrepresentation of genetically homozygous deficient CYP2D6 metabolizers in the nonfatal PMMA abuse controls could theoretically have indicated that this trait was
protective against fatal PMMA toxicity. However, the phenotypic CYP2D6 metabolic ratio in the nonfatal PMMA abuse controls was not different from that found in the fatal PMMA intoxications regardless of CYP2D6 genotype. Further, the great predominance of partly deficient CYP2D6 metabolizers among the ‘PMMA only’ fatalities also argues against deficient CYP2D6 metabolism as being a major protective trait.

As an alternative explanation, the overrepresentation of partly or completely deficient CYP2D6 metabolizers in the nonfatal PMMA abuse controls and the ‘PMMA only’ fatalities could be related to personality traits, i.e. that this trait might make individuals predisposed to PMMA or stimulant drug abuse. The CYP2D6 enzyme, which is expressed at low levels in the brain, is involved in the formation of the endogenous brain neurotransmitters serotonin and dopamine [60]. These neurotransmitters are the site of action of PMMA and other stimulant drugs. The CYP2D6 PM trait is associated with a lower serotonin and higher dopamine tone and higher impulsiveness, and PM are reported to be more sensitive than EM to methamphetamine effects on several objective and subjective measures (stimulation, euphoria, good effects) in spite of similar plasma kinetics [61–64]. However, the results of former studies with other stimulant drugs argue against deficient CYP2D6 metabolism predisposing for stimulant drug abuse. The frequency of CYP2D6 PM was underrepresented in MDMA abusers compared to the general population [65], while reduced CYP2D6 enzyme activity was found to be a negative risk factor for methamphetamine dependence [66]. We have no good overall explanation for the overrepresentation of CYP2D6 deficient metabolizers in the nonfatal PMMA abuse controls, but it may represent random variation due to the limited sample size.

4.2. CYP2C9, CYP2C19 and CYP3A5

The results of our study did not suggest any involvement of CYP2C9, CYP2C19 or CYP3A5 genetics on the risk of fatal toxicity of PMMA.

4.3. Strengths and limitations

The Norwegian PMMA epidemic in 2010–2013 offered unique biologically samples and data to investigate a possible association between genetically determined differences in CYP genotype, PMMA metabolism and fatal toxicity of PMMA. Our control group represented living PMMA abusers with similar population and drug abuse demographics as in the PMMA fatalities, hence representing those taking PMMA without a fatal outcome. We also included two large reference groups of non-abuser natural deaths and healthy blood donors, whose genotype frequencies for CYP2D6, CYP2C9, CYP2C19 and CYP3A5 were found to be similar to those reported in the general Caucasian and Norwegian population. Our findings of similar frequencies of the CYP2C9, CYP2C19 and CYP3A5 isofoms in all of the compared groups indicate that geographical selection bias is not a main cause of our findings regarding CYP2D6 genetics and metabolism.

Our initial analyses revealed that the CYP2D6 gene copy number assay based on quantitative real-time PCR was not suitable for the post-mortem blood samples. Compared to pyrosequencing, the real-time PCR assay overestimated gene copy numbers, probably due to matrix effects. This emphasizes the need for thorough optimization and validation of quantitative gene copy number assays for the analysis of post-mortem sample materials. Pyrosequencing is reported to be a robust method for gene copy number analysis of forensic samples in post-mortem whole blood [49,58], and is reportedly not affected by factors such as fluoride preservative, heparin or freezing [58].

It was a limitation of our study that the statistical power was calculated at a time point when our preliminary results indicated an unusually high frequency of CYP2D6 UM in the fatal PMMA intoxications in comparison with our controls and reference populations. This turned out to be an artefact, probably due to matrix effects in the post-mortem blood samples as mentioned above. Post hoc calculations showed that our study and sample size was still sufficient to detect toxicologically relevant differences in genotype frequencies between our study group and the controls and references, although smaller differences could go unnoticed. Given an expected frequency of CYP2D6 PM of about 0.06 in the nonfatal PMMA abuse controls and the healthy references, our study would have an 84% power to detect a significant difference if the PM frequency were above 0.31 in the fatal PMMA intoxications. Likewise, given an expected frequency of CYP2D6 UM genotype of about 0.006 in our control and reference groups (n = 335), our study would have an 85% power to detect a frequency above 0.026 in our study group.

5. Conclusions

Our study demonstrated that the CYP2D6 enzyme and genotype are important in the metabolism of PMMA to the major metabolite OH-MA in humans, but that other enzymes are also involved in this biotransformation. We could not identify any genetic CYP2D6, CYP2C9, CYP2C19 or CYP3A5 predictive marker on fatal toxicity of PMMA in humans. Our findings indicate that in most cases PMMA death occurred rapidly and at an early stage of PMMA metabolism, following the ingestion of large and toxic PMMA doses.

Conflict of interest

There are no financial relations that could lead to a conflict of interest.

Acknowledgments

The authors are grateful to the Division of Forensic Sciences, NIPH Oslo, for the skilled analytical work performed, with special thanks to Inger Hasvold and Gerd Wencle Brochmann.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2016.02.027.

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