Distribution of Methylone in Four Postmortem Cases

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Drugs derived from amphetamine, methamphetamine and their methylenedioxy-analogues, although being sold as plant food or bath salts, are being used as legal alternatives to scheduled amphetamine stimulants. These products often contain methylenedioxymethylamphetamine (MDMA)—three amphetamine derivatives shown to have strong pharmacological effects. Four postmortem cases were analyzed for methylenedioxymethylamphetamine, methamphetamine and MDPV, with drug levels quantitated in multiple biological matrices. All four cases had detectable levels of methylenedioxymethylamphetamine, with heart blood concentrations of 0.740, 0.118, 0.060 and 1.12 mg/L. Analysis of several tissue samples shows that methylenedioxymethylamphetamine does not sequester in a particular tissue type after death. The average liver-to-blood ratio was 2.68. Two cases also had MDPV present, but insufficient data were collected to formulate a hypothesis on postmortem sequestration or redistribution. Two different extraction methods, as well as analysis of derivatized and underivatized methylenedioxymethylamphetamine, show that the drug is suitable for analysis in either method. The cases are believed to show one instance of chronic methylenedioxymethylamphetamine use, with a urine concentration of 38 mg/L.

Introduction

There has been a recent increase in the number of individuals using amphetamine analogues as legal alternatives to amphetamine, methamphetamine and the methylenedioxy derivatives of these stimulants (1–3). These analogues are typically separated into four distinct chemical groups: the piperazines, pipеридines, phenethylamines and tryptamines. Drugs included in these families typically exert stimulant effects through multiple central nervous system (CNS) actions, including inhibition of serotonin, dopamine or norepinephrine reuptake transporters (SERT, DAT, and NET, respectively), or through release of these neurotransmitters into the synaptic clefts via reversal of the reuptake transporters (4). Various modifications of the functional groups present on the compounds can result in either stronger stimulant effects, or increased hallucinogenic or entactogenic effects (4–5). Three compounds considered to be “designer” derivatives of amphetamines or cathinone, which are of current interest to the law enforcement and toxicological communities, are methylenedioxymethylamphetamine (MDMA), methamphetamine and 3,4-methylenedioxy-N-methylamphetamine (MBDB). These compounds have been found to be the active ingredient of materials packaged and sold as bath salts, plant food and stain removers under names such as Cloud 9, White Lightning and Ivory Wave. The products are usually labeled as unsuitable for human consumption, but the presence of the amphetamine derivatives suggests that many customers are buying these products specifically to consume them (2, 5–8).

The drug methylenedioxymethylamphetamine (2-methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one) (Figure 1A), a derivative of the drug Ecstasy [methyleneoxymethylamphetamine (MDMA)], is a β-ketoned methylenedioxyphenethylamine, a class of drugs that also includes ethylone and butylone. It was first synthesized in 1996 as a potential anti-depressant and anti-Parkinsons drug by Peyton Jacob III and Alexander Shulgin (6). Methylone inhibits the reuptake of norepinephrine, dopamine and serotonin, but there is disagreement in the current literature as to which neurotransmitter is affected to the greatest degree (9–11). There is also slight variation in the concentration of methylenedioxymethylamphetamine required to fully inhibit SERT, DAT and NET. The IC50 values for the reuptake inhibition of serotonin for three previously published studies are shown in Table I.

The IC50 values for methylenedioxymethylamphetamine, compared to those for (+)-amphetamine and (+)-methamphetamine, indicate that methylenedioxyamphetamine is a potent inhibitor of SERT, DAT and NET. This shows that methylenedioxymethylamphetamine has a high abuse potential, because it will cause desired effects at a low concentration. Adverse effects of methylenedioxymethylamphetamine include tachycardia, hypertension, nausea, vomiting, hallucinations, psychosis and short-term memory loss (13). The psychoses and hallucinations have been especially prevalent in some reported cases, with separate acute and chronic users of “bath salts” jumping out of windows to escape imaginary pursuers, resisting medical assistance strenuously enough to require multiple individuals and electric shock (Taser) for submission, walking barefoot on broken glass, or leaving infant children in the middle of a highway (5).

The toxicity of methylenedioxymethylamphetamine is not fully understood, but several reports discuss the acute toxicity at the molecular level. Nakagawa et al. (14) studied time-dependent cell death, loss of cellular adenosine triphosphate (ATP), and reduction in glutathione levels in rat hepatocytes when exposed to N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), 3,4-(methylenedioxyphenyl)-2-butanamine (BDB), MDMA, and methylenedioxymethylamphetamine. Of these four drugs, a 4-mM solution of methylenedioxymethylamphetamine caused the lowest percentage of cell death, ATP loss and glutathione reduction compared to equally concentrated solutions of the other drugs. The authors posit that cell death results from loss of cell ATP and decrease in mitochondrial membrane potential through a mitochondrial permeability transition (MPT) induced by chemical interference from MDMA and its derivatives. However, the addition of methylenedioxymethylamphetamine alone at a concentration of 4 mM did not affect the MPT during the incubation period. The concluded order of cell toxicity from the study was MBDB > BDB > MDMA > methylenedioxymethylamphetamine. A second study conducted by Sogawa et al. (11) focused more closely on the toxicity of methylenedioxymethylamphetamine. Cell toxicity was evaluated by...
low concentrations, and were undetectable within 36 hours. Parent drug and MDC were present in the urine in HMMC and 87.6% of the 3-OH-4-MeO-MC were glucuronide conjugated. Approximately 26% of the dose excreted as HMMC and 5% as 3-OH-4-MeO-MC within 48 hours. Eighty-one percent of the dose of methylone administered to rats resulted in approximately 50/50 μM was sufficient for a spike in LDH release after a 24-hour incubation period.

Metabolism of methylone occurs through two primary pathways: (1) N-demethylation to form methylenedioxyxycathinone (MDC); and (2) demethylation of the oxymethylene ring followed by O-methylation via catechol O-methyltransferase, forming both 4-hydroxy-3-methoxymethcathinone (HMMC) or 3-hydroxy-4-methoxymethcathinone (3-OH-4-MeO-MC). Of these metabolites, HMMC is most prevalent. A single 5-mg dose of methylone administered to rats resulted in approximately 26% of the dose excreted as HMMC and 5% as 3-OH-4-MeO-MC within 48 hours. Eighty-one percent of the HMMC and 87.6% of the 3-OH-4-MeO-MC were glucuronide conjugated. Parent drug and MDC were present in the urine in low concentrations, and were undetectable within 36 hours.

Methylone is often found in conjunction with mephedrone and MDPV in the products mentioned. These two drugs have similar clinical effects as methylone—euphoria, alertness and empathogenic effects, with adverse effects including tachycardia, agitation, hallucinations and hypertension. Both drugs also have similar mechanisms of, inhibiting presynaptic monoamine reuptake, particularly dopamine. Methylone has also been reported in conjunction with 5-methoxy-N-methyl, N-isopropyl tryptamine (5-MeO-MIPT) and ethcathinone.

Few reports exist on the analysis of methylone, mephedrone and MDPV in postmortem samples. Reported drug concentrations are summarized in Table II. There have been no reported postmortem concentrations for methylone, either alone or in conjunction with other drugs. This study presents four postmortem cases that had detectable concentrations of methylone in various biological matrices; in two of the cases, MDPV was present as well. The study also allows for some examination of postmortem distribution of methylone, because at least four different matrices were analyzed with each case.

**Case Histories**

**Case 1**
The decedent, a 19-year-old male, collapsed while running during a physical fitness assessment. Once emergency medical services arrived on the scene, they initiated CPR and called for an ambulance. At some point, their resuscitation efforts ceased and the decedent was pronounced dead. The decedent was transferred to the medical examiner for autopsy and toxicological testing to determine cause of death. The cause of death was cardiac arrest associated with methylone, and the manner of death was accident.

**Case 2**
The decedent, a 38-year-old male, was found in bed with a single gunshot wound to the head. The decedent’s girlfriend was also found deceased in the bed with sharp force trauma to the neck. The decedent had a history of bipolar disorder, but was not receiving treatment. A suicide note was found with the body. Emergency services were called, but no resuscitation efforts were performed. The cause of death was contact gunshot wound to head and the manner of death was suicide.

**Case 3**
The decedent, a 21-year-old female, was involved in a high-speed chase with the police. Police were contacted by a friend of the decedent advising that the decedent had expressed suicidal ideations and left in her vehicle. Police located and

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**Table 1** Neurotransmitter Reuptake Inhibition IC50 Values for Methylone, Compared to those for (±)-Amphetamine and (±)-Methamphetamine

<table>
<thead>
<tr>
<th>Reference</th>
<th>[3H] Serotonin</th>
<th>[3H] Dopamine</th>
<th>[3H] Norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cozzi et al. (9)</td>
<td>5.75 ± 0.68</td>
<td>0.819 ± 0.168</td>
<td>1.22 ± 0.13</td>
</tr>
<tr>
<td>Nagai et al. (10)</td>
<td>2.3 ± 0.58</td>
<td>2.9 ± 0.67</td>
<td>7.4 ± 2.4</td>
</tr>
<tr>
<td>Sogawa et al. (11)</td>
<td>6.42 ± 1.01</td>
<td>2.94 ± 0.36</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>Rothman et al. (12)</td>
<td>13.9 ± 0.51</td>
<td>1.5 ± 0.08</td>
<td>0.244 ± 0.015</td>
</tr>
<tr>
<td>Rothman et al. (12)</td>
<td>2.13 ± 0.09</td>
<td>0.114 ± 0.011</td>
<td>0.048 ± 0.005</td>
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</tbody>
</table>
attempted to stop the vehicle, at which point the decedent sped up to over 100 mph, struck a second car and then came to a stop on a bridge. The decedent was subsequently located in the river below the bridge. The deceased had a history of substance abuse and had been incarcerated several times. The cause of death was drowning (Part II: multiple blunt force injuries) and the manner of death was accident.

Case 4

This case was associated with Case 2 and the decedent was a 35-year-old female who was found unresponsive in bed with her boyfriend. Emergency services were called, and both individuals were pronounced dead at the scene. The deceased had sharp force trauma to her neck and a gunshot wound at the back of her head. The cause of death was cutting wound of neck and gunshot wound of head and the manner of death was homicide.

Materials and Methods

Reagents and materials

All solvents were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA). Sodium phosphate and mepivacaine were purchased from Sigma–Aldrich (St. Louis, MO). Methylene, methylene-d₃, mephedrone and MDPV were purchased from Cerilliant (Round Rock, TX). Pentfluoropropionic anhydride (PFPA) was purchased from Aldrich (Milwaukee, WI). Concentrated hydrochloric acid (HCl) was purchased from EMD Chemicals (Gibbstown, NJ). Concentrated potassium hydroxide (KOH) pellets were purchased from Fisher Scientific. Mixed-mode silica-based solid-phase extraction columns (ZCDAU020) were purchased from United Chemical Technologies (Bristol, PA). Chem Elut columns were purchased from Agilent Technologies (Wilmington, DE).

Toxicology screening

The cases presented were analyzed at the Armed Forces Medical Examiner and Maryland Office of the Chief Medical Examiner (OCME). Different methods of analysis were developed and used at each laboratory, with both methods presented in the following. At each laboratory, tissues were analyzed as 1:5 homogenates in deionized water. Blood standards were used as a reasonable approximation of the tissue homogenates. Validation data are presented in the analysis section.

A standard postmortem toxicology panel was performed on all cases within 24 hours of death, including gas chromatography (GC) headspace analysis for volatile compounds, an alkaline drug screen using full scan GC–mass spectrometry (GC–MS), an acidic/neural drug screen using GC–nitrogen–phosphorus detection (NPD), color tests for acetaminophen and salicylate and an enzyme-linked immunosassay (ELISA) for morphine and benzodiazepines. Case 1 had an additional ELISA screen for amphetamines, barbiturates, cocaine, phencyclidine and cannabinoids.

Under a full scan GC–MS drug screen, Cases 1, 2 and 4 screened positive for methylene and Case 3 screened positive for MDPV. Confirmation and quantitation analyses for all three analytes (methylene, MDPV and mephedrone) were performed as part of a “bath salt” confirmation assay in Cases 2–4.

Methylened analysis: Case 1

A stock standard of methylene was prepared in methanol and stored at −15 °C in an amber glass vial. Calibrator samples were prepared in 2.0 mL of certified drug-free blood by spiking methylene at concentrations of 0.05, 0.10, 0.20, 0.50, 1.00 and 2.00 mg/L. A 0.50-mg/mL positive blood control was prepared from the same standard source into certified drug-free blood. Methylene-d₃ was prepared for use as the internal standard at a concentration of 10 mg/L. A negative blood control was extracted with each batch.

Two milliliters of sample, calibrators and control or 1 g of tissue (homogenized) were pipetted into clean, labeled 16 × 100 mm test tubes and spiked with the internal standard for a final methylene-d₃ concentration of 0.25 mg/L. Four drops of concentrated KOH and 5 mL of chlorobutane were added to all samples, and the samples were mixed for 20 minutes on a rotomixer. The samples were then centrifuged for 10 min at 3,000 rpm to separate the aqueous and organic layers, then the organic layer was transferred to a clean 10-mL conical test tube. Fifty microliters of 10% methanolic HCl was added to each tube. The samples were then centrifuged for 4 min, increasing at 18 °C and then at 40 °C in an amber glass vial. Calibrator samples were prepared in 2.0 mL of certified drug-free blood.

Each residue was reconstituted in 100 μL of 1:1 ethyl acetate–PFPA. Each tube was capped, vortexed and incubated at 70 °C for 30 min. The samples were evaporated to dryness under nitrogen at 55 °C, reconstituted with 50 μL of ethyl acetate and then transferred to glass autosampler vials. One microliter of sample was injected into the GC–MS.

Quantitation was performed on an Agilent Technologies 6890 GC coupled to a 5975 mass selective detector (MSD) (Palo Alto, CA) The stationary phase was a 5% J&W phenyl methyl silicone column (Rancho Cordova, CA) (20 μm × 0.18 mm i.d. × 0.18 μm) and the mobile phase was helium flowing at 0.9 mL/min. A pulsed split injector program was used as follows: 40 psi for 2.0 min at a 20:1 split and an inlet temperature of 225 °C. The oven temperature began at 100 °C for 4 min, increasing at 18 °C per minute to 300 °C and then at 40 °C per minute to 325 °C, holding for 2 min. The transfer line was set to 280 °C. The MSD source and quadrupole temperatures were held at 230 and 150 °C, respectively. For identification, full scan electron ionization data were collected over a
mass range of m/z 42–550 and a detection threshold of 100. Quantitation of targeted analytes was accomplished using selected ion monitoring (SIM). The SIM ions collected were: m/z 353, 204, 160 for methylene; m/z 356, 207 for methylone-d3, with quantitative ions in bold. Samples that contained analyte concentrations above the upper limit of the calibration curve were diluted appropriately and re-extracted.

**Methylone, MDPV and mephedrone analysis: Cases 2–4**

Stock standards of 5 and 50 mg/L of each analyte were prepared in methanol and stored at 4°C. Calibrator samples were prepared in 5.0 mL of certified drug-free blood by spiking each analyte at concentrations of 0.025, 0.05, 0.10, 0.25 and 0.50 mg/L. A 0.075-mg/L positive blood control was prepared from the same standard source into certified drug-free blood. Calibrator and control samples were prepared before each extraction. Mepivicaine was prepared for use as the internal calibrator and control samples were prepared before each extraction from the same standard source into certified drug-free blood. Stock standards of 5 and 50 mg/L were prepared in methanol and stored at 4°C.

For each case, the analytes were tentatively identified by retention time from the alkaline drug screen chromatogram. The presence of methylene and MDPV was then confirmed by full scan electron ionization GC–MS. Mephedrone was not detected in any of the cases. The results from the toxicological analyses of the postmortem samples are presented in Table III.

No other volatile substances or drugs were detected in Cases 1 and 4. Cases 2 and 3 had drugs present in addition to the amphetamine derivatives. Case 2 had hydrocodone and acetaminophen present at 0.1 and 23 mg/L, respectively. Case 3 had morphine present at 0.031 mg/L.

The stability of the analytes in various biological matrices was not directly studied, but comparison of the initial quantitation and the re-extractions performed for this study shows that there was no significant degradation of analytes over the time that the samples were in storage. All samples from Cases 2–4 were stored at 4.0°C for two weeks while routine testing was conducted, then transferred to a –20°C freezer for long-term storage. Case 3 was initially analyzed in July 2011; Cases 2 and 4 were initially analyzed in October 2011. Each of these cases was re-extracted in November 2011 to include all available biological matrices for the purposes of this study. Initial and secondary quantitations of all analytes differ in value by less than 20%.

**Results**

The intra-run percent coefficient of variation (%CV) for mephedrone, methylene and MDPV was 8.5, 6.8 and 5.5%, respectively. Inter-run %CV was 7.2, 7.4 and 10.2%, respectively. The accuracy of a 0.150-mg/L control sample of methylene, methylene and MDPV was 102, 100 and 99.1%, respectively (n = 20).

**Discussion**

Extraction of methylene and MDPV from both biological fluids and tissue homogenates was easily accomplished following alkalinization. The addition of 2 mL of 1.0 M NaOH to the

<table>
<thead>
<tr>
<th>Table III</th>
<th>Methylene and MDPV Quantitation Results</th>
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<tr>
<td>Case 1</td>
<td>Source</td>
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<td></td>
<td>Central blood</td>
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<tr>
<td></td>
<td>Per. blood</td>
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<tr>
<td></td>
<td>Urine</td>
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<td>Liver</td>
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<td>Kidney</td>
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<td></td>
<td>Spleen</td>
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<td>Bile</td>
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<tr>
<td>Case 2</td>
<td>Heart blood</td>
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<tr>
<td></td>
<td>Urine</td>
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<td>Liver</td>
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<td>Bile</td>
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<tr>
<td>Case 3</td>
<td>Heart blood</td>
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<td>Kidney</td>
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<td>Bile</td>
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<td>Case 4</td>
<td>Heart blood</td>
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<td>Urine</td>
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<td></td>
<td>Liver</td>
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<td></td>
<td>Kidney</td>
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From the urine, the highest methylone concentration is found sequestered in any biological matrix over the long term. Apart user, there is no indication that the drug is preferentially ducts containing methylone. No interviews with family or Case 1 suggests that the decedent was a chronic user of pro-

of commercially available reference standards. Were not tested in the cases presented due to the current lack in which blood is available for testing. However, in cases where the primary metabolite, HMMC, in postmortem cases or cases in which a blood specimen is unavailable for analysis. The high concentration of methylone in the heart blood, liver, and kidney relative to the urine concentration in Case 4 suggest that death occurred soon after the drug was ingested.

In each of the cases presented, the methylone was analyzed in its unmetabolized form and present in easily detectable amounts in all matrices tested. This demonstrates that in which a blood specimen is unavailable for analysis. The high concentration of methylone in the heart blood, liver, and kidney relative to the urine concentration in Case 4 suggest that death occurred soon after the drug was ingested.

In each of the cases presented, the methylone was analyzed in its unmetabolized form and present in easily detectable amounts in all matrices tested. This demonstrates that methylone can be directly assayed, without having to initially test for postmortem redistribution from the liver into the nearby blood. Moreover, in all four cases, the liver and kidney samples contain similar amounts of drug, indicating that both matrices are suitable for toxicological analysis. This is useful for cases in which a blood specimen is unavailable for analysis. The high concentration of methylone in the heart blood, liver, and kidney relative to the urine concentration in Case 4 suggest that death occurred soon after the drug was ingested.

The very large concentration of methylone in the urine in Case 1 suggests that the decedent was a chronic user of products containing methylone. No interviews with family or friends are available to corroborate the decedent’s use, but the urine concentration is significantly higher than any of the other cases analyzed. Assuming the decedent was a chronic user, there is no indication that the drug is preferentially sequestered in any biological matrix over the long term. Apart from the urine, the highest methylone concentration is found in the kidney, but is not significantly higher than the concentrations in the liver or spleen. Creatinine levels were not measured in this case.

MDPV was present in Case 3, in concentrations higher than methylone. This is most likely due to the amphetamine deriva-

tive content of the product used, although without accurate histories of drug use, it is difficult to attribute both drugs to a single source as opposed to multiple products. Both Nakagawa et al. and Sogawa et al. have correlated increasing methylone toxicity with the presence of other amphetamine-like drugs, but neither investigated the combination of methylone and MDPV (11, 14). These studies and other research show it is likely that the two drugs are more toxic when found in conjunction than used alone (8, 9).

Acknowledgment

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References


