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 methylone, mephedrone and methylenedioxypyrovalerone (MDPV)—three amphetamine derivatives shown to have strong pharmacological effects. Four postmortem cases were analyzed for methylone, mephedrone and MDPV, with drug levels quantitated in multiple biological matrices. All four cases had detectable levels of methylone, with heart blood concentrations of 0.740, 0.118, 0.060 and 1.12 mg/L. Analysis of several tissue samples shows that methylone 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 methylone, show that the drug is suitable for analysis in either method. The cases are believed to show one instance of chronic methylone 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, piperidines, 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 transports (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 methylone, mephedrone and 3,4-methylenedioxypyrovalerone (MDPV). 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 and stain removers under names such as Cloud 9, White

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Metabolism of methylone occurs through two primary pathways: (1) N-demethylation to form methylenedioxyxymethcathinone (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 MDC (2).

Methylone is often found in conjunction with mephedrone and MDPV in abused products (5). 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 (8, 19, 23). Methylone has also been reported in conjunction with 5-methoxy-N-methyl, N-isopropyl tryptamine (5-MeO-MIPT) (17) and eth-cathinone (18).

Few reports exist on the analysis of methylone, mephedrone and MDPV in postmortem samples (21). Reported drug concentrations are summarized in Table I. 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
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 Sigma–Aldrich (St. Louis, MO). Methylone, methylone-d3, mephedrone and MDPV were purchased from Cerilliant (Round Rock, TX). Pentfluoropropionic anhydride (PFPA) was purchased from Sigma–Aldrich (St. Louis, MO). Methylone at concentrations of 0.05, 0.10, 0.20, 0.50, 1.00 and 2.00 mg/L. A 0.50-mg/L 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 sample, and the samples were dried at 40°C under nitrogen. 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 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 siloxane column (Rancho Cordova, CA) (20 m × 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.

**Methylene, 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. Methvicaine was prepared for use as the internal standard at a concentration of 100 mg/L. A negative blood control was extracted with each batch. Cases 2–4 were extracted as 5.0 mL aliquots of sample with pH adjusted through the addition of 2.0 mL of 0.1 M NaOH. Bile was prepared as a 3.0-mL aliquot diluted to 7.0 mL with 0.1 M NaOH and passed through a Chem Elut extraction column, eluting after a 10-min equilibration period with two 15-mL aliquots of methylene chloride. The methylene chloride eluent was evaporated at 40°C, holding for 6 min. Samples that contained analyte concentrations above the upper limit of the calibration curve were diluted appropriately and re-extracted. Order of elution was as follows: mephedrone (3.139 min), methylone (4.150 min), mepipvacaine (5.635 min) and MDPV (5.860 min).

The limit of detection (LOD) and limit of quantitation (LOQ) for each analyte was 0.025 mg/L, and the upper limit of linearity (ULOL) was 1.00 mg/L. Correlation coefficients for all calibration curves were greater than 0.99 and all calibrators, when calculated as an unknown against the calibration curve, quantitated within 20% of the expected concentration.

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 (\(n = 20\)).

**Results**

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%.

**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 III**

<table>
<thead>
<tr>
<th>Case</th>
<th>Source</th>
<th>Methylene concentration</th>
<th>MDPV concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Central blood</td>
<td>0.74 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Per. blood</td>
<td>0.67 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>38 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.8 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2.3 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>2.1 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>1.9 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>Heart blood</td>
<td>0.11 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.25 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0.55 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.26 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>0.52 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>Heart blood</td>
<td>0.06 mg/L</td>
<td>0.47 mg/L</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0.14 mg/kg</td>
<td>0.53 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.16 mg/kg</td>
<td>0.49 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Bile</td>
<td>0.42 mg/L</td>
<td>0.58 mg/L</td>
</tr>
<tr>
<td>4</td>
<td>Heart blood</td>
<td>1.1 mg/L</td>
<td>0.03 mg/L</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.22 mg/L</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.3 mg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.91 mg/kg</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
sample aliquot effectively adjusted the pH to 10.5–11.0, which was sufficient for the creation of a neutral methylenone species that easily extracted into organic solvent. Similarly, addition of the sulfuric acid to the separated organic phase allowed for an acceptable back-extraction of the resultant ionic methylenone species. The pKa values for MDMA and its demethylated analogue methylenedioxymethamphetamine (MDA) are 10.38 and 10.04, respectively (26). The structural similarities between methylenone, MDMA and MDA indicate that the pKa of methylenone would be in a similar range.

Two different extraction methods were used in this study, as well as GC–MS analysis of methylenone in both derivatized and underivatized forms. Comparison of the total ion chromatograms and mass spectral data shows that methylenone is volatile and stable enough to be easily analyzed by GC–MS without needing prior derivatization. However, the single labile proton available on the amine moiety means methylenone is amenable to derivatization. Despite the presence of a carbonyl group, methylenone does not exhibit tautomeration, so pre-extraction reactions such as oxime formation are not necessary.

Postmortem distribution of methylenone has not been previously reported. Analysis of the cases presented here can provide some information on the distribution of the drug throughout the body. Blood specimens from multiple sites were collected in Case 1. Although the concentrations of methylenone were similar in the two specimens, there is insufficient blood data to address the issue of postmortem redistribution. The liver-to-blood ratios in the four cases averaged 2.68 (range 1.19–4.66). This suggests that there is not as significant a degree of sequestration of methylenone as for drugs such as tricyclic antidepressants, a class of drugs known to show 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 methylenone 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 methylenone was analyzed in its unmetabolized form and present in easily detectable amounts in all matrices tested. This demonstrates that methylenone can be directly assayed, without having to initially test for the primary metabolite, HMMC, in postmortem cases or cases in which blood is available for testing. However, in cases where methylenone use is suspected over 36 hours before death, the parent drug may be fully metabolized, necessitating the analysis of HMMC. Metabolites of methylenone, MDPV or methylphenidate were not tested in the cases presented due to the current lack of commercially available reference standards.

The very large concentration of methylenone in the urine in Case 1 suggests that the decedent was a chronic user of products containing methylenone. 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 methylenone 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 methylenone. This is most likely due to the amphetamine derivative 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 methylenone toxicity with the presence of other amphetamine-like drugs, but neither investigated the combination of methylenone 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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