Stability of 3,4-Methylenedioxymethamphetamine (MDMA), 4-Methylmethcathinone (Mephedrone) and 3-Trifluoromethylphenylpiperazine (3-TFMPP) in Formalin Solution

Peter D. Maskell*, L. Nitin Sectohul, Alison C. Livingstone, Alexandra K. Cockburn, Jamie Preece and Derrick J. Pounder

Centre for Forensic and Legal Medicine, University of Dundee, Dundee DD1 4HN, UK

*Author to whom correspondence should be addressed. Email: p.d.maskell@dundee.ac.uk

Introduction

Embalming of bodies and storage of organs/tissues following autopsy using formalin-based fluids are common in both the funeral industry and forensic medicine (1). Usually, autopsy samples for drugs analysis are obtained before the embalming process. Occasionally, however, the need arises to analyze either embalmed tissue or tissue retained in formaldehyde for histological studies. This problem typically arises because either there was no initial suspicion of drug involvement in the death, or because an initial drug screen failed to detect the drug. The latter problem may become more common with the rise of novel ‘designer drugs’ such as 4-methylmethcathinone (mephedrone) and 3-trifluoromethylphenylpiperazine (3-TFMPP) (2), which may not be identified on general drug screens.

The presence of formalin in a biological sample submitted for toxicological analysis presents a challenge, because any drugs present in the body at the time of death will be exposed to the highly reactive compound, formaldehyde. Previous studies have shown that many amine-containing drugs such as benzodiazepines (1), methamphetamine (3), fenfluramine (4) and various tricyclic antidepressants such as amitryptyline and desipramine (5) form N-methyl derivatives in a time-, pH- and formalin concentration-dependent manner via the Eschweiler–Clarke reaction (6, 7), in which reductive methylation of primary or secondary amines occurs in two steps. In the first step, an iminium is formed with the reaction of the amine and formaldehyde (formalin) in a pH-dependent manner. The second step is the reduction of the imine by formic acid. Formic acid is a contaminant of formalin formed by the oxidation of formaldehyde in air (8). This reactivity of formaldehyde with various drugs makes drug analysis in formalin-preserved tissues difficult, but analysis may be facilitated by the knowledge of the reaction products that can be used as alternative analytes (9).

3,4-Methylenedioxymethamphetamine (MDMA) is a secondary amine and is methylated to 3,4-methylenedioxy-N,N-dimethylamphetamine (MMDA) in the presence of formalin (10). However, the rate of the reaction, which is of importance in the interpretation of analytical results, is not documented. The secondary amines such as mephedrone and 3-TFMPP have recently become popular drugs of abuse in the UK and have been described as alternatives to MDMA. Mephedrone and 3-TFMPP have been linked to several deaths (2, 11, 12). The reactivity of formaldehyde with these two drugs has not been studied previously. We have investigated the stability of the secondary amines such as MDMA, 3-TFMPP and mephedrone over time with varying pH and formalin concentrations and identified putative N-methyl reaction products.

Materials and methods

Chemicals and reagents

All reagents and solvents were of analytical grade. Mephedrone was purchased from LGC standards (Teddington, UK). HPLC-grade acetonitrile was supplied by Fisher Scientific (Loughborough, UK). MDMA, 3-TFMPP, formaldehyde (formalin, 37% aqueous solution containing 10–15% methanol stabilizer), triethylammonium phosphate (TEAP) buffer (1.0 M, pH 3.0), formic acid, ammonium formate and potassium phosphate were purchased from Sigma-Aldrich-Fluka (Poole, UK). Deionized water was produced with a Milli-Q system (Millipore UK Ltd., Watford, UK).

Standards, calibrators and control preparation

Stock solutions of MDMA, mephedrone and 3-TFMPP were prepared at a concentration of 1 mg/mL in methanol and were stored in amber vials at 4°C. These stock solutions were used to prepare a calibration range of 0.16, 0.31, 0.62, 1.25, 2.5, 5 and 10 mg/L in deionized water. Other stock solutions of MDMA, mephedrone and 3-TFMPP were used to prepare quality control standards at concentrations of 0.5 and 5.0 mg/L. The calibrator and control samples were prepared fresh for each daily analytical run.

The formalin reaction mixtures were prepared as previously described (3). The reaction mixtures used were 5, 10 and 20%
formaldehyde in water, no pH adjustment (~pH 3.5), 10 mM K2HPO4 (pH 7) or 10 mM K2HPO4 (pH 9.5). The formic acid content of the solutions prepared was unknown. The samples were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD) to determine if drug degradation had taken place immediately upon initiation of the reaction (Day 0) and on Days 1, 3, 7, 14, 21, 28 and 60. Each experiment was conducted in triplicate. The mephedrone experiment was ceased at Day 28 due to the amount of degradation that had occurred.

Instrumental and chromatographic conditions

**HPLC analysis of MDMA and mephedrone**

Quantitative analysis for MDMA and mephedrone was performed on a Dionex (Camberley, UK) HPLC system consisting of a PDA-100 diode array detector, ASI-100 autosampler, PS80 binary pump and STH 585 column oven. The column used for analysis was a Waters Spherisorb 5 μM OD/CN 150 mm × 4.6 mm. Chromelion version 6.8 with the DAD recording spectral data between 200 and 595 nm was used for data acquisition. For quantitative analysis, a wavelength of 210 nm was used. A column temperature of 25°C was used throughout. The analysis was based on 10% MeCN isocratic elution conditions and a run time of 5 min. The flow rate was 2 mL/min and injection volume, MDMA 80 μL; mephedrone 60 μL.

**HPLC analysis of 3-TFMPP**

Quantitative determination by HPLC-DAD was based on a previously published method (11) performed using a Dionex HPLC system (P580 binary pump, STH 585 column oven, GLN 50 autosampler and a PDA-100 diode array detector). A Phenomenex (Macclesfield, UK) Synergi Fusion 150 mm × 4.6 mm column with a 3-mm Phenomenex Synergi guard column was used for analysis. Data acquisition was by a Dionex Chromelion Version 6.8 software package with the DAD recording spectral data between 200 and 595 nm. A wavelength of 210 nm was used for analysis. The quantitative analysis was based on 25% acetonitrile (with 25 mM TEAP buffer), isocratic elution conditions with a flow rate of 2 mL/min. The column was maintained at a temperature of 25°C and injection volume of 60 μL.

**Method validation**

The method validation followed previously published guidelines (13). Standard curves for mephedrone, 3-TFMPP and MDMA in deionized water were shown to be linear, over a range of 0.156–10 mg/L with an $R^2 > 0.99$. According to validation results, the limit of detection (LOD) of mephedrone was 0.06 mg/L and limit of quantitation (LOQ) was 0.29 mg/L. The LOD and LOQ for MDMA were 0.12 and 0.45 mg/L, respectively. For 3-TFMPP, the LOD was 0.1 mg/L and the LOQ was 0.4 mg/L. The LOD and LOQ were both calculated using a linear calibration curve in water ($n = 5$). The LOD and LOQ were estimated from the standard deviation of the y-intercept ($σ_y$) and the average of the slope ($m_{avg}$) as LOD = ($3.3σ_y$) / $m_{avg}$ and LOQ = ($10σ_y$) / $m_{avg}$. Accuracy and interday precision (calculated as standard deviation and bias) were <20% for MDMA and <15% for 3-TFMPP. However, accuracy and interday precision for mephedrone were >30% due to the rapid degradation of mephedrone in formalin solutions.

**LC–MS–MS analysis of MDMA, mephedrone and 3-TFMPP**

Liquid chromatography with mass spectrometry (LC–MS–MS) analysis was performed using an Applied Biosystems (Warrington, UK) MDS/SCIEX 3200 QTRAP with an Agilent (Wokingham, UK) 1200 series HPLC system. A Phenomenex...
Gemini 150 mm × 2 mm column, protected by a 4 mm × 2 mm Phenomenex Gemini guard column, was used.

Ionization was achieved with a Turbo V electrospray source. LC–MS–MS data were obtained in the positive enhance mass spectrum (EMS) mode (scanning from 50 to 800 Da) with information-dependent (>1,000 cps) enhanced product ion (EPI) scanning (between 50 and 700 Da). Product ions were formed using collision energies (CE) of 20, 35 and 50 eV in addition to collision energy spread (CES) of 35 ± 15 eV. The following parameters were used: source temperature, 700°C; curtain gas, 30.0; Ion source gas 1, 45 units; Ion source gas 2, 50 units; ion spray voltage, 5,500 V; collision gas, high; declustering potential, 20 V; entrance potential, 10 V; scan rate, 4,000 Da/s (EMS) and 4,000 Da/s (EPI) and linear ion trap fill-time, 50 ms.

LC–MS analysis was based on an acetonitrile gradient with an equilibration at 3% acetonitrile for 3 min, then increasing to 65% acetonitrile over 20 min. The overall run time was 23 min. The flow rate was 0.8 mL/min, and the column temperature was maintained at 30°C. The mobile phase consisted of Phase A buffer (1 mM ammonium formate and 0.1% formic acid) and Phase B (70% acetonitrile, 1 mM ammonium formate and 0.1% formic acid). Injection volume was 20 μL.

Figure 2. Degradation of mephedrone over 28 days in the presence of various concentrations of formalin solution (5, 10 and 20%) at (A) pH ≈ 3.5, (B) pH 7 and (C) pH 9.5. Data for each condition represent the mean (n = 3).

Figure 3. Degradation of 3-TFMPP over 60 days in the presence of various concentrations of formalin solution (5, 10 and 20%) at (A) pH ≈ 3.5, (B) pH 7 and (C) pH 9.5. Data for each condition represent the mean (n = 3).
Stability of MDMA, mephedrone and 3-TFMPP in formalin solution

We investigated the stability of the secondary amines such as MDMA, 3-TFMPP (over 60 days) and mephedrone (over 28 days) with varying pH (pH ≈ 3.5, 7 and 9.5) and formalin concentrations (5, 10 and 20%).

As can be seen from Figures 1–3, all three drugs were most stable at the more acidic pH of ≈ 3.5 with the maximum decomposition observed in any formalin concentration (5, 10 or 20%) of 22% for MDMA and 26% for 3-TFMPP, after 60 days, and 37% for mephedrone after 28 days. The control solutions of the drugs remained stable throughout the duration of the experiments. All three drugs showed the maximum decomposition (for all formalin concentrations; 5, 10 or 20%) at pH 9.5 after 60 days with 50% loss of 3-TFMPP, 95% loss of MDMA and 96% loss of mephedrone (at 28 days). Mephedrone had the fastest rate of decomposition (an 82% decrease from the starting concentration after 1 day) followed by MDMA (a 25% decrease from the starting concentration after 1 day), and the slowest was 3-TFMPP (no decrease was observed from the starting concentration after 1 day).

For MDMA on Day 7, at all formalin concentrations at pH 5, and

Figure 4. Putative product ion spectrum of [M+H]+ of N-methylated MDMA. + EPI (208.2), charge (+1), CE (35).
for 3-TFMPP on Day 21 (all samples), there were transient increases in the drug concentrations attributable to method variability.

The formaldehyde concentration, independent of pH, was also found to affect the rate of degradation although not to the same extent as pH. For mephedrone and 3-TFMPP, the largest decrease in the concentration of the drug was observed at 20% formalin for all three test pHs (≏3.5, 7 and 9.5). For MDMA (Day 60), the greatest amount of degradation was observed with 5% formalin (pH 3.5 and 9.5) and 10% formalin for pH 7, but this is likely attributable to the variability of the method.

**Decomposition products of MDMA, mephedrone and 3-TFMPP in formalin solution**

For both MDMA and mephedrone, after 1 day a secondary peak with a similar UV—Visible spectrum, but different retention time from the respective parent compound was observed on the
HPLC traces (data not shown). The size of this peak increased throughout the duration of the experiment. However, no secondary peak was observed with 3-TFMPP throughout the course of the study.

In order to further identify the unknown compounds, the Day 28 samples were run on an LC–MS–MS. The unknown peak in MDMA-spiked samples was positively identified as N-methylated MDMA based on a library match of a previously infused standard. Ions of expected protonated weight ([M+H]^+) of 209.2 m/z were identified in the samples [the molecular weight (MW) of N-methylated MDMA being 208.2]. A product ion spectrum of N-methylated MDMA was obtained using an EPI and is shown in Figure 4. For mephedrone and 3-TFMPP, no N-methylated standards were available. However, ions of the expected protonated weight ([M+H]^+) of 192.2 m/z (mephedrone) and 245.2 m/z (3-TFMPP) were identified in the respective samples (the MW

![N-methyl-mephedrone](image)

**Figure 6.** Putative product ion spectrum of [M+H]^+ of N-methyl mephedrone. +EPI (192.26), charge (+1), CE (35).
of N-methylated mephedrone being 191.2 and N-methylated 3-TFMPP, 244.2). A product ion spectrum of putative N-methylated mephedrone and N-methylated 3-TFMPP was obtained using an EPI and are shown in Figures 5 and 6. These ions putatively identify the breakdown products of mephedrone and 3-TFMPP as the N-methylated forms of both the drugs.

Discussion

Shakleya et al. (10) previously demonstrated that MDMA spiked with formalin forms 3,4-methylenedioxy-N, N-dimethylamphetamine (MDDA), which we have confirmed using LC–MS analysis. For mephedrone and 3-TFMPP, we hypothesized that, as with MDMA, the Eschweiler–Clarke reaction would occur, in which the carbonyl functional group of formaldehyde would react with the nitrogen of the secondary amine resulting in the formation of an imine or iminium ion. The imine or iminium would then be hydrogenated to the methylated amine product. We observed that there was minimal dependence on formalin concentration on the extent of degradation. At lower pH (∼3.5), an increase in formaldehyde concentration leads to an increase in the degradation of mephedrone, with the reaction occurring primarily in the first 24 h. When the different degradation trends were examined as a function of pH, time was an important factor. Of particular interest was the differing rates at which the N-methylated products were formed. For MDMA at a pH of 9.5, after 1 week, the degradation was almost double that of the 24 h sample. In general, mephedrone was the fastest to degrade, with maximum degradation of 82% in the first 24 h, followed by MDMA with a maximum degradation of 49%. 3-TFMPP did not show any change within the first 24 h. From these findings, it can be inferred that the side chain on the aromatic rings in the compounds investigated influences the extent to which degradation occurs. Both mephedrone and MDMA have an aliphatic chain of two carbon atoms attached to the secondary amine. The faster degradation observed in mephedrone could be due to the presence of the carbonyl group (C=O) that helps stabilize the iminium ion produced during the Eschweiler–Clarke reaction. These results are in agreement with previous research by Suma et al. (14), who investigated the stability of olanzapine in formalin and found it to be stable at all pH levels and at all formalin concentrations. Winek et al. (6) studied phenytoin (an antiepileptic that contains secondary amine groups in an aliphatic ring) over a 28-day period in tissue samples bathed in 5 and 8% formaldehyde solutions. Both suggested that it might be difficult to form the intermediate iminium ion by the Eschweiler–Clarke reaction due to the presence of an aliphatic ring structure. These previous observations together with our finding that 3-TFMPP, a secondary amine in an aliphatic ring structure, is more stable in formalin solutions than its counterparts that consist of an aliphatic chain attached to the secondary amine reinforce the view that the position of the amine group in an aliphatic chain or a ring may determine drug stability when exposed to formalin.

Conclusions

MDMA and mephedrone readily degrade in formalin solutions, with an increased rate of degradation occurring at basic pH. 3-TFMPP is more stable than MDMA and mephedrone when exposed to formaldehyde. We suggest that this greater stability is a consequence of the location of the amine group in the ring structure. The relative stability of amine drugs in formalin may be predictable from the drug structure. Where the Eschweiler–Clarke reaction occurs, a methylated form of the drug is produced. This methylated product of the drug may be a more important analyte than the parent drug in establishing the presence of the drug.

References