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Case Report

Case Report

Analysis of MT-45, a Novel Synthetic Opioid, in Human Whole Blood by LC–MS-MS and Its Identification in a Drug-Related Death

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Abstract

MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl)piperazine) is just one of the many novel psychoactive substances (NPS) to have reached the recreational drug market in the twenty-first century; it is however, one of the first designer opioids to achieve some degree of popularity, in a market currently dominated by synthetic cannabinoids and designer stimulants. A single fatality involving MT-45 and etizolam is described. A method for the quantitation of MT-45 in whole blood using liquid chromatography–tandem mass spectrometry was developed and validated. The linear range was determined to be 1.0–100 ng/mL with a detection limit of 1.0 ng/mL, and the method met the requirements for acceptable linearity, precision and accuracy. After analyzing the sample on dilution and by standard addition, the concentration of MT-45 in the decedent's blood was determined to be 520 ng/mL, consistent with other concentrations of MT-45 reported in drug-related fatalities. Etizolam was present at a concentration of 35 ng/mL. This case illustrates the importance of considering non-traditional drugs in unexplained apparent drug-related deaths.

Introduction

Although the first classes of drugs to appear on the novel psychoactive substances (NPS) market included synthetic cannabinoids (K2, Spice) and synthetic cathinones (popularly known as 'bath salts') such as methylenedioxypyrovalerone and methylone, novel compounds in other drug classes are also starting to appear. Examples include the NBOMe suite of designer hallucinogens, and the designer benzodiazepines, phenazepam and etizolam. Most recently, designer opioids are starting to appear, such as AH-7921 and in the present case MT-45 (1).

MT-45, also known as IC-6, was developed in the 1970s by the Dainippon Pharmaceutical Co. in Japan, as an alternative to morphine for analgesia (2–5). It is an *N*,*N*-disubstituted 4-(1,2-diphenylethyl) piperazine chemically unrelated to other opioid agonists (Figure 1). The pharmacology of MT-45 is complex and involves opioid and other non-opioid receptors that have not been fully characterized; however, it has been demonstrated to have approximately the same potency as morphine in animal studies. It is currently uncontrolled in the USA.

Internet suppliers and retailers typically sell MT-45 in its dihydrochloride salt form. It has been seized mixed with other drugs, including synthetic cannabinoids such as 5-fluoro-PB-22, pyrrolidinophenones such as 4-methoxy-α PVP and synthetic cathinones such as 4-methylbuphedrine (6).

We describe the development and validation of an analytical method for the identification and quantitation of MT-45 in human whole blood. The method was subsequently used to test blood from a suspicious death, which was ultimately attributed to the combined toxicity of MT-45 and the designer thienodiazepine etizolam.

Methods

Chemicals and reagents

All solvents were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, Pennsylvania) with the exception of formic

acid, which was purchased from Aldrich Chemical Company (Milwaukee, WI). MT-45 dihydrochloride and the internal standard acetyl fentanyl-d5 were purchased from Cayman Chemical (Ann Arbor, MI) (Figure 1).

Standards, calibrators and control preparation

A stock solution of MT-45 was prepared at a concentration of 1,000 ng/μL in methanol and stored at ≤20°C in an amber glass vial. A stock solution of the internal standard, acetyl fentanyl-d5, was purchased at a concentration of 100 ng/μL. A working solution of acetyl fentanyl-d5 was prepared by adding 20 μL of the stock solution to 50 mL of methanol.

Calibrators for MT-45 were prepared by spiking from the stock in certified drug-free human whole blood, containing potassium oxalate and sodium fluoride preservatives. Calibrators were prepared at concentrations of 1.0, 4.0, 10, 40 and 100 ng/mL.

Instrumentation

Analysis was performed by liquid chromatography–tandem mass spectrometry using a Waters Acquity Ultra Performance LC system (UPLC) with a binary solvent manager, sample manager and column manager (Waters, Milford, MA) coupled to a Waters TQD Tandem Mass Spectrometer. The instrument was operated in positive electrospray, multiple reaction monitoring (MRM) mode. The injection volume was 10 μL, run time was 3.5 min and the flow rate was 0.4 mL/min.

Mass spectrometry conditions were optimized using an infusion of 100 ng/mL solution, prepared by dilution from the stock solutions using methanol. The Q1, Q3 and MS parameters are shown in Table I.

Separation was performed on an Acquity UPLC BEH C18 column, 2.1 × 50 mm, 1.7 μm. Mobile phases were 0.1% formic acid in deionized water (A) and 0.1% formic acid in methanol (B). The elution gradient used a mixture of the mobile phases at an initial ratio of 80:20 (A:B), which was adjusted to 60:40 at 1.50 min and 5:95 starting at 2.0 min. Acetyl fentanyl-d5 was detected at a retention time of 1.72 min, and MT-45 was detected at a retention time of 2.26 min.

Sample preparation

An alkaline liquid–liquid extraction procedure was used to isolate MT-45 from whole blood. After aliquoting 500 μL of blood to an extraction tube, 25 μL of internal standard solution and 50 μL of ammonium hydroxide were added, followed by 4.0 mL of *N*-butyl chloride/acetonitrile (4:1 v/v). Samples were capped, rotated and centrifuged for 10 min. The organic top layer was removed, evaporated to dryness at 55°C and reconstituted with mobile phase of 80:20 (A:B). Analysis was performed using the method of standard addition; the sample was analyzed on a 20-fold dilution with drug-free whole blood, with 20 and 50 ng/mL spikes of MT-45. The responses for the analyte and the ISTD were plotted against the concentrations of the spikes.

Method validation

A limited fit-for-purpose validation was performed on this method, which included precision and accuracy within and between run over the course of three days. In the absence of a deuterated internal standard, the method of standard addition was applied to counteract possible matrix effects or ion suppression. Because this report revolved around a single case, it was difficult for the laboratory to justify a comprehensive method validation to include specificity and sensitivity since that is a time-consuming and costly process. Because other information was available, such as the analyses of the material found at the death scene, the level of validation performed for the purposes of this case report was deemed acceptable. This approach to validation for investigative analyses is described in the laboratory's SOPs and has been subject to external review.

Validation of the method consisted of running matrix-matched calibration curves and a set of five replicates of each control over 3 days. Increasing amounts of a 1,000 ng/μL MT-45 standard were fortified into appropriate amounts of blood, with final concentrations of 1.0, 4.0, 10, 40 and 100 ng/mL. Separate control stock solutions were made for ease of spiking the control samples; high, mid and low controls were also hand-fortified. The analytical range was specified from 1.0–100 ng/mL, and calibration performance after 3 days of replicates resulted in a slope of 0.999 and a bias of 0.5%. The calibration curve was quadratic using 1/*X* weighting regression analysis of the peak area of analyte to the peak area of the internal standard. High, mid-range and low controls were fortified at 80, 30 and 3 ng/mL. A summary of the validation results is given in Table II. None of the reagent or matrix blanks contained interfering peaks.

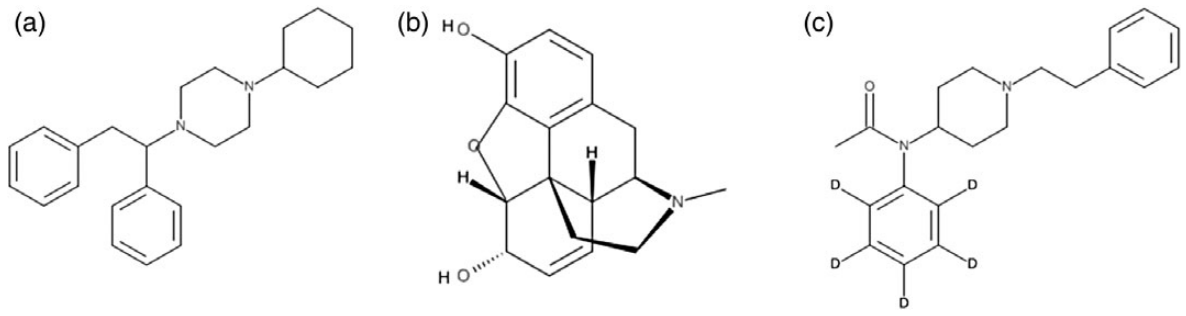

Figure 1. Chemical structures of (a) MT-45, (b) morphine and (c) acetyl fentanyl-d5 (ISTD).

Table I. Mass Transitions and Mass Spectrometer Settings

Analyte	Q1	Collision energy (eV)	Q3 Quantifier	Collision energy (eV)	Q3 Qualifier	Cone (V)
Acetyl fentanyl-d5	328.3	35	105.1	25	188.1	40
MT-45	349.3	40	181.1	30	169.2	35

Table II. Accuracy and Precision for Quantitation of MT-45 in Blood by LC–MS-MS ($N = 15$)

	Precision (% CV)		Accuracy (% Difference)	
	Between run	Total	Between run	Total
Reporting limit (1 ng/mL)	4.5	8.1	5.0	5.0
Low control (3 ng/mL)	5.2	6.9	2.0	2.0
Mid control (30 ng/mL)	3.6	4.3	16.7	16.7
High control (80 ng/mL)	3.8	6.0	3.8	3.8

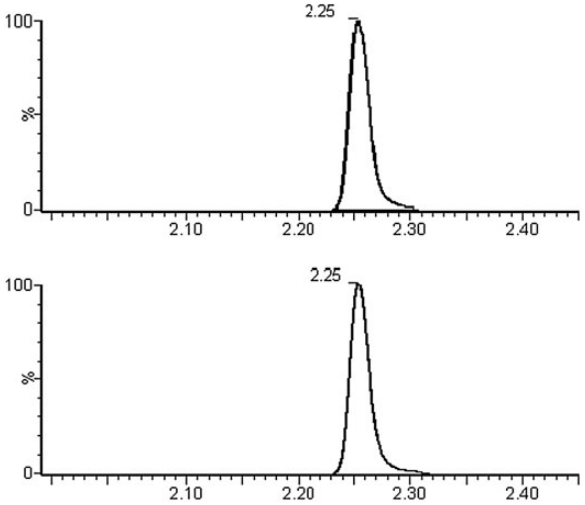

Figure 2. Example of chromatography of MT-45 from a 10-ng/mL control extracted from human whole blood. The figure shows the two transitions (m/z 349.3 > 181.1 and 349.3 > 169.2).

During limited stability testing, MT-45 was determined to be stable for up to 30 days in all conditions tested, room temperature, refrigerated and frozen.

An example of typical chromatography from the validation study is shown in Figure 2. MT-45 displayed an appropriate chromatographic peak shape under the conditions described.

In addition to the development of the LC–MS-MS assay, MT-45 and etizolam were added to the GC–MS library. Figure 3 is the mass spectrum entry for MT-45.

Case report

The method described was used to analyze a femoral blood sample from a death investigation case, sent for targeted toxicological analysis for MT-45.

The case involved a 35-year-old white male, with a known history of substance abuse, who had not been seen for several days prior to being found deceased. He appeared to have collapsed adjacent to his drug preparation area where a scale, spoon, pipe, lighter and two packets of white powder were found. There was no syringe present. The powders were tested for the presence of drugs at the Vermont Forensic Laboratory. One packet tested positive for MT-45, and the other tested positive for etizolam, an illicit thienodiazepine with benzodiazepine-like properties (7). Neither drug is prescribed in the USA, although etizolam is approved for use in certain Asian and European countries (8). Further investigation determined the decedent had purchased these materials online from a Canadian company and had

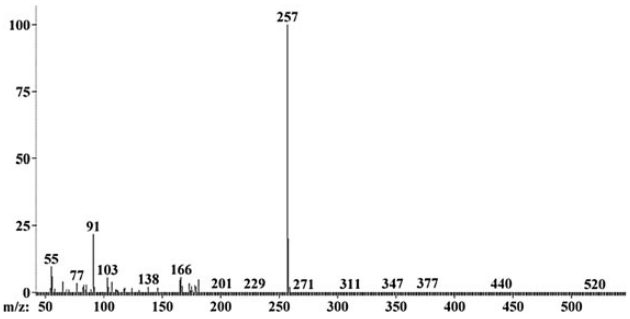

Figure 3. Mass spectrum for MT-45. Prominent base peaks include m/z 91 and 257.

been doing so on a monthly basis for some time. Autopsy revealed cerebral edema, pulmonary congestion and a possible old injection site on the dorsum of the foot. Blood samples were collected in gray top tubes and kept refrigerated. A comprehensive toxicology drug screening panel was performed on whole blood by high-performance liquid chromatography/time of flight-mass spectrometry (LC–TOF). The screen covered ~400 compounds; some of the compound classes included amphetamines, anticonvulsants, antidepressants, antihistamines, antipsychotic agents, benzodiazepines, central nervous system (CNS) stimulants, cocaine and metabolites, hallucinogens, hypnosedatives, hypoglycemics, muscle relaxants, non-steroidal anti-inflammatory agents, opiates and opioids. The only positive finding in the femoral blood was 220 ng/mL of diphenhydramine, which was confirmed and quantified by gas chromatography with nitrogen selective detection. An ELISA panel ruled out the presence of barbiturates, salicylates and cannabinoids. Further, testing by headspace gas chromatography was negative for acetone, ethanol, isopropanol and methanol. The urine screened presumptively positive for benzodiazepines and cannabinoids by Enzyme Immunoassay (EIA). Etizolam has demonstrated cross-reactivity with commercial ELISA screening kits, however, MT-45 has not demonstrated cross-reactivity with opiate immunoassay plates. The initial testing was completed within 20 days of sample collection. Further specialized testing for etizolam and MT-45 was completed within 3 months of sample collection.

Targeted analysis of the MT-45 in femoral blood confirmed its presence at a concentration of 520 ng/mL. Etizolam was analyzed by a targeted LC–MS-MS method for benzodiazepines using standard addition and found to be present in the femoral blood at a concentration of 35 ng/mL. Etizolam was tested on a method that had been also validated according to a fit-for-purpose forensic investigation and compliant with the laboratory's SOPs. The cause of death was determined to be combined toxicity of MT-45 and etizolam with manner of death determined as accidental by unintentional overdose.

The submitted samples of blood, urine, vitreous humor and bile were subsequently tested ~12 months from the original MT-45 testing using a newly validated method. The blood samples showed MT-45 degradation of ~50%; this could be due to the nature of the sample or possibly long-term instability. MT-45 was also detected in the urine, vitreous humor and bile, even after the extended period of time.

Discussion

Part of the popularity of NPS is their widespread availability via the Internet, their ambiguous legal status and the fact that they are not detected in most routine drug tests such as those used for pre-employment or random workplace testing. Opiates figure prominently among the

most frequently encountered drugs in toxic deaths (9–18). Assessing the significance of their contribution to cause of death is often complex due to issues related to therapeutic misadventure, tolerance, palliative care, drug–drug interactions and recreational versus medical use. While the most frequently encountered opioids are morphine, as a therapeutic agent or heroin metabolite, oxycodone, hydromorphone, hydrocodone and fentanyl, recently illicit 'designer opioids' have begun to appear in forensic casework. These include deaths attributed to acetyl fentanyl, a synthetic analog of fentanyl, which resulted in a series of deaths in Rhode Island and Pennsylvania, Louisiana and North Carolina (19, 20). An unrelated experimental opiate agonist, AH-7921, has been linked to deaths in Sweden and the USA (21, 22). Kratom is a Southeast Asian plant containing the drug mitragynine with opioid-like effects, which is currently uncontrolled in the USA; it has been implicated in deaths in combination with tramadol and propylhexedrine (23, 24). Mitragynine is thought to be a remedy for opiate withdrawal and is increasingly being implicated in forensic casework (25–28). Most recently, a lot of media attention has been paid to 'Krokodil' (desomorphine), a synthetic derivative of codeine, although we can find no evidence of any toxicologically confirmed cases having been documented in the USA.

MT-45 is one of the first designer opiates to have appeared as a NPS on the recreational drug market. It is a *N,N*-disubstituted piperazine compound; a cyclohexane ring is attached to one nitrogen and a 1,2-diphenylethylmoiety is attached to the other nitrogen. Although structurally unrelated to morphine, MT-45 shares analgesic and CNS depressant properties similar to that of morphine, including respiratory depression (29).

Emergency responders may have difficulty in identifying MT-45 overdose cases because MT-45 produces a low miotic effect; if an overdose is identified in a timely manner, naloxone may have some utility in the treatment of MT-45 intoxications (30).

Although MT-45 has been detected in intoxications and fatalities, it is not currently a routine part of most forensic drug screening panels. Typical routes of administration include oral, insufflation, intravenous and intramuscular, with varying reported doses; intrarectal use was also reported (31). Oral dosages commonly range from ~50 mg for opioid naive users up to ~250 mg for highly tolerant individuals; the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) also reported a wide range of doses and routes of administration and that some drug users re-dose, possibly due to an extended onset of action. Some of the potential adverse effects of MT-45 use, aside from the CNS depressant effects, include nausea, itching, bilateral hearing loss and possible withdrawal symptoms. Drug users also report some dissociative-like symptoms.

MT-45 has been detected in both non-fatal intoxications as well as fatal drug overdoses. MT-45 was typically not detected in preliminary toxicology screens, which can confound the interpretation. A recent report from Sweden listed a case series of nine non-fatal intoxications from males reporting to the emergency department, ranging in age of 17–32 years old (32). These individuals all presented to the emergency room with opioid-like adverse symptoms, including unconsciousness and respiratory depression, and three patients complained of bilateral hearing loss. MT-45 was the only substance detected in four cases; one or more psychoactive compounds were found in conjunction with MT-45 in the remaining cases. The blood concentrations reported in this case series ranged from 6 to 157 ng/mL. In a recent EMDCCA report, MT-45 was linked to 33 adverse events in Sweden, including 21 deaths between 2012 and 2014, and 2 cases of individuals who committed minor drug offenses. In these fatalities, concentrations of MT-45 in postmortem femoral blood ranged from 6 to 1,900 ng/g. In 17 of the reported cases, MT-45 was found in combination with at least one other psychoactive substance. In the six postmortem cases in which cause of death was attributed to MT-45 intoxication, the drug was reported at concentrations ranging from 200 to 1,900 ng/g (mean and median, 795 ng/g) (33). At least two cases of MT-45 have been implicated in deaths in the USA (34).

Although not marketed in the USA, the etizolam concentration is consistent with the range reported for therapeutic use; clinical studies of a single 2 mg oral dose resulted in average peak plasma concentrations of 25 ng/mL (35). A 59-year-old woman who apparently drowned in her bathtub after intentional ingestion of an overdose of etizolam had a postmortem blood concentration of 264 ng/mL (36). There have been a few other reports of accidental ingestion of etizolam by children, as well as another fatality in which etizolam was detected in conjunction with other psychoactive substances (37, 38).

The analytical result in this case for MT-45 (520 ng/mL) is consistent with those in other reported deaths. It also follows the trend that MT-45 was not the only substance identified in the decedent's blood. Etizolam was present at a concentration of 35 ng/mL, which is considered therapeutic, suggesting that MT-45 was principally responsible for this death. Combined use of opioids and benzodiazepines is a frequent finding in death investigation casework (39).

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