DISTRIBUTION OF THE SYNTHETIC CATHINONE α-PYRROLIDINOHEXIOPHENONE IN BIOLOGICAL SPECIMENS

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Keywords: α-PHP, synthetic cathinones, LC-MS/MS, tissue distribution

ABSTRACT

We report the analysis of the synthetic cathinones α-pyrrolidinohexiophenone (α-PHP) and α-pyrrolidinopentiophenone (α-PVP), both pyrovalerone derivatives, in blood, urine, gastric contents, main tissues and hair of a deceased person. Qualitative and quantitative analyses were performed by LC–MS/MS. All the biological samples were collected during autopsy and extracted/purified onto a SPE cartridge before instrumental analysis. The method was validated for blood and urine and proved to be highly sensitive and specific for both the synthetic cathinones (LOD: 0.2 ng/mL and LOQ: 0.5 ng/mL). Analyses provided negative results for α-PVP in all biological samples except for the 2-cm proximal hair segment, confirming that the young man had consumed in the last two months this compound; instead hair analysis proved that the man was a heavy α-PHP user. α-PHP was identified and quantified in biological fluids and tissues. Interestingly, bile and urine concentrations (1.2 and 5.6 ng/mL respectively) were fairly lower than blood collected into the thoracic cavity (15.3 ng/mL). The highest concentrations were measured for lung (71.1 ng/mL) and spleen (83.8 ng/mL). Concentrations of 3.5, 7.9, 4.7 and 23.6 ng/mL were measured in liver, kidney, brain and heart respectively. Even if it is not possible to evaluate the presence of this drug in biological samples as a cause of death, to our knowledge, this is the first case of α-PHP finding in postmortem samples, and its potential toxic effects should be elucidated in the future.

INTRODUCTION

Synthetic cathinones, also called “bath salts”, are new psychoactive substances (NPS) that in recent years have become popular drugs of abuse. These are phenylalkylamine derivatives and the synthesis of these compounds has been reported since 1920s; initially used for medical purposes. Years later, at the end of 2000s, the interest in synthetic cathinones as psychoactive drugs grew up due to their large availability on the Internet. The primary reason for so large spread in Europe was that these drugs were initially legal. After the introduction of regulatory measures restricting the sale of synthetic cathinones, consumers were forced to stop buying them on the Internet and began purchasing from local dealers.

Synthetic cathinones were the most frequently seized NPS in Europe in 2015, with over 25,000 seizures. Use of synthetic cathinones has been reported in 15 European countries, with a large variability of compounds by country; few people currently enter treatment in Europe for problems associated with use of these drugs, although under-reporting is likely [1]. Synthetic cathinones are the most frequently seized NPS in Italy; among them, 3-methylmethcathinone (3-MMC), 4-

methylethcathinone (4-MEC) and methylenedioxypyrovalerone (MDPV) were often identified, but also α-PHP and α-PVP findings were reported [2].

Synthetic cathinones can be ingested, snorted, smoked or, more rarely, injected. Desired effects of these compounds include empathy, increased energy and libido; negative effects, mainly cardiac and psychiatric, may occur. Some cases of intoxication associated with use of synthetic cathinones have been reported [3,4,5,6].

Synthetic cathinones have variable effects and potency levels on serotonin, dopamine and noradrenaline pathways, but typically possess sympathomimetic/amphetamine-like effects [7,8]. Instead, pyrovalerone derivatives are reported to show a cocaine-like mechanism of action (as dopamine re-uptake inhibiting agents): α-PHP is described to be more potent than MDPV and α-PVP, that show similar potency [9].

α-PHP was identified for the first time in 2014 in seized materials in Japan [10]. The molecule is chemically related to α-PVP, a compound that had been used as appetite suppressant and in the treatment of chronic fatigue, having an extra carbon on the alkyl side chain.

There are a few published data on pharmacokinetics and pharmacodynamics of α-PHP, but in a recently published article the structures of some metabolites were tentatively elucidated, analysing urine samples of a drug abuser. α-PHP is extensively metabolized (19 phase I metabolites, and 9 glucuronide conjugates were identified). Nevertheless, the parent drug is detectable in urine sample in large amount [11]. Concentrations of α-PHP in urine were measured in the range 1-300000 ng/mL and in blood in the range 4-10 ng/mL [12].

Plasmatic half-life of α-PHP was investigated by Fuyita *et al*: they analysed 14 blood samples, collected subsequently from an intoxicated patient. Due to the long-term clinical symptoms of the patient induced by α-PHP, a long half-life was suspected, and in fact it was found to be about 37 hours [13].

In recent years, few cases of pyrovalerone derivatives-related intoxications and deaths have been reported [14-16]. In these cases, blood concentrations were extremely variable, but it should be considered that exposure to the drug may be highly erratic, because users cannot know the real content of doses and their purity.

CASE REPORT

A 27-year-old man was admitted to a local psychiatric hospital for drug abuse evaluation, clinical stabilization and withdrawal treatment, after having suffered from psychomotor agitation. He had a long history of polysubstance abuse and addiction.

Upon arrival at the hospital, the patient was conscious, oriented and cooperative. He showed little postural instability and some difficulties with fine motor skills. Cardiovascular and respiratory parameters were normal. A urine sample was collected and screened for common drugs of abuse (opiates, cannabinoids, cocaine, amphetamines, benzodiazepines). The test provided positive results for benzodiazepines only.

According to self-declaration, the young man started using cannabis when he was 14 and continued until the age of 21. During the same period, he also smoked and snorted cocaine, at first rarely and then daily. He stopped using cocaine for one year, but simultaneously began to binge-drink alcohol. He also said that he often used ketamine around the age of 20. Occasionally he took LSD, but with this drug he experienced terrifying hallucinations. After these episodes, he started suffering from psychiatric disorders, requiring multiple admissions to psychiatric hospitals and being treated with antipsychotics and antidepressants. He then used amphetamines consistently for several years, too. In order to treat psychiatric disturbances, during the first day of hospitalization, the young man was treated with diazepam, clotiapine e quetiapine. His condition, however, did not improve: he reported that he was not able to leave his room because just the motion of his head gave him hallucinations.

The following day, soon after suffering withdrawal symptoms, the patient admitted that in the last few months he frequently smoked crystal-like drugs, purchased online and referred to as "cathinones". After taking those substances, he suffered from adverse effects (such as bone and muscular pains, agitation, aggression and visual hallucinations). He also developed a strong and irrepressible compulsion to redose, as well as addiction/dependence. He tried without success to reduce these symptoms firstly by taking benzodiazepines (without any medical supervision), and then by smoking heroin and using illegally obtained methadone.

Based on the clinical records provided by the patient, opioid withdrawal was suspected and physicians decided to submit the young man to a methadone treatment. The starting dose was 10 mg, which was increased to 50 mg within two days. After three days, a nurse reported that the patient showed signs of sedation. A few hours later, the man was found dead in his bed.

His body was subjected to a full autopsy by forensic pathologists. Autopsy revealed massive cerebral and pulmonary oedema, visceral congestion and left ventricular hypertrophy.

MATERIALS AND METHODS

Chemicals

α-PHP, α-PHP, mephedrone-D3 and methadone were obtained by LGC Standards (Milan, Italy). Quetiapine, clotiapine, diazepam and desmethyldiazepam standards were purchased from Sigma-Aldrich (Milan, Italy) Formic acid for mass-spectrometry was obtained from Sigma-Aldrich (Milan, Italy). HPLC-grade methanol, acetonitrile, dichloromethane, isopropanol and ammonia were purchased from Mallinkrodt Baker (Milan, Italy). Bond Elut Certify I cartridges were purchased from Agilent (Milan, Italy).
Mobile phase components were 0.1% formic acid (A) and methanol (B).

Sample treatment

Working solutions of α-PHP and α-PVP were prepared using methanol at a concentration of 1 µg/mL for mass spectrometry tuning and selectivity experiments. Calibrators were carried out by mixing the stock solutions and diluting with methanol at the following concentrations: 0.001 µg/mL, 0.005 µg/mL, 0.010 µg/mL, 0.050 µg/mL, 0.100 µg/mL. All solutions were stored at -20°C. The calibration curves were prepared using pooled blank postmortem blood and urine samples. All samples used for calibration were checked before analysis. Tissues were homogenized using a Precellys Evolution, Bertin (AlfaTech, GE, Italy) before sample treatment.
500 µL body fluids (blood, urine, bile and gastric contents) and 0.5 g homogenized tissue samples (liver, kidney, brain, lung, spleen and heart) were mixed with 2 mL phosphate buffer at pH 6.0 and 0.02 mL of mephedrone-D3 (internal standard - IS), at the concentration of 0.001 µg/mL. A hair sample (2-cm proximal) was washed with 1 mL methylene chloride and 1 mL methanol, pulverized using a Precellys Evolution and weighed (50 mg). Then, 0.02 mL of mephedrone-D3 at the concentration of 0.001 µg/mL were added, together with 1 mL HCl 0.1 N and overnight incubation at 37°C was performed. After centrifugation, samples were purified on solid phase extraction (SPE) cartridges (Bond Elut Certify, 130 mg, Agilent Technologies, MI, Italy). The columns were initially conditioned with 2 mL methanol and equilibrated with 2 mL phosphate buffer at pH 6.0; after loading the samples, the cartridges were washed with 2 mL water, 3 mL HCl 0.1 M and 5 mL methanol. Finally, synthetic cathinones were eluted from the cartridges with 2 mL dichloromethane: isopropanol (8:2) solution containing 2% ammonia. The eluates were taken to dryness under nitrogen stream at 35°C and the samples were reconstituted in 150 µL mobile phase. 10 µL were injected in the LC-MS/MS system.
Urine and blood samples were previously analyzed as blank samples.

LC-MS/MS settings

The method was developed with an Agilent 1100 Series system (vacuum degasser, binary pump, and column compartment) and an Agilent 1200 Series isocratic pump and autosampler maintained at 4°C (Agilent Technologies, Palo Alto, CA, USA) coupled with a 4000 QTrap mass spectrometer (AB/ Sciex, Foster City, CA, USA).

The LC injector needle was externally washed with methanol prior to any injection. A kinetex C18 column (100×2.1 mm i.d., 5 µm particle size, Phenomenex, Castelmaggiore, Italy) was kept at 25°C during the chromatographic run. Flow rate (0.3 mL/min) was set at gradient mode as follows: from 95% A to 5% in 6 minutes, maintained at 5% for 3 minutes and reequilibrated for 8 min. The ESI source settings were: source temperature, 500°C; nebulization and heating gas (air): 30 psig and 25 psig, respectively; curtain gas (nitrogen): 20 psig. Ion-spray voltage was set at 5000 V in positive ion mode. Mass analysis was performed in Multiple Reaction Monitoring (MRM) mode and positive polarization. All parameters, including declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were separately optimized for each analyte by infusing in the mass spectrometer a 1 µg/mL solution in 0.1% formic acid (Table 1). Two transitions for each analyte were chosen for identification while the most intense was selected for quantification purposes.

Validation

The analytical procedures have been validated according to the guidelines by Peters *et al* [17]. The following parameters were considered for method validation: linearity, sensitivity, specificity, accuracy, imprecision, recovery, carryover, matrix effects, and freeze and thaw stability.

Linearity was evaluated using a six-point calibration curve in the range 0.5-200.0 ng/mL (calibrators: 0.5, 1.0, 10.0, 50.0, 100.0, 200.0 ng/mL). Sensitivity, expressed as Limit Of Detection (LOD) and Lower Limit Of Quantification (LLOQ), was evaluated for each analyte using decreasing concentrations of analyte in drug-fortified blood and urine. LOD was defined as the lowest concentration with neat peaks and a signal-to-noise ratio of at least 3, and a relative retention time (RRT) within ± 2% of the average calibrator (10 ng/mL) RRT. LLOQ was defined as the lowest concentration with the same parameters of LOD and with signal-to-noise ratio of at least 10 and acceptable accuracy and precision as defined below. Accuracy and imprecision were measured at two quality control (QC) levels (5.0 and 200 ng/mL). The lower QC level was chosen according to the results obtained on authentic samples. Spiked blank tissues at QC levels were evaluated on calibration curve prepared with blood. Accuracy was calculated as the percentage deviation of the mean calculated concentration (n = 10 over a 4-day period; absolute value) from the corresponding nominal value. Intra-assay and inter-assay imprecision were calculated as the coefficient of

variation (CV%) of five replicates for each QC level. Specificity was evaluated by adding high concentrations (1000 ng/mL) of potentially interfering licit and illicit drugs to calibration points and QC samples. Traditional drugs of abuse, benzodiazepines, cardiovascular drugs, antidepressants, antipsychotics and metabolites were included in the drug-fortified samples. Ten blank samples of urine, blood and liver were also processed to exclude interferences from endogenous substances. Interference was excluded if samples were within ± 20% target concentration. Absolute analytical recovery was assessed for each analyte using three replicates for each QC sample concentration by comparing the peak areas obtained when samples were analyzed by adding the QC samples and the ISs in the extract of blank blood and urine prior to and after the extraction procedure. Carryover was assessed by injecting extracted blank blood and urine samples immediately after analysis of the highest concentration point of the calibration curve on each of the days of the validation protocol and measuring the area of eventual peaks, present at the relative retention times of analytes under investigation. Matrix effects were measured by comparing blank biological samples with water samples, spiked at QC levels and processed with the described procedure; absolute peak area in blood, urine and in water were compared. Experiments were carried out in triplicate, using ten different blank samples. Results were calculated by comparing the peak areas of α-PHP and α-PVP in biological samples vs those measured in water. The effect of three freeze-thaw cycles (storage at –20°C) on the compounds stability in blood and urine was evaluated by repeated analysis (n=3) of QC samples.

In consideration of the fact that the procedure for detecting α-PHP and α-PVP in hair was developed for screening purposes, validation was limited to the verification of method selectivity (7 different blanks injected), to the determination of the LODs.

Further toxicological analyses

Quantitative determination of ethanol, and systematic toxicological analysis (STA) to detect acidic, neutral and basic drugs including those that are commonly prescribed, non-prescribed and illicit drugs were performed on blood sample: STA was carried out using gas chromatography-mass spectrometry (GC-MS) technique preceded by a solid phase extraction with mixed-mode cartridges Bond Elut Certify, using the method detailed in a previously published study [18]. GC-MS data files were processed by an original procedure for the automated purification of mass spectra from the total ion chromatogram [19].

Quantitative analysis of methadone in blood was performed by GC-MS using the method routinely used in our laboratory. Quantitative analysis of diazepam, desmethyldiazepam, clotiapine and

quetiapine in blood were carried out by LC-MS/MS, using the method detailed in a previously published study [20].

RESULTS

GC-MS and LC-MS/MS analyses revealed the presence of methadone (440.0 ng/mL), diazepam (500.0 ng/mL), desmethyldiazepam (340.0 ng/mL), clotiapine (360.0 ng/mL) and quetiapine (180.0 ng/mL) in blood sample. Ethanol intake was excluded.
Linear calibration curves showed determination coefficients (R^2) higher than 0.99 for both α-PHP and α-PVP. LODs were set at 0.2 ng/mL for both α-PHP and α-PVP in urine and blood while the lowest calibration point (0.5 ng/mL) was chosen as LLOQs for the two cathinones in biological samples (Table 2). A LOD of 10 pg/mg was measured in hair for both α-PHP and α-PVP.

Accuracy, intra and inter-assay imprecision were within the established acceptance criteria (Table 2). CV% and BIAS% were lower than 15% for QC levels prepared using blank tissues. No additional peaks due to endogenous substances that could have interfered with the detection of the two synthetic cathinones have been observed. Likewise, none of the drugs of abuse or abovementioned medicines, carried through the entire procedure, interfered with the assay and with the accurate quantification of the low QC samples. Analytical recoveries obtained after extraction procedure for the two different QC samples ranged between 83.2% and 97.8% for both analytes (Table 2). No carryover was observed when blank samples were injected after the highest point of the calibration curve. Matrix effects were negligible. No relevant degradation was observed after any of the three freeze/thaw cycles, with differences in the initial concentration less than 10%.

All the biological samples collected during autopsy provided negative results for α-PVP except for the 2-cm proximal hair segment, confirming that the young man had consumed in the last two months this synthetic cathinone. α-PHP was identified and quantified in all the biological fluids and tissues. Results are listed in Table 3. The absolute peak area of α-PHP measured in hair sample was extremely high, suggesting that the subject was probably a heavy consumer of α-PHP. However, it cannot be excluded a potential contamination due to sweat [21].

DISCUSSION

Analysis of NPS in biological samples may be difficult. Few laboratories own the instrumentation required for their identification and an adequate number of analytical standards, given the large

number of molecules belonging to this category; any finding of drug specimens may indeed be of great help. In our case, suspicion that the man could have taken exactly α-PHP, came from the discovery of a plastic wrap containing some small crystals, identified as this compound, in the apartment where he lived.

The case here reported involve a young man that started using synthetic cathinones after previous experiences with cannabis, LSD and cocaine, and in a short time he could not stop taking them. After a few months of intake, he was in a recurrent state of agitation with visual hallucinations and terrifying nightmares, so his parents convinced him to go into rehab. It is recognised that synthetic cathinones have both stimulant and psychoactive effects: self-reported symptoms associated with their use include cardiovascular effects (palpitation, shortness of breath), gastrointestinal (nausea, vomiting, abdominal pain), neurologic (aggressiveness, dizziness, headache, memory loss, tremor, seizures), ophthalmologic (blurred vision, mydriasis), psychological (anger, anxiety, hallucinations, depression, dysphoria, panic, nightmares, paranoia) [22]. A significant proportion of synthetic cathinones users report tolerance, dependence or withdrawal symptoms [23]; many of them experienced strong craving to repeat doses after taking the drug. Long-term effects related to the intake of these substances are typically associated with the imbalance of a range of neurotransmitter pathways/receptors, and, consequently, with the risk of psychopathological disturbances [24].
In order to treat psychiatric disturbances, the man was given diazepam, quetiapine and clotiapine, but his condition did not improve. All these drugs were detected in blood sample at therapeutic levels. Furthermore, methadone was administered because the patient said he used to take heroin and methadone to reduce the side effects of cathinones, but unexpectedly, morphine and methadone were not detected in hair sample collected during autopsy. Methadone blood concentration (440.0 ng/ml) could justify the death in a *naïve* subject, according to current scientific knowledge, so methadone was determined to be the cause of death. Other drugs (diazepam, clotiapine and quetiapine) due to the depressing effect on the nervous system, may had contributed to the man's death.
The finding of α-PHP in biological fluids and tissues of the patient three days after the hospitalization is an interesting detail, and two different scenarios may be considered in trying to interpret analytical results. A concentration of 1078.0 pg/mg α-PHP was measured in the 2-cm proximal hair segment. Though, to the best of our knowledge, this is the very first case where α-PHP was measured in hair, similar concentrations of synthetic cathinones were already measured in other cases of chronic abusers of cathinone and amphetamine derivatives [25]. Hence, the level of α-PHP is more consistent with a continuing heavy consumption of the drug by the subject during, at

least, the last two months before death, rather than a contamination through sweat. The man experienced compulsive redosing, so it is possible that he may have brought some doses with him.Yet, qualitative analysis of gastric contents showed the presence of α-PHP, thus suggesting a very recent consumption. Likewise, the highest concentrations of the drug were found in lung and spleen samples, both affected by massive congestion, with a higher amount of blood accumulated in these tissues. Unfortunately, quantitative analysis performed on blood sample has to be considered scarcely reliable: in fact, the analysis was carried out on blood collected into the thoracic cavity, due to the lack of peripheral and cardiac blood. According to this hypothesis, the man could have taken the drug shortly before death, perhaps to counteract the narcotic properties of methadone.
The second option is that the young man could have taken the last dose before hospitalization: indeed α-PHP is thought to be eliminated from the body over a period of 150 hours. A recent study reported a blood concentration of α-PHP of 175.0 ng/mL in an intoxicated patient that slowly decreased to 15.7 ng/mL after 96 hours [13]. Authors assume that the long elimination of α-PHP may be attributed to the gradual release of the compound from other body tissues. This hypothesis could also be consistent with our results: in this instance, the finding of α-PHP in gastric contents could be interpreted as gastric secretion of the drug, and postmortem redistribution cannot be excluded.

CONCLUSION

Synthetic cathinones have been the most frequently seized NPS in Europe in the last few years, but their finding in biological samples remains an analytical challenge, especially when use is not suspected. Furthermore, there are few studies concerning distribution of these drugs in man. To our knowledge this is the first report about detection and quantification of α-PHP in body fluids and tissues in a postmortem case. Even if the drug was not directly the cause of death, our data may be useful for further analyses and interpretation of toxicological cases involving α-PHP.

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Figure Legend

Figure 1. Chemical structure of α-pyrrolidinohexiophenone (A) and α-pyrrolidinopentiophenone (B)

Figure 1.

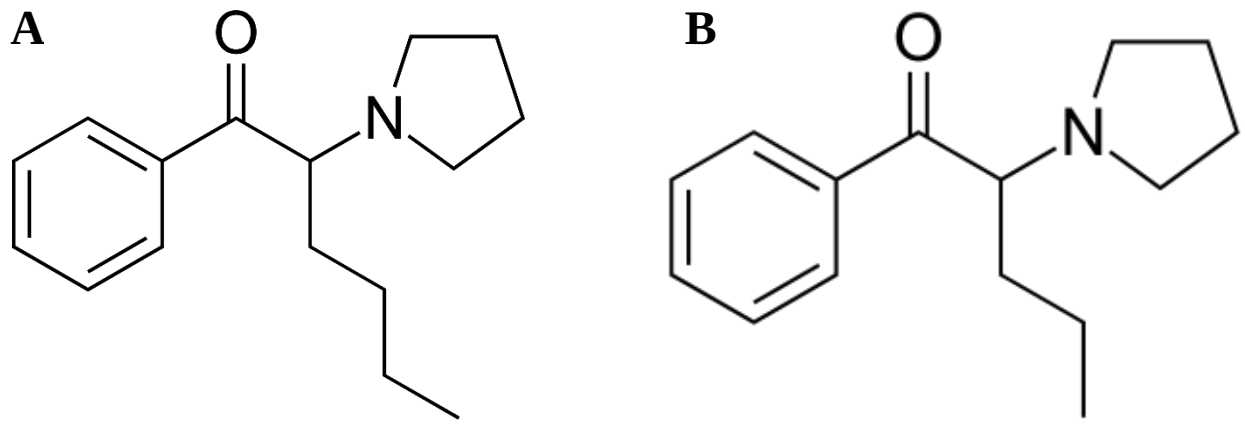

Table 1. MRM transitions and parameters

Analyte	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (eV)
α-PHP	246.4	105.2	80	10	40
α-PHP	246.4	140.2	80	10	35
α-PVP	232.1	91.3	90	10	65
α-PVP	232.1	105.3	90	10	31
Mephedrone-D3	181.2	148.1	60	10	29
Mephedrone-D3	181.2	163.3	60	10	29

Table 2. Validation parameters

Analyte	QC concentration (ng/mL)		Accuracy (RSD%)		Intraday-Imprecision (CV%)		Interday-Imprecision (CV%)		Recovery (%)		Matrix effects (%)	
	blood	urine	blood	urine	blood	urine	blood	urine	blood	urine	blood	urine
α-PHP	5.0	5.0	13.4	2.4	12.1	10.4	15.4	16.2	97.8	94.5	-17.8	-18.5
	200.0	200.0	4.8	3.5	9.7	10.2	10.3	9.5	91.0	95.4	-12.1	-10.1
α-PVP	5.0	5.0	15.7	17.9	11.5	15.3	16.3	18.4	87.4	89.6	-16.6	-18.4
	200.0	200.0	8.7	6.7	10.3	9.6	8.7	6.5	83.2	84.7	-14.6	-16.7

Table 3. Postmortem samples concentrations

Biological sample	α-PHP ng/mL(g)
Blood	15.3
Urine	5.6
Bile	1.2
Gastric contents	pos
Liver	3.5
Kidney	7.9
Spleen	83.8
Lung	71.1
Brain	4.7
Heart	23.6
Hair	1078.0*

*pg/mg