Forensic Science International 251 (2015) e1–e8

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Case report

A fatal poisoning involving 25C-NBOMe

Mette Findal Andreasen [a,*], Rasmus Telving [a], Ingrid Rosendal [a], Marlene Beyer Eg [b], Jørgen Bo Hasselstrøm [a], Ljubica Vukelic Andersen [a,c]

[a] Section of Forensic Chemistry, Department of Forensic Medicine, Aarhus University, Palle Juul-Jensens Boulevard 99, DK-8200 Aarhus N, Denmark
[b] Section of Forensic Pathology and Clinical Forensic Medicine, Department of Forensic Medicine, Aarhus University, Palle Juul-Jensens Boulevard 99, DK-8200 Aarhus N, Denmark
[c] Department of Clinical Pharmacology, Aarhus University Hospital, The Bartholin Building, Wilhelm Meyers Allé 4, 8000 Aarhus C, Denmark

ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form 4 March 2015
Accepted 17 March 2015
Available online 24 March 2015

Keywords:
25C-NBOMe
UPLC–TOFMS
LC–MS/MS
Intoxication
Multiple organ failure

ABSTRACT

This paper reports on a fatal overdose case involving the potent hallucinogenic drug 25C-NBOMe (2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine). In the present case, a young male was hospitalized after the recreational use of this potent drug. He died at the hospital at approximately 12 h after ingestion, with preceding signs of serotonin toxicity. Medico-legal autopsy was performed on the deceased, during which time peripheral whole blood, urine, vitreous humor, liver and gastric content samples were submitted for toxicological examination. Further, whole blood collected at the hospital at 2–4 h following ingestion of the drug was analyzed. 25C-NBOMe and a demethylated and glucuronidated metabolite of 25C-NBOMe were identified in the urine and blood samples using ultra-performance liquid chromatography with high-resolution time-of-flight mass spectrometry (UPLC–HRTOF–MS). Subsequently, 25C-NBOMe was quantified in the peripheral whole blood (0.60 μg/kg), urine (2.93 μg/kg), vitreous humor (0.33 μg/kg), liver (0.82 μg/kg) and gastric content (0.32 μg total) samples collected during autopsy and in the ante-mortem whole blood (0.81 μg/kg) by ultra-performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS). The autopsy findings were consistent with acute poisoning.

Based on the toxicological findings, the cause of death was determined to be a fatal overdose of 25C-NBOMe in combination with amphetamine intake. To our knowledge, the present paper reports the first quantification of 25C-NBOMe in biological specimens from a fatal intoxication case.

1. Introduction

The consumption of synthetic drugs has increased over the last decade and has become a serious public health problem in many European countries [1]. These drugs are often synthesized illegally in clandestine laboratories, where new variants may be produced by modifying the molecular structure of a known stimulant or hallucinogenic compound [2]. Recently, a new class of hallucinogenic drugs termed NBOMe has appeared on the drug market and has gained prominence [3,4]. Since their introduction, these compounds have been associated with intoxication and fatal incidents [3–10]. They have no recognized therapeutic use, and the adverse health effects reported include tachycardia, hypertension, agitation, aggression, visual and auditory hallucinations, seizures, hyperpyrexia, clonus, elevated white cell count, elevated creatine kinase, metabolic acidosis, rhabdomyolysis, and acute kidney injury [11].

NBOMe drugs are analogs of the 2C series of psychedelic phenethylamine drugs that contain an *N*-*o*-methoxybenzyl ("NBOMe") substituent, which significantly affects their pharmacological activities [12–14]. The three most frequently reported drugs in this series are 25I-NBOMe (2-(4-iodo-2,5-dimethoxy-phenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine, 25B-NBOMe (2-(4-bromo-2,5-dimethoxy-phenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine and 25C-NBOMe (Fig. 1). However, several other drugs in this class have been reported in the scientific literature, especially by neurobiological research studies, as well as on internet web pages, including 2CBCB-NBOMe, 2CBFly-NBOMe, 25TFM-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe and 25T7-NBOMe [12–18].

The synthesis of 25C-NBOMe was first reported in the scientific literature in 2011 by Ettrup et al. [14]. It is derived from the

* Corresponding author. Tel.: +45 8716 8335; fax: +45 8716 5003.
E-mail address: mfa@forens.au.dk (M.F. Andreasen).

http://dx.doi.org/10.1016/j.forsciint.2015.03.012
0379-0738/

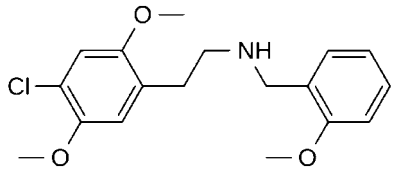

Fig. 1. Structure of 25C-NBOMe.

psychedelic drug 2C-C (4-chloro-2,5-dimethoxy-phenylethylamine) by substituting a hydrogen at the amine nitrogen with a 2-methoxybenzyl (BOMe) group. Alternative abbreviations used for 25C-NBOMe include 2C-C-NBOMe, NBOMe-2C-C and Cimbi-82 [19]. 25C-NBOMe acts as a potent partial agonist of 5-HT_{2A} (serotonin) receptors [15]. Its hallucinogenic activity has been reported by users on Internet forums, and it is considered to be a potent hallucinogen. Users report dosages of approximately 300 to 1000 μg, depending on the route of administration [20].

Prior to 2010, essentially no history of the human use of drugs of the NBOMe class had been reported on the internet [20]. Since then, reports of intoxication and deaths associated with the intake of these drugs have increased in the so-called grey literature, which include newspapers, personal drug experience websites and internet forums. 25I-NBOMe seems to be the most widely abused drug of the NBOMe derivatives, and signs and symptoms of 25I-NBOMe intoxication have been described in several scientific papers [6,8,9,21–25]. However, there are few scientific reports of intoxication with the other NBOMe-derivatives. One paper has reported a case of severe intoxication due to the ingestion of 25B-NBOMe [10], and 4 papers have reported intoxication by 25C-NBOMe [7,26–29], which resulted in fatality in one case [27]. No precise quantitation of 25C-NBOMe has been performed in any previous studies.

Recently, drugs of the NBOMe class have entered the Scandinavian drug scene, and in the present paper, we describe the first death related to the intake of 25C-NBOMe in Denmark. Since October 2013, this drug has been controlled in Denmark. To the best of our knowledge, the present paper reports the first quantification of 25C-NBOMe in biological specimens obtained from a fatal intoxication case.

2. Case history

In the present case, a 22-year-old healthy male, with a short history of alcohol and recreational drug abuse died at a hospital after the nasal ingestion ("sniffing") of 25C-NBOMe. The deceased and a friend purchased 25C-NBOMe on the internet and the drug was delivered in small transparent capsules containing small flakes. The friend reported that he and the deceased had been sniffing 25C-NBOMe on the preceding evening (between 8:00 and 10:00 pm). At approximately 9:30 pm, the deceased was talking incoherently, and at 10:15 pm, he was running around in his apartment pulling down curtains. At 11:00 pm, an ambulance was called because the deceased was hallucinating and had jerky movements and a clenched jaw. When the ambulance arrived 3 min later, he was unconscious (he scored 3 on the Glasgow Coma Scale (GCS)). At initial evaluation, the respiratory rate was 23 breaths per minute, blood pressure was 104/83, pulse was 100 bpm, and blood glucose was 13.3 mmol/L, and he exhibited mydriasis. He had convulsions that were treated with the intravenous administration of 10 mg diazepam with no effect. He was intubated at the scene and treated with 5 mg midazolam, 100 mg suxamethonium and 100 mg ketamine. The initial oxygen saturation level was 80%, but it was increased to 100% by mechanical ventilation. An episode of ventricular tachycardia and atrial fibrillation was observed, and he was treated with 300 mg amiodarone. The transit time to the emergency department was approximately 30 min. The initial investigation at the emergency department revealed the following symptoms: hyperthermia with a core temperature of 40 °C, pulse of 140 bpm, diffuse bleeding from all mucosa, respiratory and metabolic acidosis, rhabdomyolysis, high lactic acid, anuria and hyperkalemia (Table 1). His blood pressure was low at 70/45 mmHg, despite infusion of noradrenalin and phenylephrine. He was further treated with infusion of saline, human albumin, and sodium bicarbonate and blood transfusion. An additional dose of 15 mg diazepam was administrated, and the hyperkalemia was also treated. ECG showed inferior ST depression changes and increases in coronary biomarkers (Table 1). His head was actively cooled. He was transferred to the intensive care unit and held in an artificially induced coma and was additionally treated with fentanyl. His

Table 1
Biochemistry results obtained from the deceased ante-mortem by the local Department of Clinical Biochemistry.

Analysis	June 22nd 2014 (00.20)	June 22nd 2014 (01.25)	June 22nd 2014 (06.00)	Unit	Reference
B—Leukocytes	#1	#1	#1	$\times 10^9$/L	3.50–10.0
B—Hemoglobin	10.1	8.1	3.9	mmol/L	8.3–10.5
B—Platelets	#1	#1	#1	$\times 10^9$/L	145–350
P—Coagulation factor II + VII + X (INR)	#2	1.2	1.4		<1.2
P—(APTT)	#2	>150	#3	s	25–38
P—Antithrombin	#4	#4	0.51	$\times 10^3$ IE/L	0.80–1.20
P—Fibrinogen	#4	#4	<0.9	μmol/L	5.5–12.0
P—Fibrin D-Dimer	#4	#4	>20.0	mg/IFEU	<0.50
P(aB)—pH	6.69	7.21	7.31		7.37–7.45
P(aB)—pCO_2	10.4	6.9	5.2	kPa	4.7–6.0
P(aB)—pO_2	40.6	78.5	44.3	kPa	11.1–14.4
P(aB)—Potassium	8.6	7.3	7.1	mmol/L	3.5–4.6
P(aB)—Chloride	99	100	108	mmol/L	98–106
P(aB)—Calcium-ion (free)	1.39	0.93	0.93	mmol/L	1.18–1.32
B(aB)—Hemoglobin	9.9	7.7	3.6	mmol/L	8.3–10.5
P(aB)—Glucose	15.5	15.7	2.5	mmol/L	4.2–7.8
P(aB)—Lactate	28.0	16.0	9.5	mmol/L	0.5–2.5
P—Troponin I	451	1119	3513	ng/L	<24
P—Creatine kinase MB (CK MB)	#1	204.8	433.5	μg/L	<7.0
P—Creatine kinase	#4	#4	>42670	U/L	50–270
P—Myoglobin	#1	>12000	>12000	μg/L	<75
P–Alanine transaminase (ALAT)	46	108	660	U/L	10–70
P—Glucose	15.1	#4	3.1	mmol/L	4.2–7.8
P—Creatinine	188	231	217	μmol/L	60–105

#1: Hemolysis, #2: annulled, #3: technical problems, #4: not performed, B: blood, P: plasma, P(aB): arterial blood plasma.

condition continued to deteriorate with disseminated vascular coagulation (DIC) (Table 1). Despite lifesaving treatment, he died at approximately 10:00 am the following morning of multi-organ failure and a clinical picture consistent with serotonin syndrome. The friend had none of these symptoms and reported having "a good trip" for 4–6 h after sniffing the 25C-NBOMe.

3. Autopsy findings

A medico-legal autopsy was performed two days post-mortem at the Department of Forensic Medicine, Aarhus. The deceased was a 22-year-old male with normal nutritional status and physique, and only a few minor external injuries in terms of superficial bruises and abrasions. The internal examination revealed organ congestion, with free fluid in both thorax and abdomen, and diffuse mucosal hemorrhage, especially in the lower parts of the colon (Fig. 2). Post-mortem specimens submitted for toxicology included peripheral blood, urine, vitreous humor, stomach contents and liver tissue. Further histopathology revealed degeneration of the liver.

4. Initial toxicological screening

Screening for illegal and legal drugs, including ethanol, was performed using whole blood, liver and urine samples obtained from the deceased by analytical methods routinely applied in the laboratory. The laboratory is accredited by an external, independent organization, DANAK (Danish Accreditation), and participates in various screening and quantification proficiency tests. Most of the methods used were validated and accredited in accordance with the requirements of the DS/EN ISO/IEC 17025 standard. Blood, liver, vitreous humor and urine samples were screened for basic, acidic and neutral compounds using a protein precipitation method with acetonitrile and subsequent UPLC–HRTOFMS analysis as described in Section 5.4. Subsequently, the identified drugs were quantified using various validated LC–MS/MS methods [30,31]. Quantification of 25C-NBOMe is described in Section 5.3. The blood and urine samples were analyzed for the presence of ethanol using an accredited and validated head-space gas chromatography with flame ionization detection (HS–GC–FID) method.

5. Materials and methods—25C-NBOMe analysis

5.1. Chemicals and reagents

The primary reference materials 25C-NBOMe, 25H-NBOMe and 25C-NBOMe-D_6 were purchased from Cayman Chemicals and Toronto Research Chemicals Inc. as hydrochloric salts. β-glucuronidase from *Escherichia coli* K12 (EC 3.2.1.31) was supplied from Roche (140 U/mg). All other chemicals and reagents used were of the highest commercially available quality. 25I-NBOMe and 25B-NBOMe were obtained from a police seizure (identified using GC–MS and UPLC–HR–TOFMS at our department).

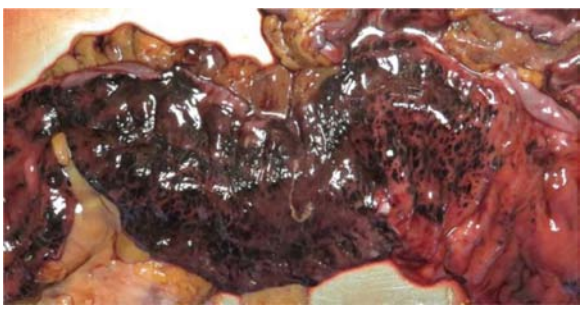

Fig. 2. Mucosal hemorrhage in colon sigmoideum.

5.2. Biological materials

Ante-mortem whole blood was collected by the local Department of Clinical Biochemistry at hospitalization and before blood transfusion was performed. The samples were collected in light blue BD Vacutainer® tubes containing sodium citrate and refrigerated for 4 days. Then the samples were send by mail (room temperature) and received one day later at the Institute of Medicine where they were stores at −18 °C until use. Post-mortem specimens were collected during autopsy 2 days after death (within 24 h after the death, the deceased was stored at approximately 4 °C). Samples of peripheral whole blood (femoral vein blood), urine, vitreous humor, liver and gastric contents were collected, mixed with NaF (5 mg/g) and stored at −18 °C until use. Whole blood and urine samples from healthy volunteers free of any xenobiotics were used as blank samples and for the preparation of calibrators and quality control (QC) samples. All samples were stored at −18 °C and were thawed just before analysis. The different analyses were performed within 1 to 21 days post-autopsy.

5.3. Quantitative analytical procedures

5.3.1. Extraction

Whole blood samples (0.50 g) and the internal standard (25C-NBOMe-d_6, 0.5 ng) were mixed with 250 μL carbonate buffer (1 M Na_2CO_3 adjusted to pH 11 with HCl) and extracted twice using 2 mL hexane/butyl chloride (50:50). The organic extracts were combined with 50 μL 0.1 M HCl in methanol and evaporated to dryness under a stream of nitrogen at 30 °C. The residue was dissolved in 150 μL mobile phase (9:1 solvent A:solvent B, Section 5.3.4), and a 10 μL sample was automatically injected onto the LC–MS/MS-system. Duplicate determination in two analytical series was performed, and calibrators containing 25C-NBOMe were prepared from drug free blood at the following concentrations: 0, 0.1, 0.5, 1.0, 5.0 and 10 μg/kg.

The autopsy urine samples (0.50 g) and 150 μL 0.5 M BIS-TRIS propane buffer (pH 6.8) was added to screw-capped tubes. β-Glucuronidase (20 μL of a stock of 140 U/mg at 37 °C) was added to each sample. The tubes were capped and incubated for approximately 16 h in a water bath (with orbital shaking) at 37 °C [32]. The hydrolyzed and non-hydrolyzed urine samples were extracted as described above for autopsy blood samples. Calibrators containing 25C-NBOMe were prepared from drug-free urine at concentrations of 0, 0.1, 0.5, 1.0, 5.0 and 10 μg/kg, and treated as above (16 h and 37 °C). The vitreous humor (0.5 g) was handled in the same manner as the autopsy blood samples. Calibrators were prepared in MilliQ-water at the following concentrations: 0, 0.1, 0.5, 1.0, 5.0 and 10 μg/kg. A liver homogenate was prepared by homogenizing the liver tissue in MilliQ water (1:2). One gram of liver homogenate was analyzed by method of standard additions [33] and extracted and analyzed as described for blood samples. The gastric contents (0.5 g) were handled in the same manner as the blood samples using the calibrators prepared in blood. Standard addition was not used to measure the concentration of 25C-NBOMe in blood and vitreous humor samples as we did not have enough material, further it was not used on gastric content and urine samples as this method is more time consuming.

5.3.2. Calibrators and quality control samples

Stock calibrator solutions (100 μg/mL) of 25C-NBOMe and 25C-NBOMe-d_6 were prepared in methanol. Working solutions at concentrations from 0.01 μg/mL to 1 μg/mL were prepared by

dilution of the stock calibrators with methanol. The internal standard (IS) working solution was prepared in a concentration of 0.1 μg/mL. Calibrators containing 25C-NBOMe in concentrations from 0.1 to 10 μg/kg were prepared daily for each analytical batch by adding suitable amounts of the methanol working solutions to 0.5 g of a pooled sample of whole blood, previously confirmed to be drug-free. The samples were extracted according to the procedure described previously (5.3.1.). The calibration curves were constructed by plotting the peak-area ratios 25C-NBOMe/IS (25C-NBOMe-d_6). A weighted (1/concentration) least-squares regression analysis was used to determine the slopes and intercepts. Quality control (QC) samples of 1 μg/kg were prepared in drug-free whole blood, aliquoted and stored at −18 °C. A QC sample was included in each analytical batch to check for calibration, trueness and precision.

5.3.3. Validation procedures

A validation was performed due to the necessity of insuring the reliability of the analytical results, by assaying the selectivity, recovery, matrix effect, linearity, precision and trueness, limit of detection (LOD) and lower limit of quantification (LLOQ) according to accreditation criteria [34]. Selectivity was tested using twenty different drug-free autopsy blood samples. These were extracted and analyzed for interfering substances. The extraction recovery (ER) was calculated by comparing the peak areas of blood standards to the peak areas of post-extracted spiked samples at 1 μg/kg (n = 20). The matrix effect (ME) was investigated in twenty post-mortem blood samples with negative drug screening results. The twenty blood samples were extracted according to the requirements of the assay and spiked after extraction with 25C-NBOMe at 1 μg/kg. The ME was calculated by comparing the response of these samples with the response of standard solutions at 1 μg/kg dissolved in mobile phase.

The calibration curves were tested over the concentration range from 0.1 to 10 μg/kg. The standard deviation (S.D.) of 10 individual blank whole blood samples was used to determine the limit of detection (LOD = 3 S.D.) and lower limit of quantification (LLOQ = 10 S.D.). QC samples (1 μg/kg) analyzed in duplicate in four independent experimental assays were used for determination of precision and accuracy. The precision (repeatability and factor-different intermediate precision) was calculated using one-way analysis of variance (ANOVA) with day as the grouping factor and expressed as the relative S.D. (R.S.D) of the concentration calculated for QC sample. The trueness (bias) was expressed as a percent deviation from the spiked concentrations.

5.3.4. Instrumentation—LC–MS/MS

Quantification of 25C-NBOMe was performed using reversed phase UPLC–MS/MS analysis. A Xevo TQ MS API mass-spectrometer (Waters Corporation, Milford, MA, USA) fitted with a Z-spray ion source was used for the analysis. Ionization was achieved using electrospray in the positive mode (ES+). LC separation was achieved using an ACQUITY UPLC system from Waters equipped with an analytical column from Waters (ACQUITY UPLC BEH C18 1.7 μm, 50 × 2.1 mm). Before the first analysis, the column was conditioned 3 min with 20% solvent B. The chromatography was performed at 45 °C at a flow rate of 0.6 mL/min using a gradient solvent system consisting of solvent A (5 mM aqueous ammonium acetate with 0.1% formic acid) and solvent B (100% methanol), with the following gradient: 0–0.5 min linear from 20 to 30% B; 0.5–2.0 min linear from 30 to 100% B; 2.0–2.7 min isocratic at 100% B; 2.7-3.0 min linear from 100 to 20% B; 3.0–5.0 min isocratic at 20% B. The retention times were: 25C-NBOMe, 1.46 min; 25C-NBOMe-d_6, 1.46 min; 25H-NBOMe, 1.34 min; 25I-NBOMe, 1.53 min; 25B-NBOMe, 1.48 min. Quantification of 25C-NBOMe was performed using the multiple reaction monitoring (MRM) acquisition mode. The following conditions were found to be optimal for the analysis of 25C-NBOMe and the IS: Capillary voltage, 3.8 kV; Source block temperature, 150 °C; desolvation gas (nitrogen) heated to 650 °C and delivered at a flow rate of 1000 L/h; collision (argon) gas flow, 0.15 mL/min; cone gas flow, 50 L/h. The cone voltages for all were 40 V. The following transition ions (m/z) were monitored in MRM mode with their corresponding collision energies (eV) in parentheses: 25C-NBOMe: $336^a > 121^a$ (20) and $336 > 91^b$; 25C-NBOMe-d_6: $342^a > 121^a$ (20); 25H-NBOMe: $302^a > 121^a$ (20) and $302 > 91^b$; 25I-NBOMe: $428^a > 121^a$ (20) and $428 > 91^b$; 25B-NBOMe: $380^a > 121^a$ (20) and $380 > 91^b$ (^{a}ion used as quantifier, ^{b}ion used as qualifier).

5.4. Qualitative analytical procedures—UPLC–TOFMS screening analysis

Chromatography was performed using an ACQUITY I-Class UPLC system (Waters Corporation, Milford, MA, USA). Mass spectrometry was conducted with a Bruker maXis Impact QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples (300 μL of blood, urine, vitreous humor and liver homogenate) acidified with 150 μL of 0.1 M hydrochloric acid were prepared by protein precipitation in 96-deepwell plates. Two hundred microliters methanol containing four deuterated internal standards (amphetamine-d_5, cocaine-d_3, phenobarbital-d_5 and diazepam-d_5) were added followed by the addition of 600 μL of acetonitrile. The mixture was centrifuged, and 600 μL of clear supernatant was transferred to a 30 kDa AcroPrep filter plate. The filtrate was evaporated to dryness and reconstituted in 15% methanol in water with 0.1% formic acid. Separation was performed in 13.5 min by gradient elution using an ACQUITY BEH C18 (100 mm × 2.1 mm, 1.7 μm) column with mobile phases A and B consisting of 0.1% formic acid and acetonitrile, respectively. The gradient was as follows: 0% B at 0 min, a linear increase to 20% B at 4.0 min, a linear increase to 80% B at 8.0 min, holding at 80% B to 8.5 min, a linear increase to 100% B at 9.0 min, holding at 100% B to 10.0 min, an immediately decrease to 0% B, and holding at 0% B to 13.5 min. The flow rate was 0.6 mL/min, and 10 μL of sample was injected. The HR-TOFMS instrument equipped with an electrospray ionization source was operated in positive mode using an m/z calibration range of 50–1000. The acquisition rate was 10 Hz, and MSMS analysis was carried out using broad-band collision-induced dissociation (bbCID) with undirected fragmentation.

6. Results and discussion

The standard toxicological investigation of the post-mortem samples resulted in the detection of several drugs, which are listed in Table 2.

The toxicological significance of the concentrations of 25C-NBOMe found in the deceased is difficult to determine because this is the first report describing whole blood concentrations of 25C-NBOMe. However, Poklis et al. [9] have reported similar levels of 25I-NBOMe in various biological specimens obtained from a 19-year-old male who died after ingestion of 25I-NBOMe (peripheral blood, 405 pg/mL; heart blood, 410 pg/mL; urine 2.86 ng/mL, vitreous humor, 99 pg/mL; bile 10.9 ng/g; brain, 2.54 ng/g; liver, 7.2 ng/g; and gastric contents, 7.2 μg total). The subject reported by Poklis et al. [9] had no prior history of alcohol or drug abuse, and no alcohol or drugs with the exception of 25I-NBOMe were detected in his body. Similar levels of 25B-NBOMe (serum, 180 pg/mL; and urine, 1900 pg/mL) have also been found in a 19-year old male with no history of drug use who had severe intoxication after ingestion of 25B-NBOMe [10]. In our fatal intoxication case by 25C-NBOMe, the police who conducted the investigation and the subject's physician reported that the deceased had a short history

Table 2
Drug quantified in biological specimens from the deceased.

Drug	Ante-mortem blood[b] (μg/kg)	Post-mortem blood (μg/kg)	Urine (μg/kg)	Vitreous humor (μg/kg)	Liver (μg/kg)	Gastric content (μg total)
25C-NBOMe	0.81	0.60	2.93	0.33	0.82[c]	0.32[d]
Amphetamine	370	470	n.a.	n.a.	n.a.	n.a.
Tetrahydrocannabinol (THC)	n.a.	1.5	n.a.	n.a.	n.a.	n.a.
11-nor-9-Carboxy-THC	n.a.	8.9	n.a.	n.a.	n.a.	n.a.
Amiodarone[a]	n.a.	120	n.a.	n.a.	n.a.	n.a.
Acetaminophene[a]	n.a.	5000	n.a.	n.a.	n.a.	n.a.
Diazepam[a]	n.a.	99	n.a.	n.a.	n.a.	n.a.
Demethyldiazepam[a]	n.a.	12	n.a.	n.a.	n.a.	n.a.
Fentanyl[a]	n.a.	0.47	n.a.	n.a.	n.a.	n.a.
Ketamine[a]	n.a.	120	n.a.	n.a.	n.a.	n.a.
Lidocaine[a]	n.a.	78	n.a.	n.a.	n.a.	n.a.
Midazolam[a]	n.a.	<6	n.a.	n.a.	n.a.	n.a.
Quetiapine	n.a.	13	n.a.	n.a.	n.a.	n.a.

[a] Drugs given at the hospital.
[b] Blood taken 2–4 h post nasal ingestion.
[c] Standard addition.
[d] 17 g gastric content, n.a.: not analyzed.

of alcohol and recreational drug use. This report was confirmed by the detection of THC (1.5 μg/kg) at a recreational level and amphetamine (470 μg/kg) at a recreational level for an addict or a high level for a non-addict [35]. Because the deceased was a recreational user of amphetamine, we presume that the level of amphetamine of 470 μg/kg would not have been lethal (the level in the ante-mortem whole blood sample was 370 μg/kg). However, because amphetamine also increases the central release of serotonin, it might have enhanced the effects of 25C-NBOMe. Because the level of 25C-NBOMe was the same as those that have been reported in the cases of intoxication with 25I-NBOMe and 25B-NBOMe, we presume that the patient died of severe intoxication by 25C-NBOMe in combination with a high concentration of amphetamine, which is also supported by the clinical symptoms and autopsy findings. Serotonin syndrome due to intoxication with 25C-NBOMe is primarily the result of the excessive stimulation of serotonin receptor 5-HT_{2A}. The classic clinical triad of serotonin toxicity is altered mental status, neurologic symptoms (e.g., coma, rigidity, and clonus) and autonomic manifestation (e.g., hypertension, tachycardia, and hyperthermia) [36]. Intoxication is evident as a continuum of clinical symptoms. Akathisia, tremor, altered mental status, clonus, muscular hypertonicity, and hyperthermia are observed in mild to moderate intoxication. Severe intoxication may further manifest as shock, marked hypertension or hypotension, tachycardia, agitated delirium, rigidity, acidosis, rhabdomyolysis, seizures, renal failure, and DIC.

25C-NBOMe was initially identified in post-mortem whole blood and urine using UPLC–HR-TOFMS. The extracted ion chromatogram (EIC) of 25C-NBOMe obtained from the post-mortem blood and urine samples is shown in Fig. 3. Identification was based on the accurate mass and retention time of the compound compared with a reference standard. However, the intensity of the peak was very low, and the identification was only considered reliable after the identification of a peak that presumably represented an *O*-demethylated glucuronidated metabolite ($C_{23}H_{28}ClNO_9$) of 25C-NBOMe (Fig. 4). This peak had a much higher intensity than that of 25C-NBOMe. The area of the peak of this metabolite in urine was approximately 40 times larger than the area in the blood. 25C-NBOMe has 3 methoxy groups (Fig. 1). Thus, there could theoretically have been more glucuronidated metabolites present; however, we only found one. The exact structure of the identified metabolite could not be established with the available methodology. Proposed structures are shown in Fig. 5.

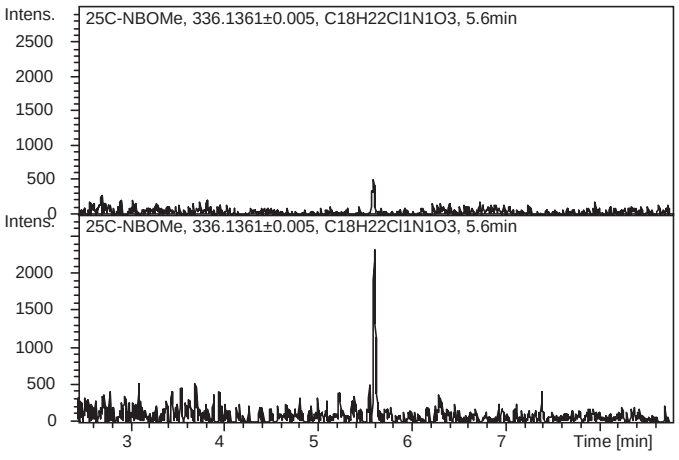

Fig. 3. Extracted ion chromatograms of 25C-NBOMe ($C_{18}H_{22}ClNO_3$) from protein precipitated blood and urine samples acquired by LC–TOFMS. Upper chromatogram: post-mortem blood (measured mass: 336.1384; calculated mass [M + H]: 336.1361; ion formula: no result; mSigma: no result). Lower chromatogram: urine sample (measured mass: 336.1376; calculated mass [M + H]: 336.1361; ion formula: $C_{18}H_{23}ClNO_3$; mSigma: 164.7).

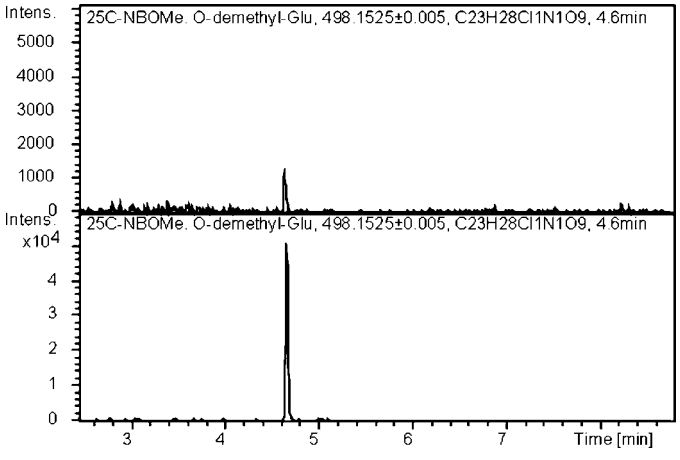

Fig. 4. Extracted ion chromatograms of a 25C-NBOMe-metabolite ($C_{23}H_{28}ClNO_9$, "demethylated-glucuronidated-25C-NBOMe") from protein precipitated post-mortem blood and urine samples acquired by LC–TOFMS. Upper chromatogram: post-mortem blood (measured mass: 498.1536; calculated mass [M + H]: 498.1525; ion formula: $C_{23}H_{29}ClNO_9$; mSigma: 60.9). Lower chromatogram: urine sample (measured mass: 498.1528; calculated mass [M + H]: 498.1525; ion formula: $C_{23}H_{29}ClNO_9$; mSigma: 39.5). The area of the peak in urine is approximately 40 times larger than in the blood.

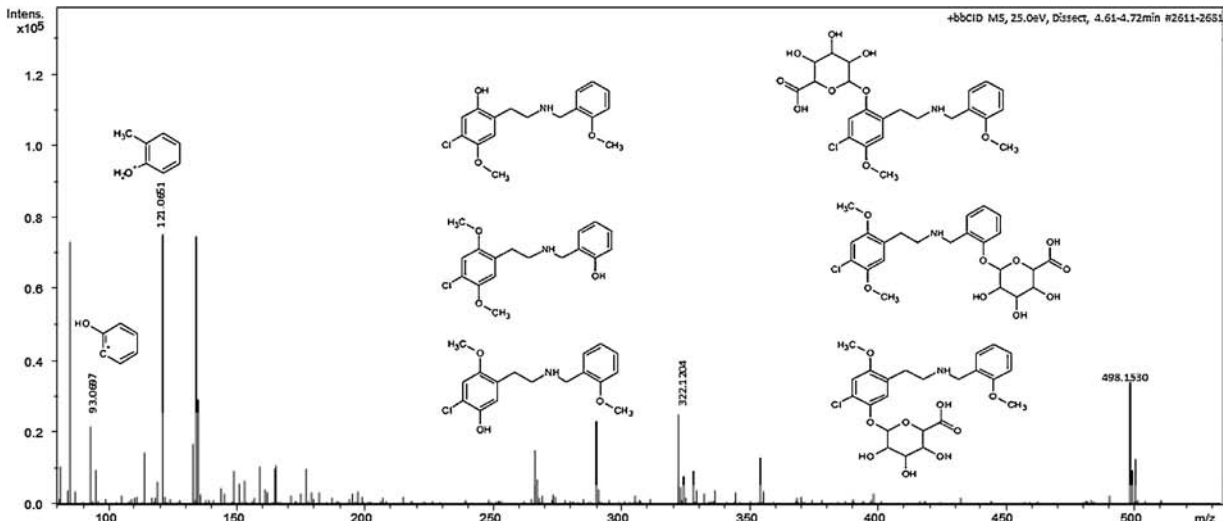

Fig. 5. Proposed structures of 25C-NBOMe-metabolites ($C_{23}H_{28}ClNO_9$, "demethylated-glucuronidated-25C-NBOMe"). Fragmentation at CE 25 eV using bbCID in a HR-QTOF system.

25C-NBOMe was subsequently identified and quantified by UPLC–MS/MS using a more specific extraction method (hexane/butyl chloride extraction, Section 5.3.1) in the following matrices from the deceased: ante-mortem blood and post-mortem blood, urine, hydrolyzed urine, vitreous humor, gastric contents and liver. The identification was based on retention time, two daughter ions and ion ratios monitored between the daughter ions. Fig. 6 shows a MRM chromatogram of 25C-NBOMe generated from UPLC–MS/MS analysis of post-mortem whole blood from the deceased.

The concentrations of 25C-NBOMe in the different biological matrices are shown in Table 2. The concentration of 25C-NBOMe in the post-mortem peripheral blood was 0.60 ± 0.01 μg/kg (duplicate determination in two analytical series), and it was 0.81 ± 0.04 μg/kg in the ante-mortem whole blood. This discrepancy may be explained by the fact that the deceased had received a blood-transfusion of at least one unit of red blood cells, one unit of platelets, and one unit of fresh frozen plasma and/or by the post-mortem redistribution of the drug.

In the untreated urine, the concentration of 25C-NBOMe was 2.93 ± 0.05 μg/kg. In the β-glucuronidase-treated urine, the measured concentration was approximately three times higher. The reason for this finding is unknown because 25C-NBOMe most likely undergoes demethylation before glucuronidation. Furthermore, we detected a large peak with the same MRM transition as 25C-NBOMe in the hydrolyzed urine with a retention time of 1.26 min (Fig. 7). Similarly, Tang et al. [28] have found an unexplained peak in a β-glucuronidase-treated urine sample. Further studies are needed to explain this peak.

In the β-glucuronidase-treated urine sample we detected a large peak that presumably represented an *O*-demethylated-25C-NBOMe ($C_{17}H_{20}ClNO_3$). The difference in the concentration of the compound between the β-glucuronidase-treated urine sample and the untreated urine sample is shown in Fig. 8. The area of the peak for the hydrolyzed urine sample was approximately 20 times larger than that for the untreated urine sample.

In the vitreous humor, the concentration of 25C-NBOMe was 0.33 ± 0.01 μg/kg. However, it should be noted that the calibration standards were prepared in Milli-Q water. The concentration of 25C-NBOMe in the liver (analyzed by standard addition to liver homogenate) was 0.82 ± 0.31 μg/kg (duplicate determination). In the gastric contents, the concentration was 18.8 ± 1.0 μg/kg (quantified against blood calibrators). The total mass of the gastric contents was 17 g; therefore, the gastric sample contained 0.32 μg 25C-NBOMe. The gastric contents appeared bloody, and because the

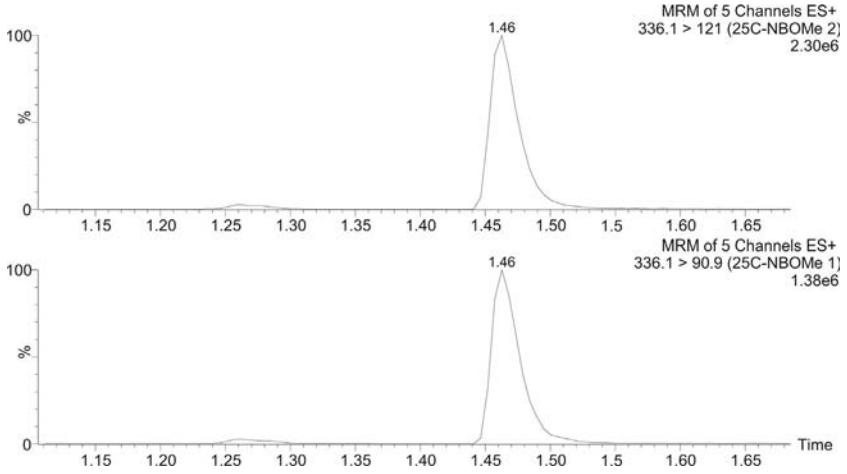

Fig. 6. MRM extracted ion chromatograms of 25C-NBOMe (0.60 μg/kg) obtained from a hexane/butyl chloride extraction post-mortem blood sample by LC–MS/MS (MRM 1: *m*/*z* 336.1/121, MRM 2: *m*/*z* 336.1/90.9).

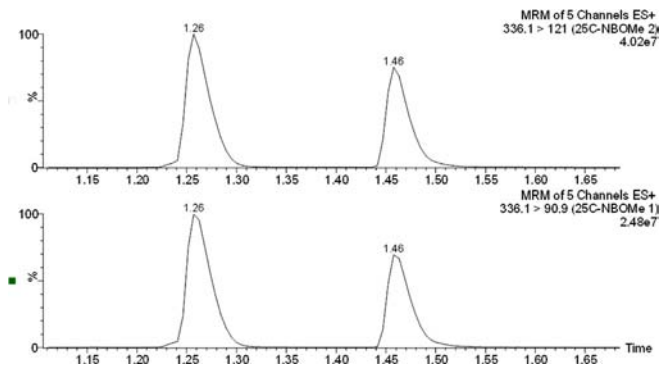

Fig. 7. MRM extracted ion chromatograms of 25C-NBOMe (Retention time, 1.46 min) obtained from a hexane/butyl chloride enzyme treated urine by LC–MS/MS (MRM 1: *m/z* 336.1/121, MRM 2: *m/z* 336.1/90.9). An unexplained peak appears in the chromatogram at retention time 1.26 min.

deceased was bleeding from all cavities, we assume that a proportion of the gastric contents were blood. However, the concentration of 25C-NBOMe in the gastric contents was approximately 30 times higher than that in the blood; thus, we propose they contained some additional 25C-NBOMe besides that in the blood. According to the friend of the deceased, they had ingested the drug by nasal administration; therefore, it is likely that some saliva from the nose entered into the gastric compartment, leading to the higher level in the gastric contents.

The method used in this study was found to be selective for 25C-NBOMe in post-mortem blood. No interfering peaks were observed in extracts of 20 different drug-free post-mortem blood samples. Interference by other compounds was minimized and was evaluated by the LC–MS/MS by monitoring the retention times, two daughter ions and ion ratios between the daughter ions. The extraction recovery varied from 45 to 90%, with a mean of 71% and a median of 74%. There is a high variation in the extraction recoveries, so the most accurate methodology of quantification 25C-NBOMe in blood would have been using standard addition. However, we did not have enough material for this type of analysis. The quantitative assessment of the matrix effect in the blood showed an ion enhancement of less than 12%, with a mean of 3% and median of 2% enhancement. The LOD and LLOQ were

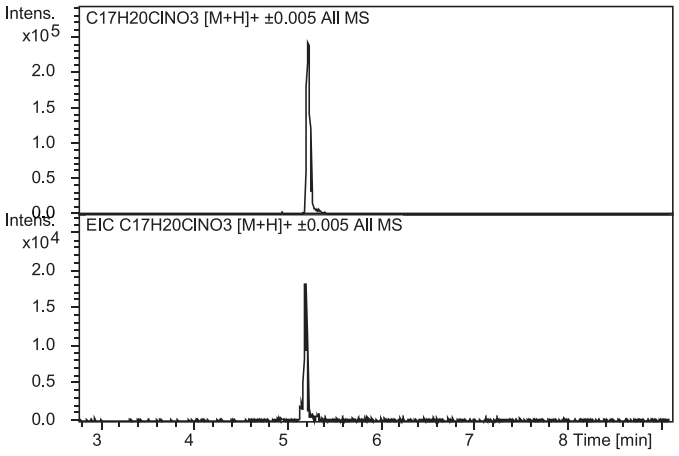

Fig. 8. Extracted ion chromatograms of a 25C-NBOMe-metabolit ($C_{17}H_{20}ClNO_3$, "demethylated-25C-NBOMe") from a hexane/butyl chloride extracted urine (500 μL) acquired by LC–TOFMS. Upper chromatogram: β-glucuronidase treated urine (measured mass: 322.1212; calculated mass [M + H]: 322.1204; ion formula: $C_{17}H_{21}ClNO_3$; mSigma: 40.4). Lower chromatogram: untreated urine (measured mass: 322.1203; calculated mass [M + H]: 322.1204; ion formula: $C_{17}H_{21}ClNO_3$; mSigma: 18.1).

0.02 μg/kg and 0.08 μg/kg, respectively. The precision was 5% and accuracy (bias) ranged from −6 to 1% for the blood quality control samples (1 μg/kg). The calibration curves were linear over the tested interval, which ranged from 0.1 to 10 μg/kg ($r^2 > 0.999$: $y = 0.77 \times -0.0017$). The stability of 25C-NBOMe was tested by Soh et al. [26]. They found that 25C-NBOMe was stable at room temperature (20–23 °C) for at least 21 days in equine blood and plasma.

The number of NBOMe related intoxications and fatal cases may well be underestimated due to the lack of proper routine analytical methods. The high potencies of 25C-NBOMe and other similar drugs challenge even highly sensitive methods, including high-resolution time-of-flight techniques, because the signals from these drugs are very low and are easily lost in the background noise of the sample. In the present case, the detection of 25C-NBOMe was made possible by information provided by the police. In the future, the detection of drugs of the NBOMe class may be improved by additionally evaluating the metabolites of these drugs, particularly the demethylated glucuronidated metabolites, by the HR-TOFMS screening method. However, new substituted phenethylamines continually appear; therefore, we and all other forensic chemists and toxicologists must remain alert.

7. Conclusion

25C-NBOMe was identified and quantified in ante-mortem and post-mortem whole blood, urine, liver, gastric content and vitreous humor samples. The clinical course and autopsy findings were consistent with serotonin toxicity. Based on these findings and the toxicological findings the cause of death was determined to be a fatal overdose of 25C-NBMe in combination with a high concentration of amphetamine. To our knowledge, this is the first report of the quantification of 25C-NBOMe in post-mortem biological specimens.

References

[1] EMCDDA, European Drug Report, Trends and Developments, EMCDDA, 2014.
[2] S.D. Brandt, L.A. King, M. Evans-Brown., The new drug phenomenon, Drug Test Anal. 6 (2014) 587–597.
[3] A. Ninnemann, G.L. Stuart, The NBOMe series: a novel, dangerous group of hallucinogenic drugs, J. Stud. Alcohol Drugs 74 (2013) 977–978.
[4] W. Lawn, M. Barratt, M. Williams, A. Horne, A. Winstock, The NBOMe hallucinogenic drug series: patterns of use, characteristics of users and self-reported effects in a large international sample, J. Psychopharmacol. 28 (2014) 780–788.
[5] D.G.E. Caldicott, S.J. Bright, M.J. Barratt, NBOMe—a very different kettle of fish, Med. J. Aust. 199 (2013) 322–323.
[6] S.L. Hill, T. Doris, S. Gurung, S. Katebe, A. Lomas, M. Dunn, P. Blain, S.H.L. Thomas, Severe clinical toxicity associated with analytically confirmed recreational use of 25I-NBOMe: case series, Clin. Toxicol. 51 (2013) 487–492.
[7] S. Grautoff, J. Kähler, Near fatal intoxication with the novel psychoactive substance 25C-NBOMe, Med. Klin-Intensivmed. 109 (2014) 271–275.
[8] P. Nikolaou, I. Papoutsis, M. Stefanidou, C. Spiliopoulou, S. Athanaselis, 2C-I-NBOMe, an N-bomb that kills with Smiles. Toxicological and legislative aspects, Drug Chem. Toxicol. 38 (2015) 113–119.
[9] J.L. Poklis, K.G. Devers, E.F. Arbefeville, J.M. Pearson, E. Houston, A. Poklis, Postmortem detection of 25I-NBOMe [2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine] in fluids and tissues determined by high performance liquid chromatography with tandem mass spectrometry from a traumatic death, Forensic Sci. Int. 234 (2014) e14–e20.
[10] J.L. Poklis, C.R. Nanco, M.M. Troendle, C.E. Wolf, A. Poklis, Determination of 4-bromo-2,5-dimethoxy-*N*-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe) in serum and urine by high performance liquid chromatography with tandem mass spectrometry in a case of severe intoxication, Drug Test Anal. 6 (2014) 764–769.
[11] Drug Enforcement Administration, Department of Justice, US, Schedules of controlled substances: temporary placement of three synthetic phenethylamines into Schedule I, Fed. Regist. 78 (221) (2013) 68716–68719.
[12] M.R. Braden, J.C. Parrish, J.C. Naylor, D.E. Nichols, Molecular interaction of serotonin 5-HT2A receptor residues Phe339(6.51) and Phe340(6.52) with super-potent *N*-benzyl phenethylamine agonists, Mol. Pharmacol. 70 (2006) 1956–1964.
[13] M.R. Braden, Towards a Biophysical Understanding of Hallucinogens Action, Purdue University, West Lafayette, Indiana, 2006 (Ph.D. Thesis).

[14] A. Ettrup, M. Hansen, M.A. Santini, J. Paine, N. Gillings, M. Palner, S. Lehel, M.M. Herth, J. Madsen, J. Kristensen, M. Begtrup, G.M. Knudsen, Radiosynthesis and in vivo evaluation of a series of substituted 11C-phenethylamines as 5-HT (2A) agonist PET tracers, Eur. J. Nucl. Med. Mol. Imaging 38 (2011) 681–693.
[15] M. Hansen, K. Phonekeo, J.S. Paine, S. Leth-Petersen, M. Begtrup, H. Brauner-Osborne, J.L. Kristensen Synthesis, Structure-activity relationships of *N*-benzyl phenethylamines as 5-$HT_{2A/2C}$ agonists, ACS Chem. Neurosci. 5 (2014) 243–249.
[16] D. Zuba, K. Sekula, Analytical characterization of three hallucinogenic *N*-(2-methoxy)benzyl derivatives of the 2C-series of phenethylamine drugs, Drug Test Anal. 5 (2013) 634–645.
[17] J.L. Poklis, D.J. Clay, A. Poklis, High-performance liquid chromatography with tandem mass spectrometry for the determination of nine hallucinogenic 25-NBOMe designer drugs in urine specimens, J Anal Toxicol. 38 (2014) 113–121.
[18] H.P.J.F. Casale, Characterization of eleven 2,5-dimethoxy-*N*-(2-methoxybenzyl)-phenethylamine (NBOMe) derivatives and differentiation from their 3- and 4-methoxybenzyl analogues—Part I, Microgram J. 9 (2012) 84–109.
[19] D. Zuba, K. Sekula, A. Buczek, 25C-NBOMe—new potent hallucinogenic substance identified on the drug market, Forensic Sci. Int. 227 (2013) 7–14.
[20] https://www.erowid.org/chemicals/2cc_nbome/2cc_nbome.shtml
[21] A. Kelly, B. Eisenga, B. Riley, B. Judge, Case series of 25I-NBOMe exposures with laboratory confirmation, Clin. Toxicol. 50 (2012) 702.
[22] S.R. Rose, J.L. Poklis, A. Poklis, A case of 25I-NBOMe (25-I) intoxication: a new potent 5-HT2A agonist designer drug, Clin. Toxicol. (Phila) 51 (2013) 174–177.
[23] S.J. Stellpflug, S.E. Kealey, C.B. Hegarty, G.C. Janis, 2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe): clinical case with unique confirmatory testing, J. Med. Toxicol. 10 (2014) 45–50.
[24] J.P. Walterscheid, G.T. Phillips, A.E. Lopez, M.L. Gonsoulin, H.H. Chen, L.A. Sanchez, Pathological findings in 2 cases of fatal 25I-NBOMe toxicity, Am. J. Forensic Med. Pathol. 35 (2014) 20–25.
[25] J.L. Poklis, J. Charles, C.E. Wolf, A. Poklis, High-performance liquid chromatography tandem mass spectrometry method for the determination of 2CC-NBOMe and 25I-NBOMe in human serum, Biomed. Chromatogr. 27 (2013) 1794–1800.
[26] Y.N. Soh, S. Elliott, An investigation of the stability of emerging new psychoactive substances, Drug Test Anal. 6 (2014) 696–704.
[27] World Health Organisation, 25C-NBOMe—Critical Review Report, 36th ECDD, World Health Organisation, 2014.
[28] M.H.Y. Tang, C.K. Ching, M.S.H. Tsui, F.K.C. Chu, T.W.L. Mak, Two cases of severe intoxication associated with analytically confirmed use of the novel psychoactive substances 25B-NBOMe and 25C-NBOMe, Clin. Toxicol. 52 (2014) 561–565.
[29] P. Armenian, R.R. Gerona, The electric Kool-Aid NBOMe test: LC–TOF/MS confirmed 2C-C-NBOMe (25C) intoxication at Burning Man, Am. J. Emerg. Med. 32 (2014) 1444.e1443–1444.e1445.
[30] L.K. Sørensen, Determination of acidic and neutral therapeutic drugs in human blood by liquid chromatography–electrospray tandem mass spectrometry, Forensic Sci. Int. 206 (2011) 119–226.
[31] L.K. Sørensen, J.B. Hasselstrom, A high-throughput multi-class liquid chromatography tandem mass spectrometry method for quantitative determination of licit and illicit drugs in whole blood, Anal. Methods 5 (2013) 3185–3193.
[32] R. Kronstrand, I. Nystrom, M. Andersson, L. Gunnarsson, S. Hagg, M. Josefsson, J. Ahlner, Urinary detection times and metabolite/parent compound ratios after a single dose of buprenorphine, J. Anal. Toxicol. 32 (2008) 586–593.
[33] DIN 32633 Chemical Analysis – Methods of Standard Addition – Procedure, Evaluation Standard by Deutsches Institut Fur Normung E.V. (German National Standard), 12/01/1998, 1-7.
[34] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224.
[35] A.W. Jones, A. Holmgren, Amphetamine abuse in Swed subject demographics changes in blood concentrations over time the types of coingested substances, J. Clin. Psychopharmacol. 33 (2013) 248–252.
[36] C.M. Stork, Serotonin reuptake inhibitors and atypical antidepressants, in: L.S. Nelson, N.A. Lewin, M.A. Howland, R.S. Hoffman, L.R. Goldfrank, N.E. Flomenbaum (Eds.), Goldfrank's Toxicologic Emergencies 9, The Mc Graw Hill Companies, New York, 2011, pp. 1041–1043.