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Identification of novel psychoactive substances 25B-NBOMe and 4-CMC in biological material using HPLC-Q-TOF-MS and their quantification in blood using UPLC–MS/MS in case of severe intoxications

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ABSTRACT

This paper describes cases of poisoning caused by new psychoactive substances such as: 25B-NBOMe (2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine) and 4-CMC (1-(4-chlorophenyl)-2-(methylamino)-1-propanone). The analytical procedure includes rapid and selective method for the extraction and determination of 4-CMC and 25B-NBOMe in blood samples using UPLC–MS/MS technique. To the best of our knowledge, this is the first report, that involves a fully validated method for quantification of new-designer drug – 4-CMC in postmortem blood samples. The biological material was also analyzed with the use of routine analytical methods: immunochemical techniques, gas chromatography with flame ionization detection and gas chromatography with electron impact mass spectrometry. The results of real samples analyses correspond to possible toxicological effects: death resulting from 25B-NBOMe - mediated hallucinations (661 ng/mL of 25B-NBOMe and 0.887 ng/mL of 4-CMC), fatal overdose of 25B-NBOMe and 4-CMC (66.5 ng/mL of 25B-NBOMe and 2.14 ng/mL of 4-CMC) and non-fatal intoxication of these drugs (38.4 ng/mL of 25B NBOMe and 0.181 ng/mL of 4-CMC). Additionally, *O*-demethylathed O, *O*-bis-demethylathed and glucuronidated metabolites of 25B-NBOMe in biological specimens were detected.

1. Introduction

Over the past several years, the number of non-controlled psychoactive substances also called as “legal highs”, “designer drugs”, “novel psychoactive substances (NPS)” or “bath salts”, constantly appearing on the illicit Internet drug market has been tremendously increasing [1]. Among these substances, a new class of hallucinogenic drugs: *N*-2-methoxybenzyl-phenethylamines named “NBOMes” has become widely distributed and its spreading throughout the world has become an important issue connected particularly with young people’s health [2–4]. As it has been reported by many researches, NBOMes act as highly potent agonists of 5-HT_{2A} receptors, that might be associated with their hallucinogenic features [5–11]. Symptoms of NBOMes intoxications include among other things: tachycardia, agitation, aggression, hallucinations and hyperpyrexia [12–16]. Additionally, due to presence of *N*-2-methoxybenzyl substitution, NBOMe substances exhibit high in vitro binding affinity to α1A –adrenergic receptors (19 times higher compared with the 2C drugs) [17]. It might contribute to the stimulant-type cardiovascular effects that are typically seen in cases of NBOMe drug intoxication [5,12,18]. 25B-NBOMe (2-(4-bromo-2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl) ethanamine) has become more and more popular derivative among NBOMes [14–16,19–22]. The preliminary clinical studies has shown that, the safe dose of 25B-NBOMe without a harmful effect for humans, is about 1 mg [23]. In the amendments to the drug abuse prevention act adopted by the Polish Parliament in April 2015, most “NBOMe” derivatives (including 25B-NBOMe) were added to the psychoactive substances group (legally called in Poland as “I-P group”). In

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the view of the above, mentioned compounds were included in the list of legally controlled substances in the USA in November 2013 [24].

Until now, there has been limited scientific evidence concerning metabolism of NBOMe designer drugs in humans. According to recently published data the main metabolic pathways of NBOMe derivatives, such as: 25B-, 25C- and 25I-NBOMe includes mainly: *O*-demethylation, followed by *O*-di-demethylation and hydroxylation, and *N*-dealkylation as well as extensive glucuronidation and sulfation of the main phase I metabolites [25]. Hydroxylation preferably occurs at the NBOMe ring. The studies about the metabolic pathways has been performed using incubation with human liver microsomes (HLMs) followed by LC-high resolution MS analysis. In vitro characterization of potential CYP-and UGT-derived metabolites by HLMs revealed that after phase I O-desmethylated metabolites were the most dominant compounds and hydroxylated and *N*-dealkylated glucuronidated metabolites were also present [19]. These studies are consistent with results obtained from rat and human urine samples analysis performed during controlled-drug administration by standard urine screening approaches (SUSA) using LC–MS [22]. The formation of these metabolites involved several CYP isoforms - CYP1A2 and CYP3A4 in hydroxylation and CYP2C9 and CYP2C19 in *O*-demethylation.

Cathinones and their synthetic derivatives appeared on the drug market between 2008 and 2012. Initially, cathinone of natural origin, which is present in the Khat plant (*Catha edulis*), was broadly used. As it has been reported, *Khat* was a substance chosen by immigrants from Somalia, Ethiopia and Yemen, used as a medicine and stimulus to achieve increased activity and psychoses during religious ceremonies [26]. In Poland, cathinone, like NBOMes is a controlled substance classified as I-P group of psychotropic substances.

Mephedrone (4-methylmethcathinone) was known in most European countries between the years 2009 and 2010 as representative cathinone derivative [27]. Due to increasing number of poisonings involving mephedrone, it became a controlled drug in the United Kingdom in April 2010 [28] and has been replaced over time by other non-controlled, federally legal derivatives such as: 4-FMC (4-fluoromethcathinone) [29], 4-BMC (4-bromomethcathinone) [30] or 4-MEC (4-methyl-*N*-ethylcathinone) [31] in order to circumvent the law. The most frequently recorded unwanted effects of these substances include sympathomimetic toxicity combined with tachycardia, hypertension, pains in the chest and palpitations, agitation, aggression, fear, psychoses, hallucinations, and sleeplessness. 4-CMC (4-chloromethcathinone, clephedrone) is a synthetic chlorine substituted methcathinone and recently available commercially on the Internet (www.deboralabs.com). Currently, there is a lack of clinical studies about the effects and toxicity of clephedrone [32]. In the recently published study, Klavž et al. identified 4-CMC in powder material from bags found at the scene, but it was neither detected in the urine and blood nor in the stomach contents [33]. Until now, there have not been written any reports that describe the precise quantitation of 4-CMC in biological specimens.

The identification and quantification of NPS, such as 25B-NBOMe and 4-CMC have become an analytical challenge, due to fact, that these substances and/or their metabolites cannot be identified or quantified by routine cost-effectiveness toxicological screening used in forensic laboratories. Moreover, their rapid transience on the drug market, initial lack of analytical reference standards and large number of possible structures are very problematic [34]. Screening of drugs with use of liquid chromatography coupled with quadrupole time of flight mass spectrometry (HPLC-Q-TOF-MS) and tandem mass spectrometry (HPLC–MS/MS), gas chromatography/mass spectrometry (GC–MS), matrix-assisted/laser desorption/ionization time of flight mass spectrometry, which overcome these problems, has become nowadays method of choice for many toxicological investigations [35–40]. It must be taken into account, that some drugs are not amenable to GC–MS analysis because of their thermolabile and hydrophilic properties [41]. Before LC–MS analysis, clean-up steps involving: solid phase extraction (SPE), liquid-liquid extraction (LLE), protein precipitation, dispersive liquid–liquid microextraction (DLLME) are required. The choice of sample preparation method for determination of drugs in biological specimens depends mainly on the sample matrix, drug properties and analytical problem to be solved (e.g. possible matrix interferences) [41].

The cases described herein concern acute intoxications of three young men that took place in Gdańsk on the 24th October 2014 at about 10:30p.m. A 23 year-old man (Subject No. 2) who jumped out of a window from the fifth floor of the block of flats was found dead at the scene. There were two other men: a 23-year old (Subject No. 1), and a 24-year-old (Subject No. 3) in the flat on the fifth floor. Both were aggressive, and very agitated. They were moving anxiously in the flat, shouting at each other, screaming incomprehensibly, gesticulating, and speaking illogically, not reacting to any orders. Verbal contact with them was impossible. When they were taken out of the flat, young man's (Subject No.1) condition deteriorated. He had strong convulsions, heavy breathing, and salivation, so an ambulance was called. On the way to the hospital, at 00:25a.m. his vital functions stopped, and the doctor confirmed his death (Subject No. 1). The third man (Subject No.3) was taken to hospital. An inspection of the flat revealed Ziploc bags with the following descriptions: "25B NBOMe 1 GR.", and "4 CMC 1 GR.".

Due to the novelty of these recreational drugs, at present there is limited research described in the published literature concerning the pharmacokinetics, pharmacological or toxicological effects of 25B-NBOMe and 4-CMC [32,42]. The cases of three intoxications due to simultaneous ingestion of 4-CMC and 25B-NBOMe that resulted in unpredicted behavior of the patients were presented.

To the best of our knowledge, there is no information in the literature concerning an identification of clephedrone by LLE followed by HPLC-Q-TOF-MS analysis and its determination by UPLC–MS/MS.

2. Materials and methods

2.1. Chemicals and biological materials

Acetonitrile HPLC gradient, methanol HPLC gradient and formic acid (FA) were purchased from Merck KGaA (Damstadt, Germany), ammonium formate was obtained from Sigma–Aldrich (Deisenhofen, Germany). Ultrapure water was produced in an HLP_5 system (Hydrolab, Poland). Sodium hydroxide, ethyl acetate and hydrochloric acid (analytical grade) were obtained from POCh (Gliwice, Poland). Standard 4-CMC and 25B-NBOMe were obtained from Cayman Chemical (Ann Arbor, USA). Internal standard rac-methamphetamine-d_5 (*rac*-mAMP-d_5) was purchased from LGC Standards (Luckenwalde, Germany). Stock standard solutions of 4-CMC and 25B-NBOMe were prepared in mixture of ACN and water (1:1) at a concentration of 100 ng/mL. Dilutions of stock solutions were prepared in mixture of 10 mM ammonium formate with 0.05% v/v of FA in water and ACN with 0.05% v/v of FA (9: 1, v/v) at concentrations of 1 and 10 ng/mL and were used to prepare calibration standards. To prepare calibration standards, blank plasma samples were spiked with appropriate stock solutions. A stock solution of internal standard was prepared at concentration of 1 μg/mL and was then diluted to 5 ng/mL and used in all analyses. All stock solutions have been stored at − 20 °C until they were used.

Post-mortem specimens (blood, urine, liver, kidney and gastric contents) were collected from two fatalities (Subject No. 1 and Subject No. 2) during their autopsies performed within 24 h after the