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Looking at flubromazolam metabolism from four different angles: Metabolite profiling in human liver microsomes, human hepatocytes, mice and authentic human urine samples with liquid chromatography high-resolution mass spectrometry

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ABSTRACT

Flubromazolam is a triazolam benzodiazepine that recently emerged as a new psychoactive substance. Since metabolism data are scarce and good analytical targets besides the parent are unknown, we investigated flubromazolam metabolism in vitro and in vivo. 10 μmol/L flubromazolam was incubated with human liver microsomes for 1 h and with cryopreserved human hepatocytes for 5 h. Mice were administered 0.5 or 1.0 mg flubromazolam/kg body weight intraperitoneally, urine was collected for 24 h. All samples, together with six authentic forensic human case specimens, were analyzed (with or without hydrolysis, in case it was urine) by UHPLC–HRMS on an Acquity HSS T3 column with an Agilent 6550 QTOF. Data mining was performed manually and with MassMetasite software (Molecular Discovery).

A total of nine metabolites were found, all generated by hydroxylation and/or glucuronidation. Besides *O*-glucuronidation, flubromazolam formed an N^+-glucuronide. Flubromazolam was not metabolized extensively in vitro, as only two monohydroxy metabolites were detected in low intensity in hepatocytes. In the mice samples, seven metabolites were identified, which mostly matched the metabolites in the human samples. However, less flubromazolam N^+-glucuronide and an additional hydroxy metabolite were observed. The six human urine specimens showed different extent of metabolism: some samples had an intense flubromazolam peak next to a minute signal for a monohydroxy metabolite, others showed the whole variety of hydroxylated and glucuronidated metabolites. Overall, the most abundant metabolite was a monohydroxy metabolite, which we propose as α-hydroxyflubromazolam based on MSMS fragmentation. These metabolism data will assist in interpretation and analytical method development.

1. Introduction

Benzodiazepines are well-known psychoactive substances with sedative, anxiolytic, muscle-relaxant and anti-epileptic effects and have a long history as prescription drugs. After years of marketing other psychoactive substances over the internet, it is not surprising that benzodiazepines are now being promoted as 'legal highs' on research chemical websites. In contrast to synthetic cannabinoids or synthetic cathinones, which are compound classes that had never been consumed by humans before, the 'new research benzodiazepines' come from widely different backgrounds: some are approved medications in some countries, e.g. phenazepam in Russia, prazepam in many European countries, while many others were developed in pharmaceutical companies and never proceeded to clinical trials, e.g. diclazepam [1], nifoxipam [2], flubromazolam [3]. Although benzodiazepines are generally considered safe (unless combined with other central nervous system suppressing drugs), all newly marketed 'research benzodiazepines' should be regarded with suspicion, even more so in

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case of high potency compounds, which carry an increased risk for severe intoxications.

Flubromazolam (8-bromo-6-(2-fluorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) is a 4-ring core 'research benzodiazepine' sold as recreational drug since 2014 and scheduled as illegal substance in Sweden since June 2015. It can be considered a triazole analog of flubromazepam containing a fluorine, bromine and methylated triazole substituent on its core benzodiazepine skeleton (Fig. 1). Drug users describe flubromazolam as highly potent, with active doses of around 0.25 mg for naïve users, and exerting mainly anxiolytic and hypnotic effects [4,5]. Flubromazolam has been detected in numerous intoxications; until September 2016, the National Board of Forensic Medicine alone counted 41 autopsy cases with confirmed flubromazolam intake and 27 cases originating other backgrounds, such as traffic, violent crimes or probation control cases.

To date, flubromazolam pharmacokinetic profile has not been fully characterized. Lukasik et al. reported a severe intoxication involving deep coma after an intake of 3 mg flubromazolam, with a peak concentration of 59 ng/mL in serum; however, the authors did not further search for metabolites [6]. Huppertz et al. performed an in vitro incubation with human liver microsomes (HLM) and detected one mono- and one dihydroxylated metabolite, but did not further elucidate their structure [7]. In a self-experiment with one volunteer, who ingested 0.5 mg flubromazolam and experienced sedation and amnesia, the same group found a serum peak concentration of 8.6 ng/mL flubromazolam and one monohydroxylated metabolite [8].

In order to better describe flubromazolam metabolism, we performed four different experiments exploiting the unique characteristics of each model: in vitro HLM experiments are robust, easy to perform and provide a preliminary overview of a compound metabolism; incubation with human hepatocytes details a more comprehensive metabolic profile; in vivo mice studies encompass further pharmacokinetic processes than mere metabolism; finally, detection of metabolites in authentic human urine samples confirms and complements the results of the aforementioned studies.

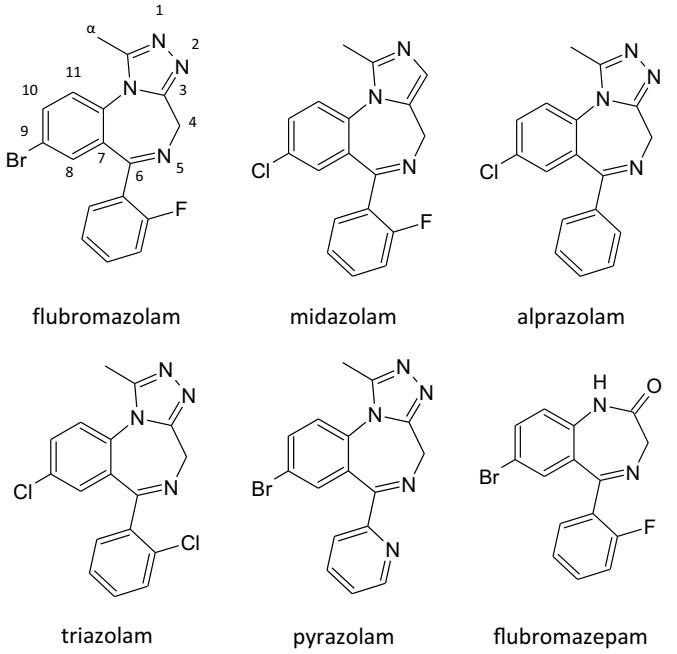

Fig. 1. Structures of flubromazolam and related benzodiazepines midazolam, alprazolam, triazolam, pyrazolam and flubromazepam.

2. Materials and methods

2.1. Chemicals and reagents

Liquid chromatography–mass spectrometry (LC–MS) grade acetonitrile (ACN), formic acid and methanol were purchased from Fisher Scientific (Gothenburg, Sweden). Ammonium formatewas obtained from Fluka (Sigma–Aldrich, Stockholm, Sweden) and β-glucuronidase/arylsulfatase (Helix pomatia) from Roche (Mannheim, Germany). Flubromazolam was acquired as powder from Chiron AS (Trondheim, Norway). Saline (9 mg/mL sodium chloride) was from Braun (Melsungen, Germany) and 99.5% ethanol from Kemetyl (Haninge, Sweden). Human liver microsomes UltraPool^{TM} (150-donor-pool) and NADPH regenerating system solution A and B were purchased from Corning (Corning, NY, US), cryopreserved human hepatocytes LiverPool^{TM} (10-donor-pool), InVitro Gro HT and InVitro Gro KHB media from BioreclamationIVT (Baltimore, MD, USA). High-purity water was produced in-house in a MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA).

2.2. Incubation with human liver microsomes

Flubromazolam was incubated in duplicate at 10 μmol/L with human liver microsomes (0.5 mg/mL) and NADPH regenerating system in potassium phosphate buffer (0.1 M, pH 7.4) at 37 °C. After 4, 10, 20, 30, 45 and 60 min samples were collected and precipitated with an equal volume of cold ACN. A time zero (precipitation before adding HLM), degradation (incubation without HLM) and negative control (incubation without substrate) were prepared separately.

2.3. Incubation with human hepatocytes

Cryopreserved human hepatocytes were thawed at 37 °C and immersed in thawing medium. After centrifugation (100 g, 4 min, room temperature) supernatant medium was removed and the pellet re-suspended in Krebs–Henseleit buffer. After a second washing step with buffer, cell viability was assessed with Trypan blue (0.4% v/v) exclusion dye method and was 96%. Flubromazolam (10 μmol/L final concentration) was incubated with hepatocytes (5×10^5 cells/0.5 mL/well) at 37 °C. Diclofenac (10 μmol/L) was incubated as positive control. Reactions were stopped with 0.5 mL ice-cold ACN after 0, 3 and 5 h. A time zero (precipitation before adding hepatocytes), degradation (incubation without hepatocytes) and negative control (incubation without substrate) were prepared separately.

2.4. Metabolism studies in mice

Animal studies were performed at the Center of Biomedical Resources at Linköping University and sample analysis at the National Board of Forensic Medicine, Linköping, Sweden. Male C57BL/6N mice aged 7–12 weeks (25 g weight) were obtained from Charles River Laboratories (Sulzfeld, Germany) and housed 4 per cage in an environmentally controlled room with a 12 h light/dark cycle. The protocol was approved by the Swedish Board of Agriculture's Animal Ethics Committee in Linköping (Dnr 49-14). Mice were injected intraperitonally with 200 μL drug solution in saline (mouse #1 and #2: 1.0 mg/kg; mouse #3 and #4: 0.5 mg/kg) or vehicle, both containing 1.25% ethanol. Standard delivery route in mice is via intraperitoneal administration, as it ensures complete diffusion of the drug throughout the body. Urine was collected over 24 h. Mice had free access to water (containing 25 g/L dextrosol to increase liquid consumption) and food.