Characterization of the designer benzodiazepines pyrazolam and flubromazepam and study on their detectability in human serum and urine samples

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Introduction

Until 2012, the offer of 'legal' alternatives to prescription-only benzodiazepines via Internet shops was limited to phenazepam and etizolam, two drugs which are marketed as pharmaceutical drugs e.g. in Russia and India. However, after these drugs were scheduled in many countries, pyrazolam and flubromazepam were offered, marking the first appearance of designer benzodiazepines. So far little is known about these new drugs, for example with regard to their pharmacological properties, their metabolism as well as their windows of detection in biological samples. As a consequence, two studies were carried out to characterize these new benzodiazepines and investigate their metabolism in humans. Additionally, an LC-MS/MS method for the quantification of pyrazolam and flubromazepam and the qualitative identification of its metabolites in serum and urine was developed. Since the availability over the Internet bears the risk of this drug being misused for drug facilitated crimes or as a substitute for prescription benzodiazepines, different immunoassays were tested to evaluate the detectability in such tests and the windows of detection.

Materials and methods

Identification

- NMR Bruker BioSpin DRX 400
- LC-Q-ToF-MS Dionex UltiMate 3000 RSLC HPLC + Bruker maXis impact Q-TOF
- LC-MS/MS Shimadzu Prominence HPLC + AB Sciex QTRAP 4000
- GC-MS Agilent 6890 GC + 5973 detector

Isolation

Extraction of the compound out of the tablet / capsule with ethanol and isolation by thin layer chromatography based on the method for alprazolam in Ph. Eur. 6.0

- Mobile phase: Acetic acid (99%), water, methanol, ethyl acetate (2:15:20:80 v/v/v/v)
- Stationary phase: Silica Gel 60, 10 x 20 cm, F256

Self-administration study

One male volunteer (42 a, 73 kg, CYP2D6 poor metabolizer) ingested 1 mg pyrazolam / 4 mg flubromazepam in two separate experiments.

Pyrazolam: 8 serum samples over 50 hours and 31 urine samples over 10 days
volunteer experienced no physical or mental effects

Flubromazepam: 15 serum samples over 31 days and 25 urine samples over 31 days
volunteer experienced fatigue and enhanced need for sleep for three days

Identification of the main metabolites

For identification of the main metabolites, selected urine samples were screened by performing enhanced product ion scan (EPI) experiments with the hypothetic masses of potential phase I and II metabolites as precursor masses and by precursor ion scan experiments with characteristic fragments of pyrazolam/flubromazepam. For further confirmation, the samples were also screened using LC-Q-ToF-MS in full scan and bbCID mode. In addition to screening the in-vivo samples for potential metabolites an in vitro experiment using human liver microsomes (HLM) was carried out.

Analysis of the serum and urine samples

LC-MS/MS

Instruments: Shimadzu LC-10 + AB Sciex API 5000
Column: Phenomenex Synergi 4u Polar RP column (150 x 2 mm, 4 µm)
Mobile Phase: A: 0.1 % HCOOH (v/v) and 1 mM ammonium formate in deionized water
B: 0.1 % HCOOH (v/v) in MeOH
Gradient: 20% B -> 95% B in 10 min; 95% B for 1.5 min; 20% B for 3 min
IS: Alprazolam-D5 (Pyrazolam); Nordazepam-D5 (Flubromazepam)
MRM transitions:

Pyrazolam:	354 -> 247, **206**, 167		
Flubromazepam:	333 -> **226**, 206, 184	Debrominated flubromazepam:	255 -> 211, 206, **180**
OH-Flubromazepam:	349 -> **303**, 273, 207	Debrominated-OH-flubromazepam:	271 -> 253, **225**, 215

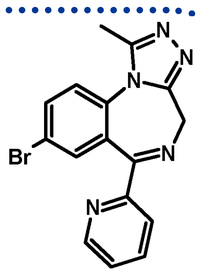
Immunochemical assays

Serum	Konelab® 30	Cloned Enzyme Donor Immunoassay (cutoff: 0 ng/ml nitrazepam equivalents; cross-reactivity for flubromazepam: 71%)
Urin	Axsym® 4602	Fluorescence Polarization Immunoassay (cutoff: 200 ng/ml nordazepam equivalents; cross-reactivity for flubromazepam: 75%)
	cobas® 8000	Turbidity Immunoassay (cutoff: 200 ng/ml nordazepam equivalents; cross-reactivity for flubromazepam: 79%)

Pyrazolam

Identification

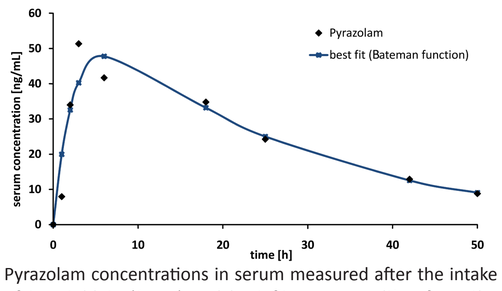
^{1}H and ^{13}C NMR analysis confirmed the compound as:
8-bromo-1-methyl-6-pyridin-2-yl-4*H*-[1,2,4]triazolo[4,3-a] [1,4]benzodiazepine



Metabolism

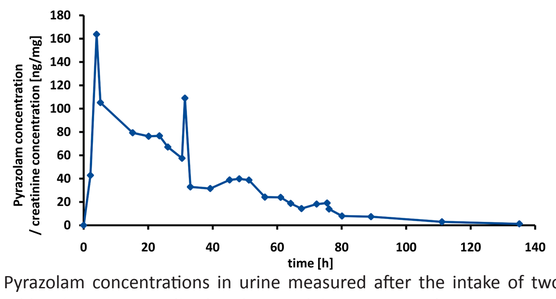
In the EPI scan experiments, none of the postulated phase I and phase II metabolites could be detected. LC–Q–ToF–MS analysis with bbCID scans also did not reveal any metabolites. Based on the genotyping and phenotyping results of the subject, a poor metabolism regarding CYP3A4 can be ruled out. However, the poor metabolizing genotype and phenotype of the volunteer for the CYP2D6 isozyme may serve as an explanation. On the other hand, no metabolites could be detected in the pooled human liver microsome assay either, strengthening the hypothesis that pyrazolam is not metabolized extensively.

Serum samples



Pyrazolam concentrations in serum measured after the intake of two tablets (1 mg) and best fit curve resulting from the calculated Bateman function with k_a and k_e values of 0.399 and 0.041 and C_0 of 62 ng/ml

Urine samples



Pyrazolam concentrations in urine measured after the intake of two tablets (1 mg), normalized to the creatinine concentrations

Estimated elimination half-life: 17 h

Immunochemical assay

- CEDIA 2 marginal (6 h; 18 h)
- FPIA 5 marginal (5.2 h; 15.2 h; 20.2 h; 31.4 h; 39.3 h)
- Turbidity IA 4/5 positive* (5.2 h; 15.2 h; 20.2 h; 39.3 h)

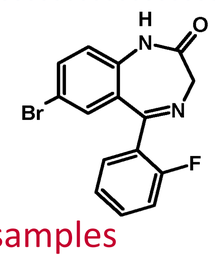
(*only the urine samples tested marginal with the FPIA were retested)

Reference: Moosmann et. al. Characterization of the designer benzodiazepine pyrazolam and its detectability in human serum and urine. Forensic Toxicol 31, 2: 263-271, **2013**

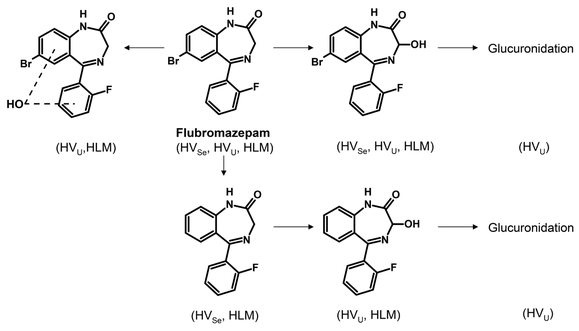
Flubromazepam

Identification

^{1}H and ^{13}C NMR analysis confirmed the compound as:
7-bromo-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one

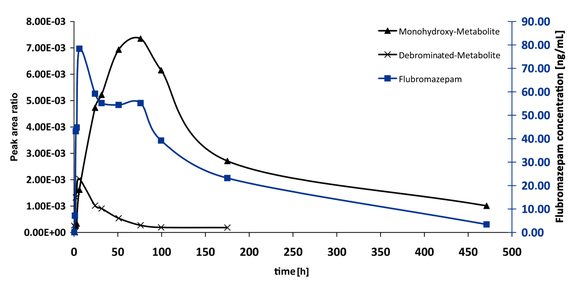


Metabolism



Proposed metabolism of flubromazepam in a human volunteer (HV$_{Se}$: serum; HV$_U$: urine) and after incubation with human liver microsomes (HLM)

Serum samples

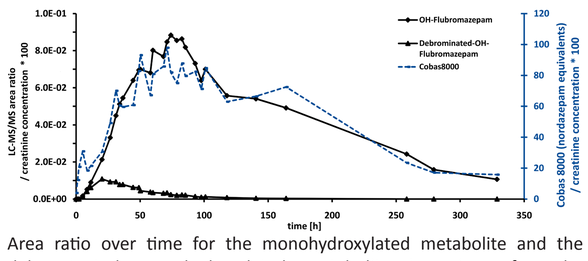


Peak area ratios (analyte/IS) of the monohydroxylated and the debrominated metabolite (left scale) and concentration of flubromazepam (right scale; 100 ng/ml equal to peak area ratio 44E-03) in serum after the consumption of 4 mg flubromazepam using LC-MS/MS analysis.

Preliminary pharmacokinetic parameters:

- Estimated elimination half-life: 106 h
- Estimated volume of distribution: 0.73 L/kg
- Estimated clearence: 0.346 L/h

Urine samples



Area ratio over time for the monohydroxylated metabolite and the debrominated monohydroxylated metabolite in urine after the consumption of 4 mg flubromazepam. The black lines indicate the peak area ratio (analyte/IS) obtained by LC-MS/MS analysis normalized to the creatinine concentration. The dotted line shows the response of the applied immunoassay (cobas® 8000 instrument) normalized to the creatinine concentration for the identical samples. **Only small amounts of unmetabolized flubromazepam could be detected in the urine samples.**

Immunochemical assay:

- CEDIA 4 positive (6 h, 31 h, 51 h, 76 h)
  3 marginal (3 h, 24 h, 99 h)
- FPIA all tested negative
- Turbidity IA 1 positive (60 h)

Reference: Moosmann et. al. Detection and identification of the designer benzodiazepine flubromazepam and preliminary data on its metabolism and pharmacokinetics. Accepted for publication in J Mass Spectrom

Conclusion

With phenazepam and etizolam being scheduled in many countries, designer benzodiazepines derived from poorly characterized pharmaceutical research drugs mark the next logical step on the 'legal highs' market. The fact that the number of new pharmacological active benzodiazepines potentially being created is immense and with tailored organic chemical synthesis available at low price we may face a similar *modus operandi* as already seen with synthetic cannabinoids, designer amphetamines and cathinones. From the data obtained from one volunteer and from HLM experiments, pyrazolam showed no detectable metabolism. Nevertheless, the long window of detection of the parent compound seems sufficient to solve forensic cases. One critical aspect regarding flubromazepam is the low detectability of its main metabolites in urine samples when applying immunochemical assays. In contrast to pyrazolam, flubromazepam could be attractive as a substitute for persons in drug withdrawal programs or other circumstances requiring regular drug testing. In addition, the typical sedating effects might lead to an instrumentalization of flubromazepam in the context of drug facilitated crimes. Furthermore, the long elimination half-life of flubromazepam could lead to an accumulation of toxic concentration levels after repeated intake. This could be particularly dangerous when combined with alcohol or other central depressant drugs such as heroine or methadone.

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