Introduction

Until 2012, the offer of ‘legal’ alternatives to prescription 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.

Identification

- **LC-Q-TOF-MS**
- **LC-MS/MS**
- **GC-MS**

**Self-administration study**

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

Flubromazepam

- **15** serum samples over 30 days and 26 urine samples over 31 days
- Volunteer experienced fatigue and enhanced need for sleep for three days

Pyrazolam

- **8** serum samples over 50 hours and 31 urine samples over 10 days
- Volunteer experienced no physical or mental effects

Analysis of the serum and urine samples

**Immunochromatographic analysis**

- **Turbidity Immunoassay**
- **CEDIA**
- **Fluorescence Polarization Immunoassay**

**LC-MS/MS**

**Instruments**:
- Shimadzu LC-10 + AB Sciex API 5500
- Phenomenex Syntery 4 μL polar RP column (300 x 2 mm, 4 μm)
- Mobile Phase: A: 0.1 % HOOC (v/v) and 1 mM ammonium formate in deionized water
- B: 0.1 % HOOC (v/v) in MeOH
- Gradient: 20 % B → 95 % B in 10 min; 95 % B for 5 min; 20 % B for 3 min
- Alprazolam-Ö5 (Pyrazolam); Narzisodam-Ö5 (Flubromazepam)

**LC-Q-ToF-MS**

- Dionex UltiMate 3000 RSLC HPLC + AB Sciex QTRAP 4000
- Agilent 6890 GC + 5973 detector

**Identification of the main metabolites**

- 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 for full scan and b CID mode. In addition to screening the in-vivo samples for potential metabolites an in vitro experiment using human liver microsomes (HLM) was carried out.

Materials and methods

**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, 25 μm

**Identification of the main metabolites**

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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 Internet market. The fact that the number of new pharmaceutical compounds potentially being created is immense and with tailored, off-the-shelf 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 immunochromatographic 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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Pyrazolam

**H** and **13C NMR analysis confirmed the compound as:**

- 8-bromo-1-methyl-6-pyridin-2-yl-4[1,2,4]triazolo[4,3-a][1,4]benzodiazepine

Metabolism

In the EPI scans, 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 polymorphism 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.

Flubromazepam

**Identification**

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

Metabolism

In an in vitro experiment using human liver microsomes (HLM) was carried out.

Serum samples

- **106 h**
- **0.399**
- **0.041**

Urine samples

- **62 ng/ml (cutoff: 200 ng/ml nordazepam equivalents)**
- **75% cross-reactivity for flubromazepam**