Research article

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Detection and identification of the designer benzodiazepine flubromazepam and preliminary data on its metabolism and pharmacokinetics

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The appearance of pyrazolam in Internet shops selling 'research chemicals' in 2012 marked the beginning of designer benzodiazepines being sold as recreational drugs or 'self medication'. With recent changes in national narcotics laws in many countries, where two uncontrolled benzodiazepines (phenazepam and etizolam), which were marketed by pharmaceutical companies in some countries, were scheduled, clandestine laboratories seem to turn to poorly characterized research drug candidates as legal substitutes. Following the appearance of pyrazolam, it comes with no surprise that recently, flubromazepam (7-bromo-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one), a second designer benzodiazepine, was offered on the market. In this article, this new compound was characterized using nuclear magnetic resonance, gas chromatography-mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS/MS) and liquid chromatography quadrupole time-of-flight MS (LC–Q–ToF–MS). Additionally, a study was carried out, in which one of the authors consumed 4 mg of flubromazepam to gain preliminary data on the pharmacokinetic properties and the metabolism of this compound. For this purpose, serum as well as urine samples were collected for up to 31 days post-ingestion and analyzed applying LC–MS/MS and LC–Q-ToF-MS techniques. On the basis of this study, flubromazepam appears to have an extremely long elimination half-life of more than 100 h. One monohydroxylated compound and the debrominated compound could be identified as the predominant metabolites, the first allowing a detection of a consumption for up to 28 days post-ingestion when analyzing urine samples in our case. Additionally, various immunochemical assays were evaluated, showing that the cross-reactivity of the used assay seems not to be sufficient for safe detection of the applied dose in urine samples, bearing the risk that it could be misused in drug-withdrawal settings or in other circumstances requiring regular drug testing. Furthermore, it may be used in drug-facilitated crimes without being detected. **Copyright © 2013 John Wiley & Sons, Ltd.**

Keywords: LC–MS/MS; benzodiazepines; immunoassay; serum; urine; flubromazepam

Introduction

Until 2012, online shops selling 'legal highs' limited their offer with regard to benzodiazepines to phenazepam and etizolam, two drugs which are approved in some countries for medication. However, with both substances now being scheduled in many countries, pyrazolam appeared on the market as a 'research chemical' in 2012 and as the first designer benzodiazepine.[1,2] In contrast to phenazepam and etizolam, pyrazolam is not approved for treatment anywhere in the world and derives from drug candidate research.[3] Additionally, in most countries without an analog act, it is not legally regulated in the respective narcotics laws.

Most recently, flubromazepam (7-bromo-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one), a second designer benzodiazepine, started to emerge in online shops. The synthesis of this compound was first described in 1962 by Sternbach *et al*.,[4,5] and it is structurally related to phenazepam, with the chlorine atom substituted by a fluorine atom, or it could alternatively be described as N-desmethylflunitrazepam after exchange of the nitro moiety by a bromine atom (Fig. 1). Regarding the pharmacological activities of this drug, Sternbach *et al*. carried out various animal studies with mice and cats investigating the sedative and muscle relaxant effects, the anticonvulsant activity as well as the taming effect on fighting mice of various 1,3-Dihydro-2*H*-1,4-benzodiazepin-2-ones.[6] On the basis of these studies, it seems likely that flubromazepam might have a higher potency as compared with bromazepam, diazepam and nitrazepam, as it showed the same effects as the aforementioned drugs at lower doses in the study animals.

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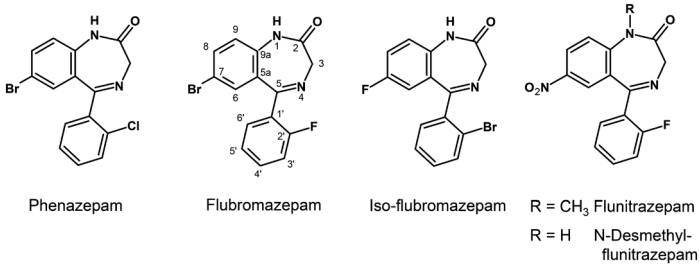

Figure 1. Structures of phenazepam, flubromazepam, flunitrazepam, N-desmethylflunitrazepam as well as the structure of iso-flubromazepam as claimed by the vendor.

With the appearance of this second designer benzodiazepine, further substances of this class can be expected. As described by Griffiths *et al.*,[7] the identification of new substances and interpretation of results is complicated by the lack of reference standards and the lack of data regarding their metabolism, potency or toxicity.

The aim of the present study was to characterize this new benzodiazepine and investigate its metabolism in humans. Additionally, a liquid chromatography–mass spectrometry (LC–MS/MS) method for the quantification of flubromazepam and qualitative identification of its metabolites in serum and urine is presented. Because 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 window of detection.

Materials and methods

Chemicals, reagents and blank serum

Methanol [HPLC grade] was purchased from Th. Geyer (Renningen, Germany), and formic acid (Rotipuran® ≥ 98%, p.a.), monopotassium phosphate and potassium chloride (≥99.5%, p.a., ACS) were obtained from Carl Roth (Karlsruhe, Germany). 1-Chlorobutane (LiChrosolv®) and sodium carbonate were from Merck (Darmstadt, Germany). Boric acid was purchased from J.T. Baker (Deventer, The Netherlands), and ammonium formate (99.995%), ethanol (analytical grade), ethyl acetate (analytical grade) and potassium hydroxide from Sigma Aldrich (Steinheim, Germany). Acetic acid (AnalaR NORMAPUR 100%) was from VWR International (Darmstadt, Germany) and deuterated chloroform ($CDCl_3$) from Euriso–Top (Saint–Aubin, France). Deionized water was prepared using a cartridge deionizer from Memtech (Moorenweis, Germany). Nordazepam–D5 (1.0 mg/ml) was obtained from LGC Standards GmbH (Wesel, Germany) and β–Glucuronidase (Escherichia coli, 140 U/mg at 37 °C) as well as β–glucuronidase/arylsulfatase (*Helix pomatia*, β–glucuronidase 5.5 U/ml and arylsulfatase 2.6 U/ml at 38 °C) from Roche Diagnostics (Mannheim, Germany). Flubromazepam capsules (declared amount: 4 mg per capsule) were ordered from an online shop selling 'research chemicals'. Human blank serum and urine samples were provided by volunteers and stored at −20 °C prior to use.

Borate buffer (pH 9) was prepared by mixing 630 ml of solution 1 (61.8 g/l H_3BO_3 and 74.6 g/l KCl in deionized water) with 370 ml of solution 2 (106 g/l Na_2CO_3 in deionized water). The pH was adjusted to 9 by addition of solution 2. 0.1 M phosphate buffer (pH 6) was prepared by dissolving 13.61 g KH_2PO_4 in 1 l deionized water and adjusting the pH to 6 by addition of KOH.

Isolation and identification of flubromazepam

Reference material for quantification was obtained by dissolving the capsule content in ethanol and removal of the insoluble additives by centrifugation at 2860 × g for 5 min (Heraeus Megafuge 1.0, Thermo Scientific, Schwerte, Germany). Afterwards, the supernatant was loaded on a thin layer chromatography plate (Silica Gel 60, 10 × 20 cm, F256, Merck, Darmstadt, Germany) and separated using acetic acid 99%, deionized water, methanol and ethyl acetate (2 : 15 : 20 : 80 v/v/v/v) based on the method published for aprazolam in the European Pharmacopoeia.[8] After separation, the band was scraped off and flubromazepam was again extracted with ethanol. The extract was finally used for identification and characterization of the compound by GC–MS, LC–MS/MS, liquid chromatography quadrupole time-of-flight MS (LC–Q–ToF–MS) and nuclear magnetic resonance (NMR) analysis.

The GC–MS, LC–MS/MS and LC–Q–ToF–MS methods and instruments applied were as described before for the characterization of pyrazolam.[2] Briefly, for GC–MS analysis a 6890 series GC system with a 5973 series mass selective detector and 7683 B series injector were used with Chemstation G1701GA version D.03.00.611 software (Agilent, Waldbronn, Germany). Similar GC parameters as published by Maurer *et al.*[9] were applied: splitless injection; column HP–5–MS capillary (30 × 0.25 mm i.d., 0.25 μm film thickness; Agilent); injection port temperature, 270 °C; carrier gas, helium; flow rate, 1 ml/min; oven temperature, 100 °C for 3 min, ramped to 310 °C at 30 °C/min, 310 °C for 10 min; transfer line heater, 280 °C; ion source temperature, 230 °C; EI mode; and ionization energy, 70 eV. Analysis was performed in scan mode from 50 to 550 amu at a scan speed of 1.5 scans/s. The solvent delay was set to 3.5 min. All the obtained spectra were compared with the spectra published in the Maurer Pfleger Weber 2011 Mass Spectral and GC Library, the National Institute of Standards and Technology Mass Spectral Library 08 and the Wiley Registry of Mass Spectral Data (sixth edition).

For LC–MS/MS analysis, a Shimadzu Prominence HPLC system (two LC-20AD SP isocratic pumps, SIL–20AC autosampler, CTO–20AC column oven, DGU–20A3 degasser and CBM–20A controller; Shimadzu, Duisburg, Germany) and a QTRAP 4000 triple-quadrupole linear ion trap with a TurbolonSpray interface were used, together with Analyst® software version 1.5.2 (both ABSciex, Darmstadt, Germany). The LC–MS/MS conditions were as follows: mobile phase A consisted of 0.1% HCOOH (v/v) and 1 mM ammonium formate in deionized water, and mobile phase B consisted of 0.1% HCOOH (v/v) in MeOH. Separation took place on a Synergi 4u Polar RP column (150 × 2 mm, 4 μm) with a corresponding guard column (Polar RP 4 × 2 mm) (Phenomenex, Aschaffenburg, Germany). Gradient elution was applied with a flow rate of 0.4 ml/min, starting at 20% mobile phase B and

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increased to 95% mobile phase B in 10 min, followed by a 1.5 min hold. Starting conditions were restored within 0.5 min, and the system equilibrated for 3 min. The autosampler and column oven temperatures were set to 4 and 40 °C respectively. The injection volume was 20 µl.

LC–Q–ToF–MS analysis was carried out on a Dionex UltiMate 3000 RSLC HPLC system (HPG–3400RS binary pump with solvent selection valve, WPS–3000TRS autosampler, TCC–3000RS column compartment and SRD–3600 solvent rack degasser; Thermo Fisher Scientific, Dreireich, Germany) coupled to a maXis impact Q–ToF instrument (Bruker Daltonik, Bremen, Germany). For data acquisition and evaluation, HyStar and Data Analysis software were used (Bruker Daltonik). LC conditions were as follows: mobile phase A deionized water/MeOH 90 : 10 (v/v) with 5 mM ammonium formate and 0.01% HCOOH, mobile phase B MeOH with 5 mM ammonium formate and 0.01% HCOOH. Separation took place on a Dionex Acclaim RSLC 120 C18 column (100 × 2.1 mm, 2.2 µm). Gradient elution was applied starting at 1% mobile phase B for 1 min with a flow rate of 0.2 ml/min, increased to 39% mobile phase B in 2 min and increased to 99.9% mobile phase B at a flow rate of 0.4 ml/min in 9 min followed by a 2-min hold at a flow rate of 0.48 ml/min. Starting conditions were restored within 0.1 min with a flow rate reduction to 0.2 ml/min after 3 min; these conditions were held for 0.9 min prior to the injection of the next sample. The autosampler and column oven temperatures were set to 5 and 30 °C respectively. For broadband collision-induced dissociation (bbCID) collision energy stepping from 24 to 36 eV was applied.

Nuclear magnetic resonance spectra were recorded in $CDCl_3$ using a DRX 400 instrument (Bruker BioSpin GmbH, Rheinstetten, Germany). 1D–^{1}H at 400 MHz, ^{13}C–NMR at 100 MHz as well as selective ^{15}N heteronuclear single quantum coherence (HSQC), 2D ^{1}H/^{13}C HSQC, ^{1}H/^{1}H correlation spectroscopy and ^{1}H/^{13}C HMBC spectra were recorded for full characterization of the compound.

Self-administration study

One of the authors (42 years old, 73 kg) ingested one capsule containing flubromazepam (declared amount 4 mg). Following the intake, the volunteer experienced some fatigue and an enhanced need of sleep for three consecutive days. No other effects were observed. One serum and urine sample prior to and 15 serum samples (over 31 days) as well as 25 urine samples (one aliquot every time the volunteer had to urinate) over 4 days and 10 additional urine samples up to 31 days post-ingestion were obtained. All samples were stored at −20 °C until analysis. In Germany, approval by an ethics committee is not required for self experiments.

Identification of the main metabolites

For the identification of the main metabolites of flubromazepam, the LC–MS/MS systems described previously were used. In addition to five urine samples collected at various timepoints over the first 4 days after consumption, three serum samples (6, 24 and 76 h) were investigated. The samples were screened by performing enhanced product ion (EPI) scan 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 flubromazepam. For further confirmation, the samples were also screened using LC–Q–ToF–MS in full scan and bbCID mode. In analogy to the metabolism of phenazepam[10–12] and various other benzodiazepines,[13] monohydroxylation, dihydroxylation, dehalogenation, methoxylation, formation of an aminobenzophenone or quinazoline-2-one and combinations of these reactions were included. For the investigation of phase I metabolites, the urine samples were incubated with ß–glucuronidase/arylsulfatase prior to a liquid–liquid extraction with chlorobutane. The samples analyzed for phase II metabolites were precipitated with cold acetonitrile.

In addition to screening the *in-vivo* samples for potential metabolites, an *in vitro* experiment using human liver microsomes (HLM) (1 mg/ml) was carried out. For this purpose, the HLM were incubated with 20 µM flubromazepam at 37 °C for 30 min. The suitability of the applied assay was proven by incubation of alprazolam and detection of its characteristic metabolites.

Analysis of serum samples, urine samples and capsule content

Sample preparation

Serum samples. Serum samples were prepared by fortifying 100 µl of serum with 20 µl of internal standard (IS) solution (5 µg/ml Nordazepam-D5). Afterwards, chlorobutane extraction under alkaline conditions was carried out. Therefore 900 µl of borate buffer (pH 9) and 1 ml of 1–chlorobutane were added. After 5 min in an overhead shaker and centrifugation at 2860 × g, the supernatant was transferred into an HPLC vial, evaporated to dryness under a gentle stream of nitrogen and reconstituted in 100 µl of mobile phase. Finally, the samples were analyzed using LC–MS/MS.

Urine samples. Urine samples were prepared similar to the serum samples; however, 500 µl of phosphate buffer (pH 6) were added after the addition of IS, and an enzymatic hydrolysis for 2 h at 45 °C using 50 µl β–Glucuronidase preceded the liquid–liquid extraction.

Flubromazepam capsules. To determine the amount of flubromazepam per capsule, the content of one capsule was dissolved in 1 ml of ethanol, centrifuged at 2860 × g for 5 min, and the supernatant was transferred into an HPLC vial. The whole procedure was repeated with the remnant for two more times, and the combined solutions evaporated to dryness under a gentle stream of nitrogen. After reconstitution in 4 ml methanol, a 1 : 20 000 dilution in mobile phase (A/B 80 : 20) was analyzed using LC–MS/MS and a five-point calibration curve (10–100 ng/ml in spiked mobile phase).

Limit of detection and Linearity. For the determination of the limit of detection (LOD) and calibration range, blank serum and urine samples were spiked with the purified compound in the concentration range of 0.1–100 ng/ml. The LOD criteria were fulfilled by obtaining a signal-to-noise ratio of at least 3 : 1 for the target ion as well as for one qualifier ion. To account for heteroscedasticity, a weighted calibration model ($1/x^2$) was applied for calibration.

LC-MS/MS analysis

For analysis of the serum samples, urine samples and capsule content, a modification of an LC–MS/MS method described elsewhere was applied.[2] The instrumentation was identical consisting of two LC–10AD VP pumps, a SCL–10A VP controller and a CTO–10AC column oven (Shimadzu, Duisburg, Germany)

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as well as an ERC–3415a degasser (ERC, Rimerling, Germany) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 100 µl syringe (Hamilton, Reno, NV, USA). For MS detection, an API 5000 triple quadrupole instrument equipped with a TurboIonSpray interface and Analyst® software version 1.5.2 (AB Sciex, Darmstadt, Germany) was applied. The LC–MS/MS conditions, column and guard column, were as described previously for the identification of the compound apart from the storage of the samples in the autosampler at room temperature. The transitions recorded for flubromazepam, its metabolites and the deuterated IS in multiple reaction monitoring (MRM) mode are listed in Table 1. The declustering potentials, collision energies and cell exit potentials were optimized for the parent compound and Nordazepam–D5. The ion source temperature was set at 400 °C and the ion source voltage at +2000 V. The gas pressures were as follows: curtain gas (N_2) 40 psi, ion source gas 1 (compressed air) 60 psi, ion source gas 2 (compressed air) 70 psi and the collision gas (N_2) 2 psi. The total cycle time was 0.33 s with a minimum dwell time of 20 ms for each transition.

Immunochemical assays

All samples obtained from the self-administration study were tested by immunoassay screenings. An Axsym® 4602 instrument and nordazepam calibrators (Abbott Laboratories, Abbott Park, IL, USA) were applied for urine samples [fluorescence polarization immunoassay (FPIA)] and a Konelab® 30 instrument (Thermo Fisher Scientific, Waltham, MA, USA) with nitrazepam calibrators (Microgenics Corporation, Femont, CA, USA) for serum samples [cloned enzyme donor immunoassay (CEDIA)]. For both assays, an in-house protocol and in-house cut-offs were applied (FPIA: calibrators at 0, 200, 400, 800, 1200 and 2400 ng/ml; cut-off 200 ng/ml nordazepam equivalents and CEDIA: calibrators at 0, 300 and 800 ng/ml; cut-off 0 ng/ml nitrazepam equivalents). In addition to the aforementioned data, all urine samples were further tested on a cobas® 8000 instrument with nordazepam calibrators (turbidity assay, Roche Diagnostics, Mannheim, Germany) applying the standard protocol (single-point calibration) and a cut-off suggested by the manufacturer (200 ng/ml nordazepam equivalents). For evaluation of the cross-reactivity of the two immunochemical assays applied for the urine samples (Axsym® 4602 and cobas® 8000), a blank urine sample was fortified with flubromazepam at 200 ng/ml. Afterwards, the sample was analyzed using both assays. For the evaluation of the cross reactivity of the assay applied for the serum samples (Konelab® 30), a blank serum sample was spiked with flubromazepam at 100 ng/ml. Finally, the sample was analyzed using the aforementioned immunochemical assay. The cross reactivity of flubromazepam to the polyclonal antibodies used in the assays applied for the urine samples was 75% (Axsym® 4602) and 79% (cobas® 8000) as compared with nordazepam. In case of the polyclonal antibodies used in the CEDIA assay for the serum samples (Konelab® 30), a cross reactivity of 71% could be determined as compared with nitrazepam.

Table 1. Multiple reaction monitoring transitions and mass spectrometry parameters of flubromazepam, selected metabolites and internal standard nordazepam–D_5

Analyte	Q1 mass (amu)	Q3 mass (amu)	CE (V)	CXP (V)
Flubromazepam	333	**226**	43	23
		206	45	25
		184	45	21
OH-Flubromazepam	349	**303**	45	25
		273	45	25
		207	45	25
Debrominated flubromazepam	255	211	45	25
		206	45	25
		180	45	25
Debrominated-OH-flubromazepam	271	253	45	25
		225	45	25
		215	45	25
Nordazepam–D_5	276	140	52	13

Declustering potential was set to 60 V, and entrance potential to 8 V.
Data in bold are ion transitions used for quantification.
CE, collision energy; CXP, collision cell exit potential.

Results and discussion

Identification and characterization of flubromazepam

Different vendors of 'research chemicals' declare flubromazepam as either 7-bromo-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one [Fig. 1 (flubromazepam)] or 5-(2-bromophenyl)-7-fluoro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one [Fig. 1 (iso-flubromazepam)]. However, using ^{1}H and ^{13}C NMR analysis, the obtained compound could clearly be identified as the first mentioned [Fig. 1 (flubromazepam)] as ^{1}H-^{1}H-correlation spectroscopy experiments implemented by 1D-total correlation spectroscopy measurements allowed the identification of two distinct spin systems in the aromatic region. One of the spin systems consists of the protons H-6, H-8 and H-9, and the other is established by the protons H-3′, H-4′, H-5′ and H-6′. By means of subsequent ^{1}H-^{13}C-HSQC experiments, it could be confirmed that the protons accounting for the three spin system are connected to singlet carbons. Thus, the fluorine can not be part of the benzodiazepine ring system. The chemical shifts recorded in the experiments are presented in Table 2. The GC–EI–MS (Fig. 2) of the compound showed next to the peak of the parent ion at m/z 332 (relative intensity 74%) the most intense peak for the fragment ions at m/z 305 (100%), 223 (20%) and 313 (19%). The LC–MS/MS EPI spectrum of flubromazepam is shown in Fig. 3(A), and the three most abundant ions (226, 206 and 184) were included as MRM transitions into the LC–MS/MS method for analyzing the serum and urine samples as well as the capsule sample. The results from LC–Q–ToF–MS analysis are summarized in Table 3 and affirmed the proposed molecular formula.

Isolation of flubromazepam

Flubromazepam could be isolated using the previously described procedure with a purity of approximately 97% (determined by NMR and GC–MS analysis) and was used for the optimization of the declustering potentials, collision energies and cell exit potentials of the MRM transitions and as reference material for the quantification of serum, urine and capsule samples.

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Table 2. Nuclear magnetic resonance (NMR) data for flubromazepam in $CDCl_3$ (for numbering, refer to Fig. 1)

Carbon no.	δ ^{13}C NMR (ppm)		δ ^{1}H NMR (ppm)	
2	170.8		—	
3	56.5		4.39	s
5	166.4		—	
5a	129.7		—	
6	132.3		7.36	d
7	116.7		—	
8	134.7		7.61	d
9	122.7		7.05	d
9a	136.4		—	
1′	127.1		—	
2′	160.3	d	—	
3′	116.2	d	7.10	ddd
4′	132.2	d	7.49	dt
5′	124.4	d	7.27	m
6′	131.3	d	7.60	dt

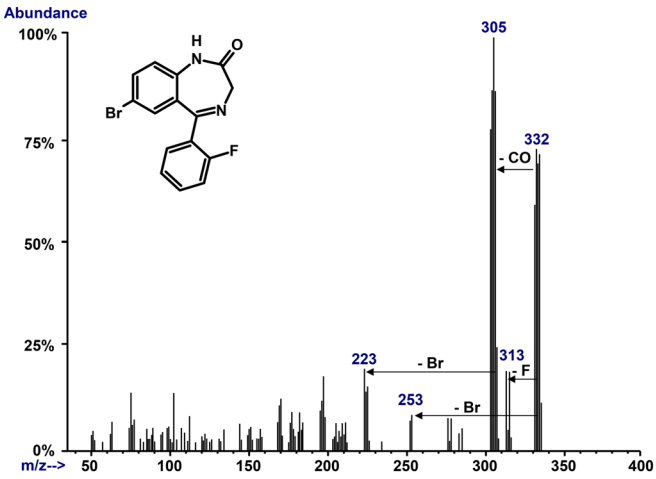

Figure 2. Electron impact ionization gas chromatography–mass spectrometry spectrum and proposed fragmentation of flubromazepam.

Quantification of the capsule

The investigated capsule contained approximately 4.2 mg of flubromazepam, which is in accordance with the amount declared by the distributor. Nevertheless, a larger number of capsules would need to be analyzed to evaluate the consistency between different capsules and especially between batches, which was not done within this study. Because the adjuvant composition of the capsule is unknown, no comments with regard to their effects toward the extraction efficiency can be made or evaluated.

Metabolism

Enhanced product ion scan experiments of urine samples showed signals at the m/z corresponding to two monohydroxylated metabolites and one debrominated monohydroxylated metabolite. In analogy to structurally related compounds like phenazepam and nordazepam,[10–13] the predominant hydroxylation most likely occurs at the 3-position of the molecule leading to 3-hydroxy-flubromazepam. However, for unambiguous assessment of the position of hydroxylation, NMR techniques would need to be applied. Considering the 3-hydroxy-metabolite of phenazepam[12] or oxazepam, it seems likely that the monohydroxylated metabolite of flubromazepam might also be pharmacologically active and thus contribute to the activity of the drug. In serum samples, signals corresponding to the debrominated metabolite could be detected next to a weak signal of the main monohydroxylated metabolite. Signals at the m/z matching the protonation and reversible ring-opening product of flubromazepam could be detected in both matrices. The same phenomenon was also observed with other benzodiazepines.[14,15] In the precipitated urine samples, signals at the m/z corresponding to the glucuronidated main monohydroxy metabolite and the glucuronidated debrominated monohydroxylated metabolite were detected. However, no signals at the m/z corresponding to the aglycones could be detected in these samples, indicating that the detected metabolites occurring in urine are present in their glucuronidated form only. The EPI scans of the main metabolites are shown in Fig. 3(B)–(D), the proposed metabolic pathway is depicted in Fig. 4 and the results of the LC–Q–ToF–MS experiments affirming the proposed main phase I metabolites are listed in Table 3 (because of the low quantity of the second monohydroxylated compound in the samples, it could not be confirmed using LC–Q–ToF–MS analysis). Analysis of the samples resulting from the *in vitro* metabolism studies with HLM also confirmed the formation of the same two monohydroxylated, the debrominated as well as of the debrominated monohydroxylated compound, suggesting the observed metabolic phase I reactions being catalyzed by CYP450 enzymes.

Analysis of the serum samples

The calibration curves showed good linearity for both matrices in the range of 1–100 ng/ml ($r^2_{serum} = 0.9968$, $r^2_{urin} = 0.9948$). Because for this study semi-quantitative results were sufficient; a full method validation was not performed. However, comparing the calibration functions for serum, urine and mobile phase, no pronounced differences in the area ratios (analyte/IS) were observed, suggesting only minor matrix effects or full compensation by the IS. In terms of its metabolites, matrix effects could not be assessed because of the lack of reference standards. Therefore, the comparability of the 'concentration profiles' obtained in the different matrices by plotting area ratios over time is limited.

The resulting serum concentrations of flubromazepam after the intake of one capsule (4 mg dose) are shown in Fig. 5. The highest concentration of approximately 78 ng/ml was detected in the serum sample collected 6 h after consumption. For an estimation of C_0 (hypothetical concentration of flubromazepam in serum at time of application), the absorption rate constant (k_a) and the elimination rate constant (k_e), a one compartment model was applied, using the excel software add-in 'solver' (Microsoft, Redmond, WA, USA) to find the best fit values for these three parameters. Basis for the calculations was the Bateman function and the analytical results in the serum samples (Fig. 5). Additionally, a demo version of the software KINETICA 5.1 (Thermo Fisher Scientific, Waltham, MA, USA) was applied for the calculation of common pharmakoninetic parameters as well as for testing different compartment models (one–three compartments) and non-compartmental analysis. However, two and three compartment models could not be matched with the experimental data. The different parameters obtained from the different approaches are summarized in Table 4. On the basis of the approximate elimination half-life of 106 h in all applied models, flubromazepam can be regarded as a rather long-acting

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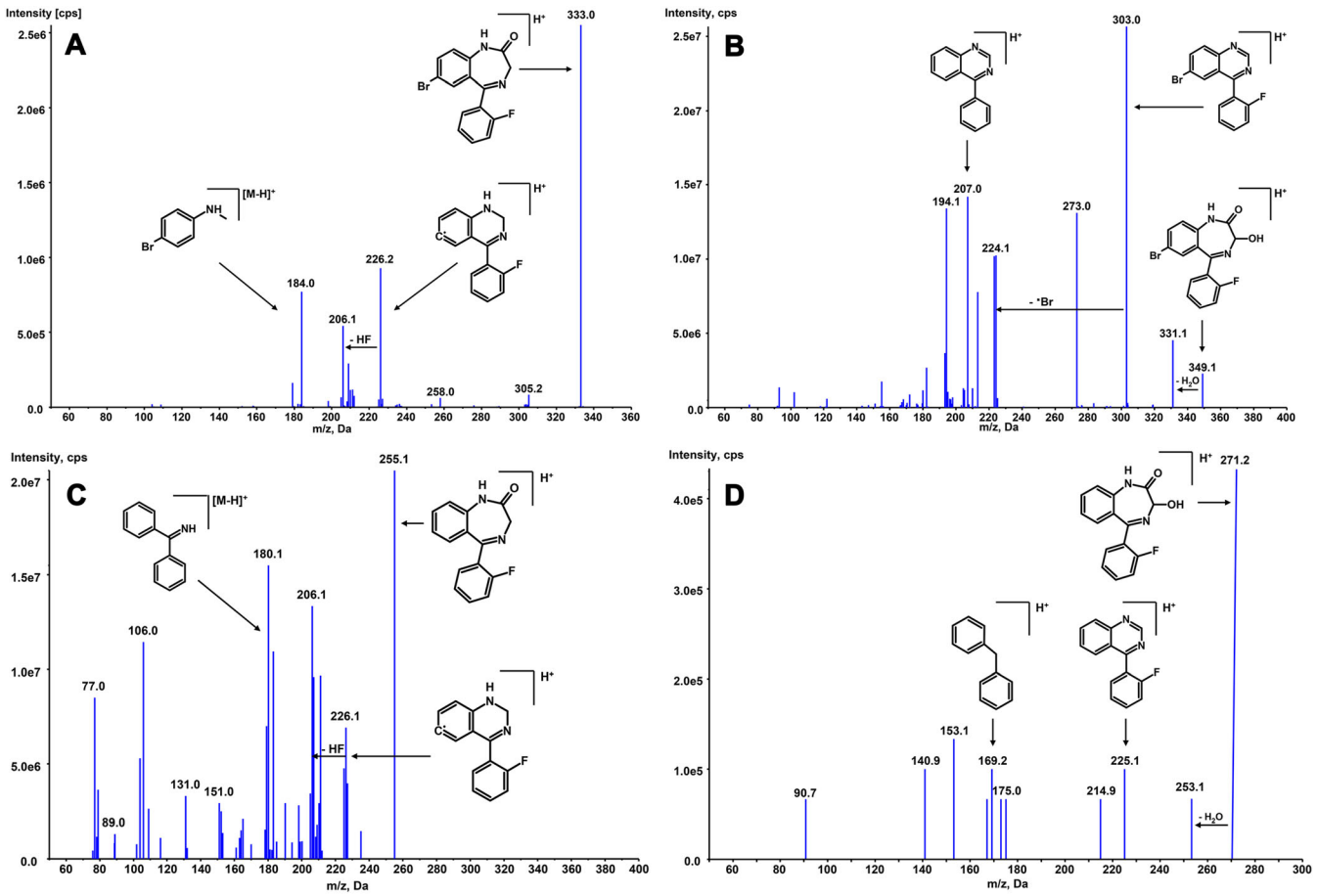

Figure 3. Enhanced product ion scans of flubromazepam and its main metabolites and their proposed fragmentation (A: flubromazepam; B: 3-hydroxy-flubromazepam; C: debrominated flubromazepam; D: debrominated 3-hydroxy-flubromazepam). The EPI scans of the glucuronides were similar to the non-glucuronidated compounds and are therefore not shown separately.

Table 3. Monoisotopic accurate masses measured with a maXis impact quadrupole time-of-flight of flubromazepam as well as its main metabolites and their characteristic fragment ions

	Monoisotopic accurate masses	Elemental composition	Electron configuration	Error (ppm)
Molecular ion $[M+H]^+$ (m/z = 333)	333.0041	$C_{15}H_{11}BrFN_2O$	Even	2.3
Fragment (m/z = 305)	305.0084	$C_{14}H_{11}BrFN_2$	Even	0.1
Fragment (m/z = 226)	226.0901	$C_{14}H_{11}FN_2$	Odd	0.2
Fragment (m/z = 184)	185.9737	C_7H_7BrN	Even	1.5
Molecular ion $[M+H]^+$ (m/z = 349)	348.9982	$C_{15}H_{11}BrFN_2O_2$	Even	0.1
Fragment (m/z = 331)	330.9877	$C_{15}H_9BrFN_2O$	Even	0.7
Fragment (m/z = 303)	302.9928	$C_{14}H_9BrFN_2$	Even	0.7
Fragment (m/z = 273)	273.0020	$C_{13}H_9BrN_2$	Even	0.7
Molecular ion $[M+H]^+$ (m/z = 255)	255.0927	$C_{15}H_{11}FN_2O$	Even	0.4
Molecular ion $[M+H]^+$ (m/z = 271)	271.0878	$C_{15}H_{11}FN_2O_2$	Even	0.2

benzodiazepine (elimination half-life > 24 h) similar to e.g. diazepam, nordazepam[13] and phenazepam,[11] which is in good agreement with the long-lasting effects in the self experiment. The volume of distribution and the clearance are also in the same range as for structurally related benzodiazepines such as nordazepam.

Interestingly, the serum samples obtained 24, 31, 51 and 76 hours after the intake show similar flubromazepam concentrations. A possible explanation for this plateau phase could be an enterohepatic circulation as it has been suggested and shown for e.g. diazepam.[16,17] This relatively long elimination half-life along with the prolonged duration of effects particularly bear health risks in cases of consumers driving a vehicle or operating heavy machinery in the days following consumption.

The concentration profile of the main monohydroxylated metabolite and the debrominated metabolite in serum was evaluated by calculation of the peak area ratio (analyte/IS) showing its maximum 76 h after the intake for the main monohydroxylated and after 6 h for the debrominated metabolite (Fig. 6). Applying a highly sensitive LC–MS/MS method, the

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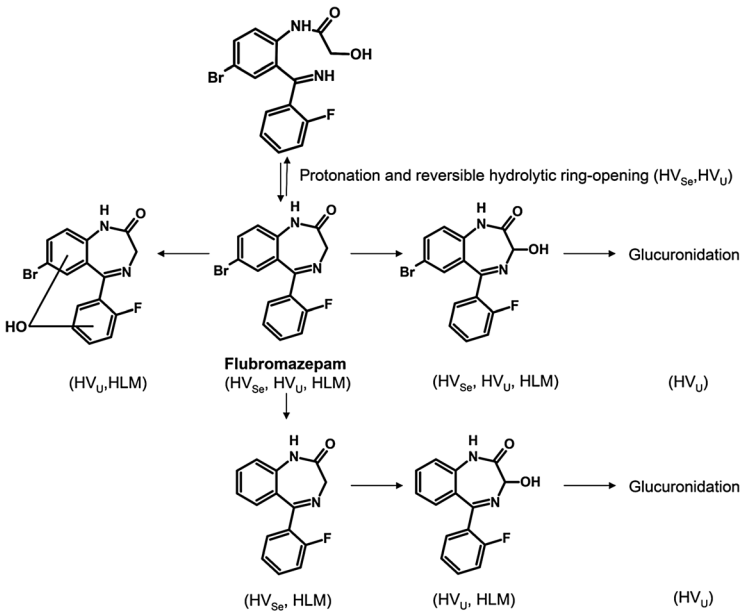

Figure 4. Proposed metabolism of flubromazepam in a human volunteer (HV_{Se}: serum; HV_U: urine) and after incubation with human liver microsomes (HLM). Ring opening may occur under acidic conditions in solution.

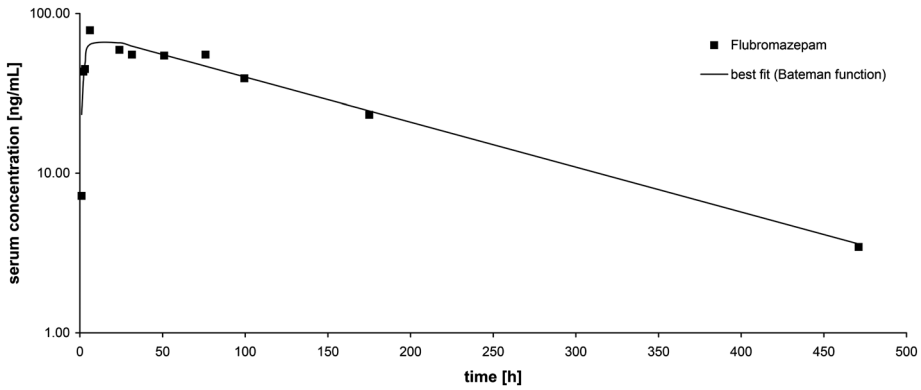

Figure 5. Flubromazepam concentration in serum after the consumption of 4 mg flubromazepam next to the best fit curve resulting from the calculated Bateman function with k_a and k_e values of 0.3412 and 0.0065 as well as an C_0 of 75.1 ng/ml.

Table 4. Pharmacokinetic parameters of flubromazepam using different approaches for calculation

	One-compartment model (Kinetica)	Non-compartmental analysis (Kinetica)	One-compartment model (excel solver)
AUC (µg/l × h)	11538.5	11343.6	
AUMC (µg/l × h^2)	1.81		
MRT [h]	154	154	
C_{max} (µg/l)	69.5	78.4	
C_0 (µg/l)			75.1
T_{max} (h)	11.8	6.1	
K_{el} (h^{-1})	0.00651	0.00652	0.00649
T_{abs} (h)	10.2		
$T_{1/2}$ (h)	106.4	106.3	106.7
V_z (l/kg)	0.73		
Cl (l/h)	0.346		
K_a (h^{-1})	0.3356		0.3411
HVD (h)		97.5	

AUC, area under the curve; AUMC, area under the moment curve; MRT, mean residence time; C_{max}, maximum concentration; C_0, hypothetical concentration of flubromazepam in serum at time of application; T_{max}, time to reach maximum concentration; K_{el}, elimination rate constant; T_{abs}, duration of absorption; $T_{1/2}$, elimination half-life; V_z, apparent volume of distribution during the terminal phase; Cl, clearance; K_a, absorption rate constant; HVD, half value duration.

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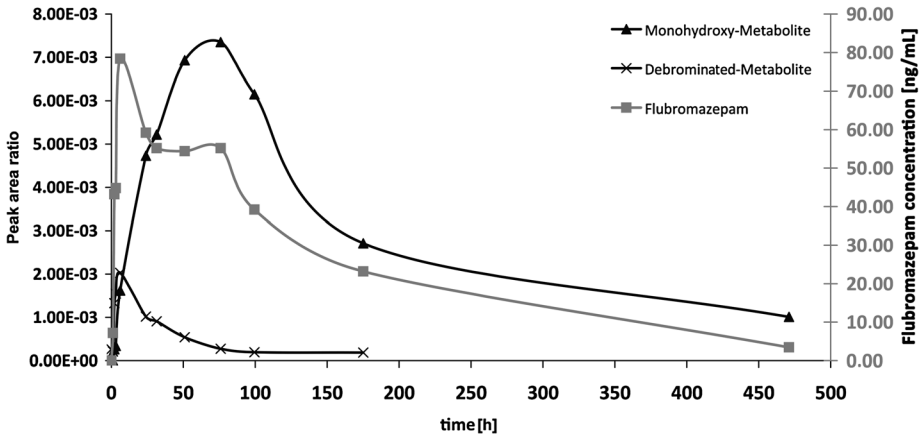

Figure 6. Peak area ratios (analyte/IS) of the monohydroxylated and the debrominated metabolite (left scale) and concentration of flubromazepam (right scale; 100 ng/ml equal to peak area ratio 44E-03) in serum after the consumption of 4 mg flubromazepam using liquid chromatography–mass spectrometry analysis.

consumption of 4 mg flubromazepam could be detected for 23 days targeting the parent compound and for 19 days or 7 days targeting the more abundant monohydroxylated or the debrominated metabolite. Considering the different timepoints of the maximum concentration of these two metabolites, it may be speculated that the enzyme catalyzing the debromination is inhibited within the first days as, after an early onset, only the monohydroxylated metabolite can be detected in the samples collected in the later stages of the study. However, both the type of inhibition (reversible or irreversible) and the compound causing it remain unknown and would need separate comprehensive experiments. Nevertheless, the debrominated metabolite might have the potential to indicate a more recent consumption as it was only detectable for a few days in the case of a single 4 mg dose.

The applied CEDIA assay tested four serum samples positive (6, 31, 51 and 76 h) and marked three further samples marginal (3, 24 and 99 h). The other eight samples (0, 1, 2, 175, 471, 563, 655 and 727 h) were reported negative by this assay. Comparing these results with the concentration profiles of flubromazepam and its monohydroxylated and debrominated metabolite obtained by LC–MS/MS analysis (Fig. 6), it seems likely that the used antibody shows a higher cross reactivity toward the parent compound or the debrominated metabolite rather than towards the monohydroxylated metabolite.

Analysis of the urine samples

Only small amounts of unmetabolized flubromazepam could be detected in the urine samples (normalized to the creatinine concentration: 0.55–4.0 ng/mg), starting from the sample obtained 2 h and 15 min after intake up to the sample collected 6 d and 20 h post-ingestion. The main monohydroxylated metabolite could be detected in the urine samples obtained between 5 h and day 28 post-consumption of the capsule with its maximum after 74 h. The debrominated monohydroxylated metabolite was detectable in the urine samples collected 5–164 h after consumption showing its maximum concentration 20 h after the intake. Because no reference standards are available for these metabolites, concentration profiles based on the peak area ratios (analyte/IS) were used for further evaluation (Fig. 7). The second hydroxylated metabolite could only be detected in traces. By using the previously described LC–MS/MS method, the window of detection in this particular study was 5 days longer in the urine samples than in serum

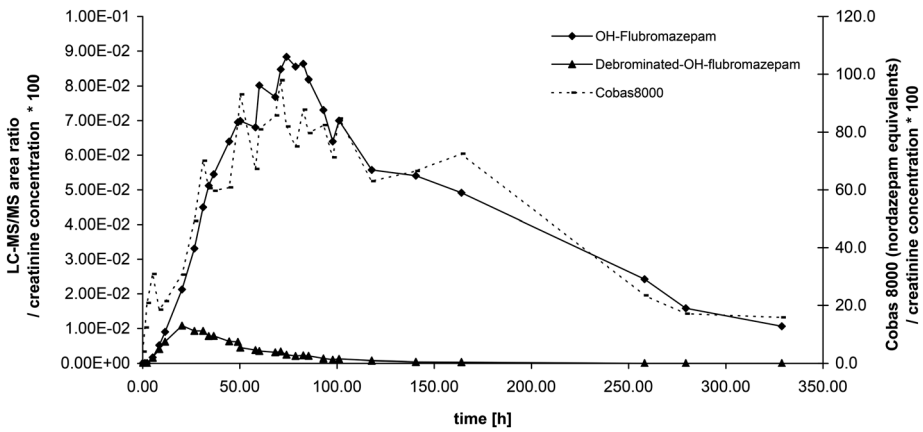

Figure 7. Peak Area ratio (analyte/IS) over time for the monohydroxylated metabolite and the debrominated monohydroxylated metabolite in urine after the consumption of 4 mg flubromazepam. The black lines indicate the peak area ratio (analyte/IS) obtained by liquid chromatography–mass spectrometry analysis 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.

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samples, allowing a detection of a consumption for 28 days when targeting the monohydroxylated metabolite. However, the debrominated monohydroxylated metabolite could only be detected for 6 days post-ingestion and might therefore be a suitable marker for a recent consumption (similar to the debrominated metabolite in serum).

Regarding the detectability by immunoassay, the observations made with serum samples were confirmed. All urine samples tested negative for benzodiazepines using the Axsym® 4602 (FPIA) instrument and all but one (60 h) using the cobas® 8000 instrument (turbidity assay). This is compatible with a relatively high cross reactivity of the assays for the parent compound (>70% for both assays), which was present in very low concentrations only. However, the applied antibodies seem to show a rather low cross reactivity toward the monohydroxylated metabolite as the LC–MS/MS area ratio over time for this compound and the immunoassay response profile show a good match (Fig. 7).

Nevertheless, the insufficient cross reactivity toward the monohydroxylated metabolite bears the risk that a consumption can go undetected in cases when urine samples are screened solely using immunochemical assays.

Conclusions

Flubromazepam, a new designer benzodiazepine, could be identified and was analytically characterized using NMR, LC–Q–ToF–MS, GC–MS and LC–MS/MS techniques. After pyrazolam, this compound is the second designer benzodiazepine marketed by Internet retailers in Europe.[2] Applying a highly sensitive LC–MS/MS method, the drug could be detected both in serum and urine samples after oral ingestion of a 4 mg dose. The monohydroxylated, debrominated and monohydroxylated debrominated compound could be identified as the major phase I metabolites, and the corresponding glucuronides of the hydroxylated compounds were detected in urine as phase II metabolites. It remains unclear whether the producing laboratories and their wholesale customers made use of published literature[4–6] and chose this particular benzodiazepine on the basis of its relatively high pharmacological activity in animal studies or by reasonable modification of its 'legal-high' predecessor phenazepam. Considering both of these approaches, 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.[7]

One critical aspect regarding flubromazepam is the low detectability of its main metabolites in urine samples when applying immunochemical assays. In contrast to the first designer benzodiazepine pyrazolam, this drug 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 the use of flubromazepam in the context of drug-facilitated crimes. Because potential consumers do not have experience with this specific benzodiazepine, the long elimination half-life of approximately 106 h 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 heroin or methadone.[18–21] Additionally, the metabolites could show pharmacological activity, resulting in even higher health risks. When driving a vehicle or operating heavy machinery in the days following consumption, the long-lasting sedative effects as seen in the self experiment could lead to severe traffic or occupational safety risks. However, the prolonged elimination half-life leads to the possibility to prove consumption of this new drug for up to 28 days post-ingestion in our case (4 mg dose) using a sensitive LC–MS/MS method (LOD: 1 ng/ml). This could be advantageous for analytical investigations in cases of drug-facilitated crimes. Although, in this study, we identified flubromazepam (7-bromo-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) as the active ingredient, iso-flubromazepam (5-(2-bromophenyl)-7-fluoro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) may also occur in such products. This isomer will most probably show significant differences in the pharmacological profile without necessarily being analytically distinguishable.

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