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SHORT COMMUNICATION

Characterization of the four designer benzodiazepines clonazolam, deschloroetizolam, flubromazolam, and meclonazepam, and identification of their in vitro metabolites

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Abstract In 2012, the first designer benzodiazepines were offered in Internet shops as an alternative to prescription-only benzodiazepines. Soon after these compounds were scheduled in different countries, new substances such as clonazolam, deschloroetizolam, flubromazolam, and meclonazepam started to emerge. This article presents the characterization of these four designer benzodiazepines using nuclear magnetic resonance spectroscopy, gas chromatography–electron ionization-mass spectrometry, liquid chromatography–tandem mass spectrometry, liquid chromatography–quadrupole time-of-flight-mass spectrometry, and infrared spectroscopy. The major in vitro phase I metabolites of the substances were investigated using human liver microsomes. At least one monohydroxylated metabolite was identified for each compound. Dihydroxylated metabolites were found for deschloroetizolam and flubromazolam. For clonazolam and meclonazepam, signals at mass-to-charge ratios corresponding to the reduction of the nitro group to an amine were observed. Desalkylations, dehalogenations, or carboxylations were not observed for any of the compounds investigated. Furthermore, for clonazolam and meclonazepam, no metabolites formed by a combination of reduction and mono-/dihydroxylation were detected. This knowledge will help to analyze these drugs in biological samples.

Keywords Designer benzodiazepines · Clonazolam · Deschloroetizolam · Flubromazolam · Meclonazepam · Phase I metabolism

Introduction

Benzodiazepines play an important role in forensic and clinical toxicology [1], because they are widely used for treatment of sleeping and anxiety disorders [2], seizures [3], and as drugs of abuse [4]. For other classes of designer drugs, like synthetic cannabinoids or designer stimulants, it is already necessary to keep analytical methods up to date [5, 6]. More recently, next to the first designer benzodiazepines, pyrazolam [7], flubromazepam [8], and diclazepam [9], four new successors have been offered via online trading platforms, thus compelling analytical laboratories to update their analytical methods to prove or exclude consumption or administration of these new drugs. Clonazolam and flubromazolam are triazolo-analogs of the registered drug clonazepam and of the designer benzodiazepine flubromazepam, respectively. Deschloroetizolam (other name: etizolam 2) is the dechlorinated analog of the thienodiazepine etizolam, and meclonazepam (other names: methylclonazepam, Ro-11-3128), a further analog of clonazepam (for chemical structures of the four new drugs, see Fig. 1). Synthesis of clonazolam was first

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reported in 1971 by Hester et al. [10] and the drug was described as the most active compound in the series tested. Meclonazepam was extensively investigated for its schistosomicidal properties [11] and has a rather long plasma half-life of approximately 40 h. Based on animal tests for anxiolytic activity, meclonazolam is far more potent than diazepam or lorazepam and shows an in vivo potency similar to that of flunitrazepam [12]. In an investigation with volunteers, sedation, incoordination, and muscle relaxation were observed after a single dose of 8 mg of meclonazepam [13]. Synthesis of deschloroetizolam is described in a patent from 1988 [14]. Based on general structure–activity relationships of benzodiazepines, it can be assumed that the loss of the chlorine atom of deschloroetizolam leads to reduced potency as compared to etizolam [15]. Regarding flubromazolam, no data could be found in the literature. However, it seems likely that this compound was chemically designed based on its predecessor flubromazepam, assuming that this triazolo-analog is more potent than flubromazepam [10, 16, 17]. The aim of the present study was to characterize these new benzodiazepines and investigate their metabolism in vitro using pooled human liver microsomes.

Materials and methods

Samples for analysis

All products were obtained via the Internet in September and October 2014 in Germany; two as research chemicals (**2** and **3**), one as tablets (**4**), and one as capsules (**1**).

Chemicals and reagents

Formic acid (Rotipuran® ≥98 %, p.a.) and 2-propanol (Rotisolv® ≥99.95 %, LC–MS grade) were purchased from Carl Roth (Karlsruhe, Germany); methanol (HPLC grade) from JT Baker (Deventer, The Netherlands); acetonitrile (ACN) (LC–MS grade), ammonium formate (99.995 %), ethanol (analytical grade), and ethyl acetate (analytical grade) from Sigma-Aldrich (Steinheim, Germany). Deionized water was prepared using a cartridge deionizer from Memtech (Moorenweis, Germany). Pooled human liver microsomes (pHLMs) were obtained from BD Biosiences (Woburn, MA, USA). Deuterated chloroform ($CDCl_3$) was purchased from Euriso-top (Saint–Aubin, France).

Preparation of sample solutions

Compounds **1** and **4**, obtained as capsules and tablets, respectively, were homogenized, weighed, and dissolved in methanol according to the stated amount of active ingredient to yield a final concentration of 1 mg/ml each. One milligram of the compounds that were obtained as research chemicals (**2** and **3**) was weighed and dissolved in 1 ml of methanol.

Identification of compounds

For identification and characterization of compounds **1**–**4**, nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–electron ionization-mass spectrometry (GC–EI-MS), liquid chromatography–tandem mass spectrometry (LC–MS-MS), and liquid chromatography–quadrupole time-of-flight-mass spectrometry (LC–QTOF-MS) were applied. Infrared (IR) spectra were also recorded.

One-dimensional ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra, and selective two-dimensional ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC), ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMBC) were recorded in $CDCl_3$ at room temperature using a DRX 400 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany). The chemical shifts are reported in ppm relative to $CHCl_3$ (^{1}H: $\delta = 7.28$) and $CDCl_3$ (^{13}C: $\delta = 77.0$).

GC–MS spectra were recorded in EI mode as described elsewhere [7–9]. The recorded mass spectra were compared with those from an in-house library, the Cayman Spectral Library [18], and the Maurer Pfleger Weber 2011 Mass Spectral and GC Library [19].

For LC–QTOF-MS analysis, we used a maXis impact II QTOF instrument (Bruker Daltonik, Bremen, Germany) equipped with an Apollo II electrospray ionization source coupled to an UltiMate 3000 RSLC high-performance liquid chromatography system, consisting of an SRD-3600 solvent rack degasser, an HPG-3400RS binary pump with solvent selection valve, a WPS-3000TRS thermostatted autosampler, and a TCC-300RS thermostatted column compartment (Thermo Scientific, Dreieich, Germany). Chromatographic separation was carried out on a Kinetex® C18 column (2.6 µm, 100 Å, 100 × 2.1 mm i.d.; Phenomenex, Aschaffenburg, Germany) with a matching guard column and gradient elution. The elution used water with 1 % ACN, 0.1 % formic acid, and 2 mM ammonium formate (A), and ACN with 0.1 % formic acid and 2 mM ammonium formate (B) at a flow rate of 0.5 ml/min as follows: held at 1 % B for 1 min, linearly increased to 95 % B within 7.0 min and held for 1.0 min; initial conditions were restored within 0.1 min and the column was re-equilibrated for 1.9 min. The autosampler and column oven temperature were set at 6 and 40 °C, respectively. Two microliters were injected into the LC–QTOF-MS system. HyStar ver. 3.2 and DataAnalysis ver. 4.2 [including the software tool SmartFormula] software (Bruker

Daltonik, Bremen, Germany) were used for data acquisition and evaluation, respectively. The MS instrument was operated in positive ionization mode acquiring spectra in the range of *m/z* 30–1000. The dry gas temperature was set at 200 °C with a dry gas flow of 8.0 l/min. The nebulizer gas pressure was 200 kPa. Full scan and broadband collision-induced dissociation (bbCID) data was acquired in one run. The sample solutions (10 µg/ml each) were diluted with the eluent A/B (50:50, v/v or 1:10 v/v). pHLM samples were diluted twofold in eluent A/B (50:50, v/v). The injection volume was 2 µl.

The LC–MS-MS system used was a Shimadzu Nexera UHPLC (Shimadzu, kyoto, Japan) coupled to a QTrap 5500 (AB Sciex, Darmstadt, Germany) triple-quadrupole linear iontrap mass spectrometer fitted with a TurboIonSpray interface. Analyst® Software ver. 1.6.2 was used for data acquisition and evaluation (AB Sciex). The same column, guard column, eluents, and gradient elution were used as described above. The injection volume was 10 µl.

For the research chemical compounds **2** and **3** (crystalline specimens), IR spectra were recorded on a Nicolet 380 Fourier-transform IR spectrometer with Smart Golden Gate Diamond attenuated total reflectance (Thermo Fisher Scientific, Waltham, MA, USA). The wavelength resolution was set to 4 cm^{-1}. The IR spectrum was collected in a range of 650–4000 cm^{-1} with 32 scans/spectrum.

Clonazolam (**1**) and meclonazepam (**4**) were extracted with $CHCl_3$. The extracts were analyzed on a GC–solid-phase IR system consisting of an Agilent GC 7890B with an Agilent G4567A probe sampler (Agilent, Santa Clara, CA, USA) and a DiscovIR-GC (Spectra Analysis, Marlborough, MA, USA). Separated compounds were frozen out on a liquid-nitrogen-cooled spirally rotating ZnSe plate and IR spectra were directly recorded through the ZnSe disk with a nitrogen-cooled mercury cadmium telluride detector.

GC parameters were: splitless injection; injection port temperature, 220 °C; column, DB-1 fused silica capillary (30 m × 0.32 mm i.d., 0.25 µm film thickness; Agilent); carrier gas, helium (flow rate, 2.5 ml/min); oven temperature, 80 °C for 2 min, ramped to 280 °C at 15 °C/min for meclonazepam (**4**) or to 290 °C at 15 °C/min for clonazolam (**1**), and held at the final temperature for 15 min; transfer line heater, 280 and 290 °C, respectively. IR conditions were: oven temperature, restrictor temperature, disk temperature, and dewar cap temperature, 280, 280, −40, and 35 °C, respectively; vacuum, 0.2 mTorr; disk speed, 3 mm/s; spiral separation, 1 mm; wavelength resolution, 4 cm^{-1}; IR range 650–4000 cm^{-1}; acquiring time, 6 s/file and 64 scans/spectrum. Data was processed with GRAMS/AI Ver. 9.1 Grams Spectroscopy Software Suite (2009, Thermo Fisher Scientific).

Identification of the main in vitro metabolites

To investigate the metabolism of compounds **1**–**4**, in vitro experiments with pHLMs (1 mg/ml) were performed. The pHLMs were incubated with 20 µM of each substance for 30 min at 37 °C. Blank pHLM samples, used as negative controls, were processed the same way.

Enhanced product ion (EPI) scan experiments, with the hypothetical masses of potential phase I metabolites selected as precursor masses, as well as QTOF analysis (fullscan/bbCID), were conducted. Mono- and dihydroxylation, carboxylation, desalkylation, dehalogenation (not for deschloroetizolam), reduction of the nitro group to an amine (for clonazolam and meclonazepam), and combinations of these reactions were anticipated as possible phase I biotransformations.

Results and discussion

Identification of compound 1

LC–QTOF-MS measurements confirmed the molecular formula of $C_{17}H_{12}ClN_5O_2$. Details on the parent compound and its fragments are shown in Table 1. The major ion signals in GC–EI MS (Fig. 2a) were: *m/z* 353, 324, 249, and 203.

The ^1H NMR spectrum clearly showed the benzodiazepine core structure together with the disubstituted phenyl moiety, and the ^{13}C and ^1H–^{13}C HSQC, and ^1H–^{13}C HMBC NMR data (Supplementary Table S1) further confirmed the compound as clonazolam (6-(2-chlorophenyl)-1-methyl-8-nitro-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) (Fig. 1). The IR spectrum of the free base is shown in Fig. S1.

Identification of compound 2

For compound **2**, the suggested formula for the protonated molecular ion was $C_{17}H_{17}N_4S$ at *m/z* 309.1170 (neutral: $C_{17}H_{16}N_4S$). Details on the parent and the corresponding fragments are given in Table 2. The GC–EI-MS spectrum (Fig. 2b) shows the most abundant signals at *m/z* 308, 279, 239, and 225.

The two protons of the diazepine ring exhibited unusual, broad signals. The monosubstituted phenyl ring and the ethyl and methyl substituents were identified upon further ^1H NMR analysis (Table S2). With the results from the ^{13}C and ^1H–^{13}C HSQC measurements, the structure was confirmed as deschloroetizolam (2-ethyl-9-methyl-4-phenyl-6*H*-thieno[3,2-f][1,2,4]triazolo[4,3-*a*][1,4]diazepine) (Fig. 1). The IR spectrum of the free base is shown in Fig. S2.

Table 1 Monoisotopic accurate masses and elemental compositions of compound **1** and its main metabolites together with their characteristic fragment ions

		Monoisotopic accurate mass	Elemental composition	Electron configuration	Error (ppm)
Parent	Molecular ion $[M + H]^+$ ($m/z = 354$)	354.0752	$C_{17}H_{13}ClN_5O_2$	Even	0.7
	Fragment ($m/z = 326$)	326.0565	$C_{16}H_{11}ClN_4O_2$	Odd	1.0
	Fragment ($m/z = 308$)	308.0823	$C_{17}H_{13}ClN_4$	Odd	1.1
	Fragment ($m/z = 280$)	280.0636	$C_{16}H_{11}ClN_3$	Even	−0.2
	Fragment ($m/z = 171$)	171.0791	$C_{10}H_9N_3$	Odd	0.2
Mono-OH	Molecular ion $[M + H]^+$ ($m/z = 370$)	370.0701	$C_{17}H_{13}ClN_5O_3$	Even	0.2
	Fragment ($m/z = 352$)	352.0596	$C_{17}H_{11}ClN_5O_2$	Even	0.8
	Fragment ($m/z = 325$)	325.0487	$C_{16}H_{10}ClN_4O_2$	Even	−1.8
NH_2[a]	Molecular ion $[M + H]^+$ ($m/z = 324$)	324.1011	$C_{17}H_{15}ClN_5$	Even	0.2

OH hydroxy

[a] Fragments not evaluated because of low intensity

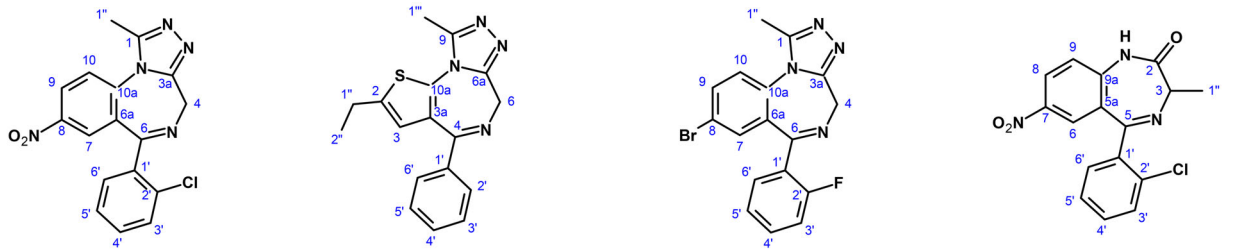

Fig. 1 Structures of the detected compounds (**1**–**4**)

Identification of compound 3

The suggested formula for the protonated molecular ion ($[M + H]^+$) of compound **3** was $C_{17}H_{13}BrFN_4$ (neutral: $C_{17}H_{12}BrFN_4$); see Table 3. The corresponding fragments and the NMR experiments (Table S3) confirmed the compound as flubromazolam (8-bromo-6-(2-fluorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine) (Fig. 1). The major ion signals in GC–MS were: *m/z* 370, 343, and 222 (Fig. 2c), further confirming the structure. The IR spectrum of the free base is shown in Fig. S3.

Identification of compound 4

In GC–EI-MS analysis, the major ion signals for compound **4** were: *m/z* 328, 294, 286, 248, and 240 (Fig. 2d). No major signal for the molecular ion at *m/z* 329 was observed, but one at *m/z* 328 was observed. The same phenomenon is known for clonazepam and flunitrazepam [19]. From the acquired exact mass of *m/z* 330.0643 for the protonated molecular ion ($[M + H]^+$) the ion formula of $C_{16}H_{13}ClN_3O_3$ (neutral: $C_{16}H_{12}ClN_3O_3$) was deduced. Table 4 lists details of the parent compound and its fragments.

The aromatic region of the ^1H NMR spectrum of compound **4** was identical to that of clonazolam. The signal groups in the aliphatic region confirmed the methyl-substituted diazepine core. Based on the QTOF and NMR data (Table S4) and supported by the GC–EI-MS analysis, the structure of compound **4** was affirmed as meclonazepam [5-(2-chlorophenyl)-3-methyl-7-nitro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one] (Fig. 1). Because chiral separation was not conducted, absolute configuration of the carbon in the 3-position could not be determined. The IR spectrum of the free base is shown in Fig. S4.

Identification of clonazolam metabolites

Signals at the mass-to-charge ratios corresponding to monohydroxylation and reduction of the nitro group of clonazolam were found in the performed QTOF analysis. A combination of hydroxylation and reduction of the nitro

Table 2 Monoisotopic accurate masses and elemental composition of compound **2** and its main metabolites together with their characteristic fragment ions

		Monoisotopic accurate mass	Elemental composition	Electron configuration	Error (ppm)
Parent	Molecular ion $[M + H]^+$ ($m/z = 309$)	309.1168	$C_{17}H_{17}N_4S$	Even	0.4
	Fragment ($m/z = 280$)	280.0777	$C_{15}H_{12}N_4S$	Odd	−0.3
	Fragment ($m/z = 255$)	255.0950	$C_{15}H_{15}N_2S$	Even	−0.3
	Fragment ($m/z = 226$)	226.0685	$C_{14}H_{12}N_2$	Even	2.2
	Fragment ($m/z = 206$)	206.0747	$C_{10}H_{12}NS$	Even	−0.1
	Fragment ($m/z = 165$)	165.0481	$C_8H_9N_2S$	Even	−0.5
Mono-OH A	Molecular ion $[M + H]^+$ ($m/z = 325$)	325.1118	$C_{17}H_{17}N_4OS$	Even	−0.3
	Fragment ($m/z = 307$)	307.1012	$C_{17}H_{15}N_4S$	Even	−1.3
	Fragment ($m/z = 297$)	297.0930	$C_{16}H_{15}N_3OS$	Odd	−0.2
	Fragment ($m/z = 281$)	281.0855	$C_{15}H_{13}N_3OS$	Even	−1.7
	Fragment ($m/z = 271$)	271.0900	$C_{15}H_{15}N_2OS$	Even	−1.3
	Fragment ($m/z = 248$)	248.1056	$C_{15}H_{12}N_4$	Odd	0.2
Mono-OH B	Molecular ion $[M + H]^+$ ($m/z = 325$)	325.1118	$C_{17}H_{17}N_4OS$	Even	−0.4
	Fragment ($m/z = 307$)	307.1012	$C_{17}H_{15}N_4S$	Even	3.8
	Fragment ($m/z = 271$)	271.0900	$C_{15}H_{15}N_2OS$	Even	−2.4
Mono-OH C	Molecular ion $[M + H]^+$ ($m/z = 325$)	325.1118	$C_{17}H_{17}N_4OS$	Even	−0.6
	Fragment ($m/z = 307$)	307.1012	$C_{17}H_{15}N_4S$	Even	−0.9
	Fragment ($m/z = 280$)	280.0903	$C_{16}H_{14}N_3S$	Even	−0.2
	Fragment ($m/z = 266$)	266.0746	$C_{15}H_{12}N_3S$	Even	0.0
Di-OH	Molecular ion $[M + H]^+$ ($m/z = 341$)	341.1067	$C_{17}H_{17}N_4O_2S$	Even	0.0
	Fragment ($m/z = 287$)	287.0849	$C_{15}H_{15}N_2O_2S$	Even	0.9

group or dihydroxylation as further possible biotransformations were not observed. Table 1 lists the detected metabolites of clonazolam and the main fragments of the parent and the monohydroxylated metabolite. Because of the rather low MS intensity for 8-aminoclonazolam, evaluation of the fragments was not possible. EPI scanning showed a monohydroxylated metabolite (Fig. S5b), but no signals corresponded to the mass-to-charge ratio of 8-aminoclonazolam or any other predicted metabolites. By analogy with other triazolobenzodiazepines, hydroxylation is most likely to occur at the α- and 4-positions. Even though the assumed α-hydroxy- and 4-hydroxy-clonazolam metabolites may undergo different fragmentation in the conducted bbCID experiments, we could not distinguish which of the two compounds was predominately formed in vitro. Consequently, to unambiguously assess the position of hydroxylation, NMR techniques are needed.

Identification of deschloroetizolam metabolites

For deschloroetiziolam, signals of four possible metabolites (three monohydroxylated and one dihydroxylated) were detected by the QTOF analysis (Table 2) and in the EPI experiments (Fig. S6b–e). Assuming similarities in the metabolism of deschloroetizolam and etizolam, hydroxylation most likely occurs at the 9-methyl and 2-ethyl moeities or at the 6-position of deschloroetizolam [20]. Whichever combination of the aforementioned hydroxylation reactions leads to the dihydroxylated metabolite remains unclear. Again, NMR experiments are required to definitely identify the position of hydroxylation.

Identification of flubromazolam metabolites

In the QTOF data and EPI scans (Fig. S7b–c), signals corresponding to mass-to-charge ratios of a monohydroxylation and dihydroxylation were observed. Table 3 lists the accurate masses of the compounds and their respective main fragments. By analogy with other triazolobenzodiazepines, hydroxylation most likely occurs at the α- and 4-positions of the molecule. Accordingly, the dihydroxylated metabolite is most likely 8-bromo-6-(2-fluorophenyl)-1-(hydroxymethyl)-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepin-4-ol. A distinction on which of the

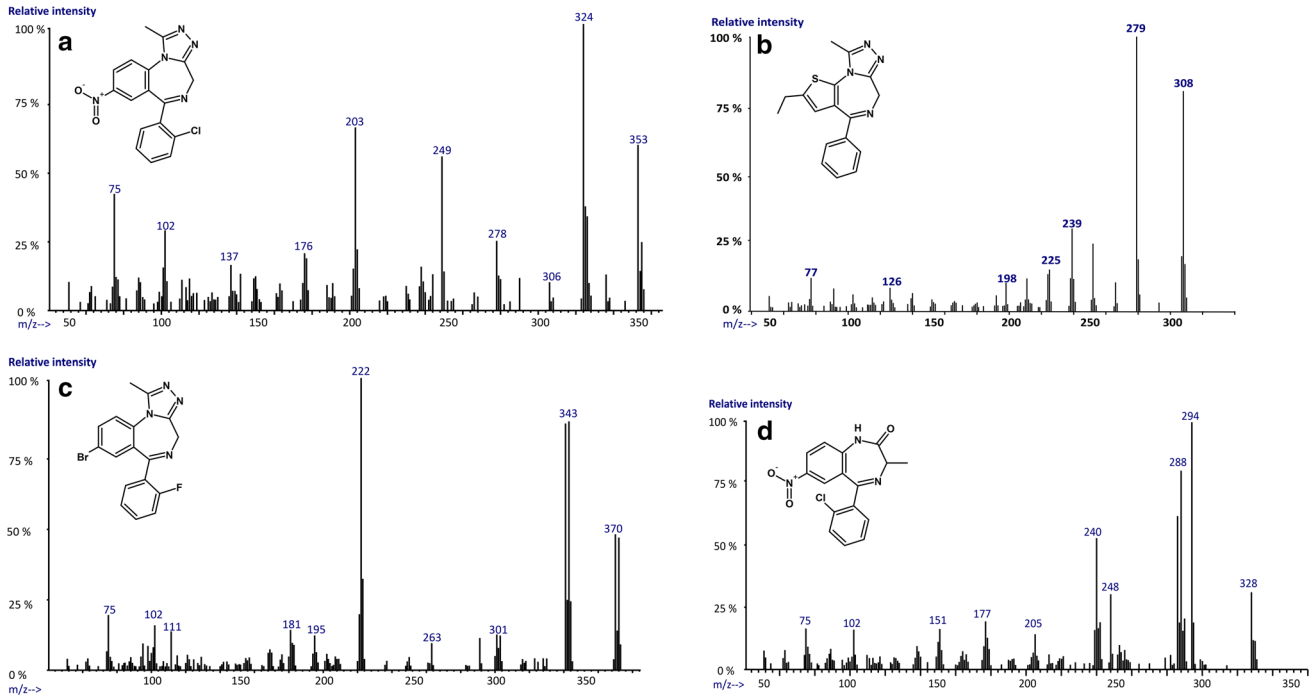

Fig. 2 Gas chromatography–electron ionization-mass spectra of clonazolam (**a**, compound **1**), deschloroetizolam (**b**, compound **2**), flubromazolam (**c**, compound **3**), and meclonazepam (**d**, compound **4**)

Table 3 Monoisotopic accurate masses and elemental composition of compound **3** and its main metabolites together with their characteristic fragment ions

		Monoisotopic accurate mass	Elemental composition	Electron configuration	Error (ppm)
Parent	Molecular ion $[M + H]^+$ ($m/z = 371$)	371.0302	$C_{17}H_{13}BrFN_4$	Even	0.1
	Fragment ($m/z = 343$)	343.0115	$C_{16}H_{11}BrFN_3$	Odd	−0.1
	Fragment ($m/z = 292$)	292.1119	$C_{17}H_{13}FN_4$	Odd	0.0
	Fragment ($m/z = 171$)	171.0791	$C_{10}H_9N_3$	Odd	−0.3
Mono-OH	Molecular ion $[M + H]^+$ ($m/z = 387$)	387.0251	$C_{17}H_{13}BrFN_4O$	Even	0.8
	Fragment ($m/z = 369$)	369.0145	$C_{17}H_{11}BrFN_4$	Even	−0.4
	Fragment ($m/z = 359$)	359.0064	$C_{16}H_{11}BrFN_3O$	Odd	0.3
	Fragment ($m/z = 290$)	290.0692	$C_{17}H_{11}FN_4$	Odd	1.2
Di-OH	Molecular ion $[M + H]^+$ ($m/z = 403$)	403.0200	$C_{17}H_{13}BrFN_4O_2$	Even	0.6
	Fragment ($m/z = 385$)	385.0095	$C_{17}H_{11}BrFN_4O$	Even	0.8

predicted monohydroxylated metabolites was formed was not possible based solely on the obtained mass spectrometric data. To unambiguously identify the site of hydroxylation, NMR measurements are required. The same is also true for the assumed 1,4-dihydroxy-flubromazolam metabolite.

Identification of meclonazepam metabolites

A signal at the mass-to-charge ratio corresponding to a monohydroxylation was found in the performed QTOF analysis and the EPI scan (Fig. S8b). In addition, a signal indicating reduction of the nitro group of meclonazepam was found in the QTOF data. A combination of these two reactions was not observed with either analysis. The MS signal indicating reduction of the nitro group most likely represents 7-amino-5-(2-chlorophenyl)-3-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one. For the formation of monohydroxylated metabolites of meclonazepam, the 3-methyl position can be presumed as the preferred site of hydroxylation. However, NMR spectroscopy is needed to verify the proposed metabolite. In addition, no conclusions

Table 4 Monoisotopic accurate masses and elemental composition of compound **4** and its main metabolites together with their characteristic fragment ions

		Monoisotopic accurate mass	Elemental composition	Electron configuration	Error (ppm)
Parent	Molecular ion $[M + H]^+$ ($m/z = 330$)	330.0640	$C_{16}H_{13}ClN_3O_3$	Even	−0.1
	Fragment ($m/z = 316$)	316.0609	$C_{16}H_{13}ClN_2O_3$	Odd	1.6
	Fragment ($m/z = 302$)	302.0691	$C_{15}H_{13}ClN_3O_2$	Even	0.4
	Fragment ($m/z = 284$)	284.0711	$C_{16}H_{13}ClN_2O$	Odd	1.1
	Fragment ($m/z = 239$)	239.0496	$C_{15}H_{10}ClN$	Odd	2.3
Mono-OH	Molecular ion $[M + H]^+$ ($m/z = 346$)	346.0589	$C_{16}H_{13}ClN_3O_4$	Even	0.3
	Fragment ($m/z = 300$)	300.0660	$C_{16}H_{13}ClN_2O_2$	Odd	1.4
NH_2	Molecular ion $[M + H]^+$ ($m/z = 300$)	300.0898	$C_{16}H_{15}ClN_3O$	Even	2.1
	Fragment ($m/z = 255$)	255.0684	$C_{15}H_{12}ClN_2$	Even	7.2

can be drawn on the absolute configuration of the carbon at the 3-position.

Conclusions

The four benzodiazepines clonazolam, deschloroetizolam, flubromazolam, and meclonazepam were structurally characterized, and their respective in vitro main phase I metabolites were tentatively identified. Certainly, all described metabolites are prone to undergo further phase II metabolic transformations in vivo, such as *O*- and *N*-glucuronidation, and acetylation of the amino moiety of the respective metabolites of clonazolam and meclonazepam. Future studies should include verification of the proposed positions of hydroxylation, comparison of the identified metabolites with metabolites formed in vivo, and assessment of basic pharmacokinetic data.

Because clonazolam and flubromazolam are triazolobenzodiazepines and the amounts of active ingredients in the marketed drug formulations are relatively low (e.g., 0.25 mg flubromazolam on blotters and 0.5 mg clonazolam capsules), we assume that these compounds show rather high pharmacological potencies. Consequently, this makes it difficult to accurately dose the drug when obtaining these compounds as research chemicals, thus bearing a high risk for potential consumers to overdose. Furthermore, the assumed blood concentrations of these potent and low-dosed drugs can be expected to be relatively low, making it particularly difficult to detect and identify these drugs in biological samples. This poses a challenge for toxicologists analyzing samples of suspected drug-facilitated crime victims or driving-under-the-influence offenders. This problem can be addressed by the use of up-to-date MS-based screening and quantitation procedures, but probably persists for the widely used immunoassays. Furthermore, with benzodiazepines having become an established substance class on the designer drug market, designer "Z-drugs" might follow. Benzodiazepines are a rather safe class of drugs; however, for most of the designer benzodiazepines, no clinical trials are published and severe side effects or unexpected toxicities cannot be ruled out.

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