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***In vitro* Phase I and Phase II metabolism of the new designer benzodiazepine cloniprazepam using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry**

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Abstract

Designer benzodiazepines have recently emerged as a class of new psychoactive substances. These substances are used in recreational settings and as alternatives to prescription benzodiazepines as self-medication for patients suffering from anxiety or other mental disorders. Due to the limited information available on the metabolic fate of these new substances, it is challenging to reliably detect their usage in bioanalytical (e.g. clinical and forensic) settings. The objective of this study was to investigate the *in vitro* Phase I and Phase II metabolism of the new designer benzodiazepine cloniprazepam and identify potential biomarkers for its detection in human biological fluids. Cloniprazepam was incubated with human liver microsomes and cytosolic fractions to generate both Phase I and II metabolites. The extracts were analysed using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. Identification of the metabolites was performed using two complementary workflows, including a suspect screening based on *in silico* predictions and a non-targeted screening. A total of nine metabolites were identified, eight Phase I metabolites and one Phase II metabolite, of which five were specific for cloniprazepam. Clonazepam was the major metabolite of cloniprazepam. Hydroxy-cloniprazepam, dihydroxy-cloniprazepam, 3-keto-cloniprazepam, 7-amino-cloniprazepam, hydroxy-clonazepam, 7-amino-clonazepam and 3-hydroxy-7-amino-clonazepam were formed through oxidation, hydroxylation, and/or reduction of the nitro-group. Glucuronidated hydroxy-cloniprazepam was the only Phase II metabolite detected. Five metabolites were specific for cloniprazepam. This study provided a set of human *in vitro* biotransformation products which can assist specific detection of cloniprazepam consumption in future studies.

34 Keywords: cloniprazepam, designer benzodiazepines, *in vitro* biotransformation, human liver
35 microsomes, cytosolic fractions, liquid chromatography-mass spectrometry, high resolution-mass
36 spectrometry

1. Introduction

Designer benzodiazepines have recently emerged as a class of new psychoactive substances (NPS), readily available on the internet or in “smart shops”. Due to legislation issues it is legal to use or possess these substances. Unlike other classes of NPS, benzodiazepines have received relatively limited attention although their use has increased significantly in the past few years [1-5]. As a sedative, anti-convulsant, muscle relaxant and anxiolytic, benzodiazepines are among the most prescribed drugs around the world for the treatment of certain forms of sleep disorders, epilepsy, different anxiety disorders and other psychiatric conditions [5-7]. Self-medication is regarded as common behaviour among individuals suffering from these disorders and given the accessibility and low price of these designer drugs; they offer an alternative to prescription benzodiazepines [2, 8]. Besides for the treatment of different mental conditions, benzodiazepines are also used in drug-facilitated crimes or in combination with other drugs to enhance or prolong the delirium and/or to reduce possible withdrawal effects [2, 9].

Since designer benzodiazepines have not been approved for medicinal use in any country, the majority have not undergone clinical trials and there is limited or no information available regarding their pharmacokinetics and biotransformation. Consequently, the detection of their use through the analysis of biological matrices is challenging. Phase I and Phase II biotransformation products are very likely to be the urinary biomarkers of consumption [10, 11]. Moreover, metabolites can also be responsible for the activity or toxic effects of the substance [12]. The two main biotransformation pathways for benzodiazepines are generally oxidation and glucuronidation [13]. To date, there is only little information available on the human *in vitro* metabolism of cloniprazepam.

Cloniprazepam is a designer benzodiazepine and a potential prodrug of the high-potency and extensively metabolized clonazepam, a prescription benzodiazepine [7, 10, 14-17]. Considering the known metabolism pathways of other 7-nitro-benzodiazepines (e.g. clonazepam, flunitrazepam and nitrazepam) cloniprazepam is expected to be converted to its respective 7-amino metabolite by CYP3A4 and subsequently to the 7-acetamido metabolite by the cytosolic enzyme N-acetyl-transferase 2 (NAT2) [10, 18-23]. Moosman *et al.* studied the *in vitro* biotransformation of cloniprazepam, but this study was limited to Phase I microsomal metabolism by using low resolution mass spectrometry [2, 10, 13] that does not provide a full, comprehensive screening with high mass-accuracy for the purpose of metabolite identification and characterisation nowadays [11, 24-26]. While benzodiazepines are largely excreted as Phase II metabolites (glucuronide conjugates among others) [27], it is not known whether cloniprazepam would undergo similar biotransformation. Overall, there is a clear need to better understand the metabolism of cloniprazepam, as well as the use of high-resolution mass spectrometry (HRMS) to perform full screening and structural elucidation for such purpose.

Pooled human liver microsomes (HLMs) are an appropriate source of enzymes for studying *in vitro* human metabolism [12], as they contain the major drug-metabolizing enzymes, particularly the cytochrome P450s (CYPs) and UDP-glucuronosyltransferases (UGTs) [28-31]. The use of HLMs is advantageous over other preclinical biotransformation experiments since biotransformation characteristics are species-dependent. In comparison to *in vitro* hepatocyte cultures, HLMs do not require a sterile environment, making them an easy, cheap and quick tool for a first metabolism screening [32].

The key objective of this study was therefore to investigate the *in vitro* Phase I and Phase II metabolism of cloniprazepam and examine which metabolites can serve as specific biomarkers for the detection of cloniprazepam use through the analysis of biological matrices. Liquid chromatography coupled to accurate-mass quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) was used to screen, identify and structurally elucidate metabolites that were formed by incubating cloniprazepam with HLMs and human liver cytosol fractions.

2. Materials and methods

2.1. Chemicals and reagents

The analytical reference standard of cloniprazepam ((5-(2-chlorophenyl)-1-(cyclopropylmethyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one), purity 99.5±0.5 %) was purchased from Chiron AS (Trondheim, NO). It was dissolved in methanol (MeOH) to obtain a stock solution at the concentration of 1004 ng/μL. The analytical reference standard of clonazepam (5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one, purity 99.95 %) was purchased from LGC GmbH (Luckenwalde, GER). The analytical reference standard of 7-amino-clonazepam (7-Amino-5-(2-chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one, purity 99.9±0.5 %) was purchased from Chiron AS (Trondheim, NO). Pooled human liver microsomes (HLMs, mixed gender, n=50) were acquired from Tebu-Bio (Boechout, Belgium). Pooled human liver cytosol (HLCYT), theophylline (anhydrous, > 99 %), 2,5-uridinediphosphate glucuronic acid (UDPGA), adenosine-3'-phosphate 5'-phosphosulfate (PAPS, > 60 %) lithium salt hydrate, alamethicin (neat, > 98 %), dimethyl sulfoxide (DMSO), and 4-nitrophenol (4-NP) were obtained from Sigma-Aldrich (Missouri, US). NADPH tetrasodium salt hydrate (> 96 %) was purchased from Acros (Geel, Belgium). Acetonitrile (ACN, HPLC-grade) was acquired from Fisher Chemical (Loughborough, GB), formic acid (> 98 %) from Merck KGaA (Darmstadt, DE). A 100 mM TRIS-buffer was prepared by dissolving 12.11 g Trizma base (Janssen Chimica, Beerse, BE) and 1.02 g MgCl₂ (Merck KGaA, Darmstadt, DE) in 1 L ultrapure water. The pH was adjusted to 7.4 by adding 1 M HCl solution. Ultra-pure water was produced in-house with a PURELAB-purifier system of Elga Labwater (Tienen, BE).

2.2. *In vitro* metabolism assay

This study employed the *in vitro* assay optimised and used in our previous studies [24, 33-35]. An overview of the experimental setup can be found in the supporting information (SI). All tested sample sets contained three replicates. Briefly, Phase I metabolites were generated using pooled HLMs. Hereto the reaction mixture with 945 μL of TRIS-buffer (pH 7.4, 100 mM), 25 μL of HLM (20 mg/mL) and 20 μL of cloniprazepam stock solution (0.5 mM) was incubated in a 1.5 mL Eppendorf tube at 37 °C. A 10 μL of NADPH (0.1 M) was added after 5, 60 and 120 min. Three negative control samples (without cloniprazepam, HLM or NADPH) were prepared in parallel. The reaction was stopped after one or three hours by the addition of 250 μL ice-cold ACN with 1% formic acid (Phase I metabolites) or by putting the samples on ice for 3 min (samples for further Phase II metabolism). The internal standard, theophylline, was prepared in the ice-cold ACN with 1% formic acid at 5 $\mu\text{g/mL}$. A positive control was obtained by incubating 10 μL phenacetin (5 $\mu\text{g/mL}$). Three Phase I metabolites of phenacetin, including N-(4-hydroxyphenyl)-acetamide (P1), N-(4-ethoxy-2-hydroxyphenyl)-acetamide (P2) and 4-ethoxy-benzenamine (P3), were monitored.

Following the *in vitro* Phase I metabolism, samples were exposed to Phase II conjugation by either glucuronidation (GLU) or sulfation (SUL). Phase I samples were centrifuged for 5 min at 8000 rpm. For the GLU samples, 935 μL of the supernatant originating from the Phase I samples was incubated with 25 μL of HLM and 10 μL of alamethicin (1 mg/mL in DMSO). 10 μL of UDPGA (100 mM) was added after 5, 60 and 120 min of incubation. SUL samples consisted of 965 μL of supernatant and 25 μL of cytosol (20 mg/mL), with addition of 10 μL of PAPS (10 mM) after 5, 60 and 120 min of incubation. Negative control samples were prepared by omitting respectively cloniprazepam or the cofactor (UDPGA and PAPS) in order to exclude false-positive results. Positive control samples were spiked with 1 μL of 4-NP (10 mM) and the formation of 4-nitrophenol glucuronide and 4-nitrophenol sulfate was monitored. The reaction was stopped by adding 250 μL of ice-cold 1 % (v/v) formic acid in ACN with internal standard theophylline (5 $\mu\text{g/mL}$).

In preparation for LC-QTOF-MS analysis, all samples were centrifuged for 5 min at 8000 rpm. The supernatant was transferred to a clean glass tube, evaporated under nitrogen at 38 °C, and reconstituted in 200 μL of a 10:90 (v/v) ACN/water solution.

2.3. LC-QTOF-MS analytical method

The samples were analysed using an Agilent 1290 Infinity UPLC hyphenated to an Agilent 6530 QTOF (Agilent, Santa Clara, US). Chromatographic separation was performed on a Kinetex C8 (150 x 2.1 mm; 1.7 μm particle size, Phenomenex, the Netherlands) using a mobile phase composed of ultra-pure

water with 0.04 % (v/v) formic acid (A) and 80/20 (v/v) acetonitrile/ultrapure water with 0.04 % formic acid (v/v on total volume) (B). The injection volume was 1 μ L. All samples were analysed in positive and negative ionisation mode. The eluent was directed to the waste during the first minute of each run to protect the ion source from extensive contamination. For the analysis in positive ionisation mode, the chromatographic run started 2 min isocratic at 5 % B, then a gradient was used to increase the percentage B to 40 % at 6 min, a second gradient augmented the percentage of B to 85 % at 15 min. The gradient was adjusted for the analysis in negative mode: after 2 min at 5 % B, a gradient increased the concentration of B to 85 % at 21 min. Both methods used a flow rate of 0.3 mL/min. For both gradients, the column was rinsed with 95 % of B for 3 min and re-equilibrated at 5 % B for 6 min before the next injection. Column temperature was kept constant at 40 °C.

In all the analyses, the QTOF-MS instrument was operated in the 2 GHz (extended dynamic range) mode, providing a Full Width at Half Maximum (FWHM) resolution of approximately 5100 at m/z 118.0862 and 10000 at m/z 922.0098. The ions 112.0508 and 922.0097 for positive mode and 112.9855 and 966.0007 for negative mode were selected for a constant recalibration throughout the chromatographic run to ensure good mass accuracy. The eluting compounds were ionised using Agilent Jet-Stream electrospray ionisation (AJS-ESI) under the following parameters in both positive and negative ionisation mode. Drying gas temperature and flow were at 300 °C and 8 L/min, respectively. The sheath gas temperature was 350 °C at a flow of 11 L/min. Nebulizer pressure was set at 25 psig (172.37 Pa). Capillary, nozzle and fragmentor voltages were at 3500 V, 500 V and 120 V, respectively. The acquisition parameters were set for the m/z values to range from 75 to 1000 at a scan rate of 2.5 scans/s and 6.67 scans/s for MS and MS/MS spectra, respectively. Collision energies were applied at 10, 20 and 40 V. Signals were detected using a data-dependent acquisition method with an isolation width of 1.3 amu. An active exclusion of 0.2 min was applied to prevent repetitive acquisition of MS/MS spectra for the same ion. Additional injections in targeted MS/MS using selected precursors were necessary in order to obtain high-quality MS/MS spectra for some biotransformation products. Collision energies and isolation width were equal to the data-dependent acquisition method. All data were stored in centroid mode and exported for analysis.

2.4. Data processing and analysis

This study followed the data processing workflow developed in our previous studies [11, 24, 25]. In brief, data interpretation was conducted with two complementary workflows for enhanced metabolite detection and identification. First, a suspect screening approach using *in silico* metabolite prediction was applied. A list of possible metabolites was generated using the Meteor Nexus (v2.1, Lhasa Limited, Leeds, UK) software. For Phase I metabolism, all redox and non-redox biotransformations were

selected. For Phase II metabolism, *O*- and *N*-glucuronidation, *O*- and *N*-sulfation, acetylation and conjugation with amino acids were selected. The metabolism prediction was processed with human CYP enzymes, the maximum depth set on 3 and the maximum number of metabolites set at 1000. The generated list of possible metabolites with the molecular formula, exact mass, structure and related enzyme for the corresponding metabolism was stored as a csv-database. Furthermore, the metabolites of other similar benzodiazepines (e.g. clonazepam, meclonazepam etc.) described in the literature that were not predicted were added to the list as to obtain a database for the suspect screening.

Secondly, a non-targeted screening approach was conducted with the metabolomics software MZmine 2.29 [36]. This allowed a comparison between different sets of samples for the detection and differentiation of MS features (e.g. spectra and masses) in the treated *in vitro* samples from the control samples through various algorithms. First, *m/z* features were detected using the centroid algorithm followed by a chromatogram building step. Resulting chromatograms were deconvoluted using the noise amplitude algorithm. Further simplification occurred by deisotoping, keeping the lowest *m/z* value as the representative isotope. During the deisotoping, chromatographic peaks were also filtered according to peak width: only peaks with a width between 0.05 and 1 min were retained. Next, peaks were aligned across samples using the Random Sample Consensus (RANSAC) alignment algorithm. Finally, using the *Same RT and m/z range gap filler* algorithm any missing peaks were re-iteratively extracted. The obtained *m/z* features were then visualised through a volcano plot using the GraphPad Prism software (version 7.0), for which an example can be found in the Supplementary Information. From the plot, potential *m/z* values were selected based on a fold-change of the peak intensity of the replicate samples versus their respective negative controls. Significance of the fold change was tested with a student t-test and all *m/z* features in which a fold change > 10 in the treated samples than the control samples and *p*-value < 0.05 were selected.

The relevant *m/z* values generated from the two approaches were extracted using MassHunter Workstation software (Agilent Technologies). The Find by Formula algorithm was used to detect the predicted metabolites obtained in the suspect screening approach. The *Find by Auto-MS/MS* and *Generate Formulas* algorithms were used to extract the molecular features for the masses sorted out from the non-target screening approach.

Identification of the metabolites was based on the accurate mass and isotopic profile obtained in the MS mode and also the fragmentation pattern of product ions and their accurate mass, with the following criteria set in our previous studies [34, 35, 37]: (a) a maximal mass variation of ± 10 ppm between the measured and theoretical parent ions; (b) a maximal mass variation of ± 25 ppm for product ions; (c) the measured isotope pattern matched with the predicted ones with an isotope abundance score of at least 70; (d) the measured double bond equivalent values (DBE) agreed with the postulated structure; (e) the identified metabolites were not present in all the negative control

samples; and (f) the detected metabolites had to be present in at least two out of three replicates. Where possible, further confirmation of the identified metabolites was also conducted by injection of analytical standard reference solutions on the LC-QTOF-MS system.

3. Results and discussion

3.1. Experimental quality controls

Phenacetin was used as the positive control compound for Phase I experiments. Three Phase I metabolites of phenacetin resulting from oxidative dealkylation, hydroxylation and hydrolysis were monitored (see MS and MS/MS spectra in SI-2.1). 4-nitrophenol glucuronide and 4-nitrophenol sulfate were monitored as the positive control for Phase II experiments (See SI-2.2). The obtained results from the positive control samples confirmed the performance of the experimental assays in our study. Also, using the internal standard response in the samples throughout the analysis, the instrumental variation was assessed at about 20 % (relative standard deviation). All identified metabolites were cross-checked with the negative control samples to eliminate false positive results.

3.2. Cloniprazepam

Cloniprazepam was detected in the positive ionisation mode as the protonated molecule ($[M+H]^+$, m/z 370.0953) and eluted at 12.21 min (Fig. 1a). Fig. 1b and Fig. 1c showed the fragmentation pattern of cloniprazepam. The most intense product ion at m/z 316.0430 corresponded to the loss of the methylcyclopropyl side chain. Further loss of NO_2 led to the second most intense product ion at m/z 270.0559. The fragments at m/z 241.0548 and 214.0452 were explained by further ring modifications. Identification was further confirmed with the injection of an analytical reference standard.

3.3. Phase I metabolite identification

An overview of all identified metabolites with the confidence levels as proposed by Schymanski *et al.* can be found in Table 1. [38] Chromatograms of all identified metabolites can be found in Fig. 2. MS-MS spectra of all identified metabolites can be found in Supplementary Information.

Hydroxy-cloniprazepam (M7)

Three hydroxylation products (M7-A, B and C) of cloniprazepam were detected in positive ionization mode as the protonated molecule ($[M+H]^+$, m/z 386.0823) at the retention time between 9.7 and 11.3

min. This suggested the presence of different isomers representing multiple positions of the hydroxy groups.

The most abundant metabolite (M7-C) eluted at 11.23 min and was the only compound showing an ion representing the loss of water (m/z 368.0756). As Ramanathan *et al* have shown, the loss of water from analytes in LC-MS/MS is predominant for aliphatic hydroxylation, whereas this loss was not favored when the hydroxylation occurred on the phenyl groups [39, 40]. Presence of the m/z 55.0540 product ion, corresponding to the methylcyclopropyl side chain, demonstrated that hydroxylation did not occur on this side chain leaving the 3-position as the only aliphatic site for the hydroxylation. Ions representing losses from the protonated molecule confirmed the identification of this compound as the 3-hydroxy metabolite. Loss of NO_2 resulted in m/z 340.0776. Loss of the methylcyclopropyl side chain resulted in m/z 332.0320, with a sub-sequential loss of NO_2 resulting in m/z 286.0316.

The other two hydroxylated cloniprazepam metabolites (M7-A and M7-B) eluted at 10.56 and 10.91 min, respectively. Both metabolites showed m/z 332.0457 as the most intense product ion, corresponding to the hydroxy-clonazepam. This excluded the methylcyclopropyl position for the hydroxylation. The remaining possible positions for the hydroxylation were the phenolic 6, 8, 9 or 16-19 positions (Figure 1A). The exact position of the hydroxylation of M7-A and M7-B could not be elucidated with the acquired MS/MS data.

Clonazepam (M5)

M5 eluted at 9.43 min and was detected as the protonated molecule ($[\text{M}+\text{H}]^+$) at m/z 316.0483. The mass difference of 54.047 Da compared to cloniprazepam corresponded to the absence of the methylcyclopropyl side chain. The m/z 270.0581, 241.0548 and 214.0452 fragments were observed both for this metabolite and cloniprazepam. The absence of the fragment at m/z 55.0553 confirmed the lack of the methylcyclopropyl chain. Based on the MS/MS fragmentation pattern, obtained DBE of 12, isotope pattern and mass accuracy (2.92 ppm mass difference), M5 was identified as clonazepam. This finding was confirmed with the analysis of clonazepam analytical standard.

Hydroxy-clonazepam (M3)

Two hydroxylated clonazepam compounds (M3-A and B) were detected in positive ionization mode as the protonated molecule ($[\text{M}+\text{H}]^+$, m/z 332.0401). Retention times for M3-A and M3-B were 8.37 and 8.55 min respectively. M3-B was the most abundant compound of the two and was the only one that showed a product ion according to the loss of water (m/z 314.0301), suggesting 3-hydroxy-clonazepam. Subsequent loss of the aromatic CO resulted in m/z 286.0356 and further loss of NO_2 resulted in m/z 240.0753. For M3-A, the acquired MS/MS data could not provide further information on the exact position of the hydroxylation.

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7-Amino-clonazepam (M2)

M2, with m/z 286.0749 ($[M+H]^+$) eluted at 6.25 min. The 84.03 Da mass difference compared to cloniprazepam corresponded with the loss of the methylcyclopropyl side chain and the reduction of the nitro group to an amine function. Loss of HCl resulted in the product ion with m/z 250.1016. Subsequent loss of the CO on the 2-position resulted in m/z 222.1031. Based on the isotope pattern, DBE of 11, mass accuracy (<1 ppm mass difference) and the retention time of an analytical standard, the identification of M2 was confirmed.

3-keto-cloniprazepam (M8)

The protonated molecule of 3-keto-cloniprazepam ($[M+H]^+$, m/z 384.0750) was identified in positive ionization mode and eluted at 10.62 min. The presence of the product ion with m/z 55.0165 confirmed the presence of the methylcyclopropyl side chain. Loss of NO_2 and CO resulted in the product ions m/z 338.0302 and 356.0404 respectively. A DBE of 14 for this feature was obtained. This matched with the formula and structure of 3-keto-cloniprazepam. In combination with the isotope pattern and mass accuracy (2.6 ppm mass difference), we could identify M8 as 3-keto-cloniprazepam.

Dihydroxy-cloniprazepam (M6)

The dihydroxy-product of cloniprazepam gave rise to multiple peaks in positive ionization mode due to different isomers. The peak at 9.82 min showed the highest intensity and only for this peak MS/MS spectra were available. These MS/MS spectra showed the loss of water resulting in product ion m/z 384.0633. As mentioned above, Ramanathan *et al.* showed that the loss of water is favored when the hydroxylation is on an aliphatic position. Presence of the methylcyclopropyl side chain was confirmed by m/z 55.0541. Loss of this side chain resulted in the product ion with m/z 348.0414. Subsequent loss of NO_2 gave rise to fragment ion at m/z 302.0465. The fragment at m/z 332.0510 corresponded with hydroxycloclonazepam. M6 was the only metabolite identified by the non-target screening method.

3-hydroxy-7-amino-clonazepam (M1)

M1 eluted at 6.25 min as the protonated molecule ($[M+H]^+$, m/z 302.0691). The MS/MS spectrum was recorded and showed the loss of water (m/z 284.0648) and ClOH (m/z 250.1018). A DBE of 11 was calculated, which corresponded with the structure of 3-hydroxy-7-amino-clonazepam.

7-amino-cloniprazepam (M4)

Reduction of the nitro-group of cloniprazepam caused the formation of 7-amino-cloniprazepam. M4 eluted at 9.30 min ($[M+H]^+$, m/z 340.1232). The calculated DBE of 12 was correct according to the structure of 7-amino-cloniprazepam. No MS/MS spectra or analytical reference standard were available to confirm this identification.

3.4. Phase II metabolite identification

Hydroxy-cloniprazepam-glucuronide (M9)

M9 eluted at 13.61 min in negative ionization mode ($[M-H]^-$, m/z 561.1150) and at 9.51 min in positive ionisation mode ($[M+H]^+$, m/z 562.1227). The mass difference of 193.0404 Da corresponded to the hydroxylation of cloniprazepam and subsequent glucuronidation. MS/MS data were acquired in positive ionisation mode. Loss of glucuronic acid resulted in the product ion with m/z 386.0984 which corresponds with hydroxy-cloniprazepam. Further loss of NO_2 resulted gave rise to m/z 340.0887. Mass accuracy (5.58 ppm difference), isotope pattern and the presence of the compound in the glucuronidation replicates, but not in any of the negative controls provide extra certainty to the identification of the metabolite.

3.5. Biotransformation of cloniprazepam compared to other benzodiazepines

Cloniprazepam appears to be mainly metabolized to clonazepam, a benzodiazepine that undergoes extensive biotransformation to its major metabolite 7-amino-clonazepam [19, 22, 23, 41]. Some of the biotransformation products of cloniprazepam identified in our study are in line with those found by Moosmann *et al.*, including 7-amino-cloniprazepam, hydroxy-cloniprazepam, 3-keto-cloniprazepam, clonazepam, hydroxy-clonazepam and 7-amino-clonazepam [10]. However, the current study, using HRMS full screening, identified three additional biotransformation products of cloniprazepam that have not been previously reported: dihydroxy-cloniprazepam (M6), 3-hydroxy-7-amino-clonazepam (M1) and the glucuronidated conjugate of hydroxy-cloniprazepam (M9). Dihydroxy-cloniprazepam (M6) was identified by applying the non-target data analysis while the two other new biotransformation products were identified through the suspected screening using *in silico* prediction. Two of them, dihydroxy-cloniprazepam and the glucuronidated metabolite, are specific to cloniprazepam and are thus candidate biomarkers.

The biotransformation reactions identified for cloniprazepam appear to be similar to other benzodiazepines. N-dealkylation is a major metabolic pathway for cloniprazepam, which is comparable to other benzodiazepines such as prazepam or diazepam [42-44]. The aliphatic hydroxylation is also commonly observed with other 1,4-benzodiazepines such as diazepam [44]. A third important pathway

for cloniprazepam and related benzodiazepines is the reduction of the 7-nitro group to form a 7-amino metabolite [41, 45]. In this study, however, 7-amino-cloniprazepam and 7-amino-clonazepam were found to be only minor metabolites of cloniprazepam and clonazepam. A possible explanation for these results could be that the experiments were carried out in presence of air, which could cause a strongly decreased reduction of the 7-nitro group to a 7-amino group [18, 41]. According to a previous study by Vikingsson *et al.* the 7-amino biotransformation products can be further acetylated to 7-acetamido species by N-acetyltransferase 2 (NAT2), which were not detected in our study. A possible reason could be the absence of this enzyme in HLM opposed to hepatocytes [18]. Also, the necessary cofactor acetyl-CoA for NAT2 was not added in this study.

3.6. Proposed biotransformation pathway

The experimental design in this study allowed to reveal the potential biotransformation pathway of cloniprazepam (Fig. 3) using the profiles for cloniprazepam and the identified metabolites over 1 h and 3 h incubation (Fig. 4). For cloniprazepam, a clear decrease in the response was observed after 1 h with almost no signal left after 3 h. The ratio response was calculated as the peak area of the biotransformation product divided by the peak area of the internal standard. Clonazepam (M5) was the major metabolite after loss of the methylcyclopropyl side chain from cloniprazepam. Clonazepam and cloniprazepam were hydroxylated to hydroxy-clonazepam (M3) and hydroxy-cloniprazepam (M7) respectively. 7-amino-cloniprazepam (M4) and 7-amino-clonazepam (M2) were formed after reduction of the nitro group of cloniprazepam and clonazepam respectively. M2 can be further transformed in 3-hydroxy-7-amino-clonazepam (M1) by hydroxylation. M1, M2 and M3 showed a continuous increase over 3 h incubation. In contrast, the ratio response of M4, M5, M7 and M8 raised after 1 h incubation followed by a decrease after 3 h, suggesting that they are potential intermediate metabolites. This could be explained by the loss of the methylcyclopropyl side chain from M4 and M7, leading to the formation of M2 and M3, respectively. M7 can also be further metabolized by hydroxylation and oxidation resulting in M6 and M8. Glucuronidation of hydroxy-cloniprazepam further resulted in the formation of M9.

Some of the proposed biotransformation products, obtained through a HRMS setup, showed agreement with earlier results of Moosmann *et al.* [10]. In addition to these biotransformation products, the new identified biotransformation products of this study can help forensic laboratories in distinguishing abuse of cloniprazepam from prescribed clonazepam ingestion using dihydroxy-cloniprazepam as an additional biomarker for cloniprazepam. Also, the identified glucuronide in this study can aid in the detection of use of cloniprazepam in studies where no deconjugation step is applied (e.g. wastewater or human pooled urine analysis).

4. Conclusions

This study identified nine *in vitro* metabolites of cloniprazepam with eight Phase I metabolites and one Phase II metabolite. Clonazepam, formed after removal of the methylcyclopropyl side chain, appeared to be the most prominent *in vitro* metabolite of cloniprazepam. Dihydroxy-cloniprazepam (M6), 3-hydroxy-7-amino-clonazepam (M1) and the glucuronidated conjugate of hydroxy-cloniprazepam (M9) are reported for the first time. M4, M6, M7, M8 and M9 were specific for cloniprazepam and can be potentially used as biomarkers in bioanalytical (e.g. forensic and clinical) settings to detect cloniprazepam consumption through the analysis of biological matrices. The detection of one of these five compounds assists in distinguishing consumption of cloniprazepam from clonazepam.

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Conflicts of interest

The authors declare they have no conflicts of interest.

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Fig. 1 Chromatogram (a), MS/MS spectrum (b) and fragmentation pattern (c) of cloniprazepam. Carbon atoms were numbered for easier identification of metabolites.

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506 **Fig. 2** Chromatographic peaks of the detected metabolites. M1: 3-hydroxy-7-amino-clonazepam, M2:
 507 7-amino-clonazepam, M3: hydroxy-clonazepam, M4: 7-amino-cloniprazepam, M5: clonazepam, M6:
 508 dihydroxy-cloniprazepam, M7: hydroxy-cloniprazepam, M8: 3-keto-cloniprazepam.

Fig. 3 Proposed biotransformation pathway of cloniprazepam.

Fig. 4 Ratio response of cloniprazepam (P) and the phase-I metabolites. Ratio response was calculated as the peak area of the parent compound/metabolite divided by the peak area of the internal standard. P: Cloniprazepam, M1: 3-hydroxy-7-amino-clonazepam, M2: 7-amino-clonazepam, M3: hydroxy-clonazepam, M4: 7-amino-cloniprazepam, M5: clonazepam, M6: dihydroxy-cloniprazepam, M7: hydroxy-cloniprazepam, M8: 3-keto-cloniprazepam.

518 **Table 1.** Confidence levels, tentative formula, DBE, measured accurate masses, exact masses, mass measurement errors (Δ , in ppm), precursor
519 ions (^a[M+H]⁺; ^b[M-H]⁻), product ions and proposed structures for cloniprazepam and its biotransformation products after incubation with HLMs

Metabolite ID	Confidence level	RT [min]	Precursor ion (MS-TOF) [m/z]	Exact mass [Da]	Δ [ppm]	Tentative formula	DBE	Product ions (MS/MS-TOF) [m/z]	Proposed structure
P	L1	12.21	370.09573 ^a	369.0880	-1.35	C ₁₉ H ₁₆ ClN ₃ O ₃	13	55.0553; 214.0452; 241.0548; 270.0559; 316.0430	
M1	L3	6.25	302.0689 ^a	301.0618	1.63	C ₁₅ H ₁₂ ClN ₃ O ₂	11	250.1018; 269.0530; 284.0648	
M2	L1	6.25	286.0752 ^a	285.0669	-0.62	C ₁₅ H ₁₂ ClN ₃ O	11	222.1031; 250.1016	
M3-A	L3	8.37	332.0401 ^a	331.0360	-1.02	C ₁₅ H ₁₀ ClN ₃ O ₄	12	277.0328; 260.0291	
M3-B	L2b	8.55	332.0434 ^a	331.0360	1.03	C ₁₅ H ₁₀ ClN ₃ O ₄	12	240.0753; 286.0356; 314.0301	
M4	L4	9.30	340.1231 ^a	339.1138	7.05	C ₁₉ H ₁₈ ClN ₃ O	12	NA	

M5	L1	9.43	316.0492 ^a	315.0411	2.92	C ₁₅ H ₁₀ ClN ₃ O ₃	12	214.0452; 241.0548; 270.0581
M6	L3	9.82	402.0854 ^a	401.0778	1.38	C ₁₉ H ₁₆ ClN ₃ O ₅	13	55.0541; 274.0261; 302.0465; 348.0414; 384.0633
M7-A	L3	10.56	386.0879 ^a	385.0829	-4.18	C ₁₉ H ₁₆ ClN ₃ O ₄	13	332.0457
M7-B	L3	10.91	386.0880 ^a	385.0829	-1.34	C ₁₉ H ₁₆ ClN ₃ O ₄	13	332.0457
M7-C	L2b	11.23	386.0903 ^a	385.0829	<0.1	C ₁₉ H ₁₆ ClN ₃ O ₄	13	55.0540; 368.0756
M8	L2b	10.62	384.0751 ^a	383.0673	2.55	C ₁₉ H ₁₄ ClN ₃ O ₄	14	55.0165; 338.0332; 356.0404
M9	L4 or L5	13.61	562.1233 ^a 560.1100 ^b	561.1150	5.58	C ₂₅ H ₂₄ ClN ₃ O ₁₀	15	340.0887; 386.0984