The AAPS Journal, Vol. 19, No. 3, May 2017 (© 2017)
DOI: 10.1208/s12248-016-0040-x

Research Article

Identifying Metabolites of Meclonazepam by High-Resolution Mass Spectrometry Using Human Liver Microsomes, Hepatocytes, a Mouse Model, and Authentic Urine Samples

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Received 20 October 2016; accepted 28 December 2016; published online 13 January 2017

Abstract. Meclonazepam is a benzodiazepine patented in 1977 to treat parasitic worms, which recently appeared as a designer benzodiazepine and drug of abuse. The aim of this study was to identify metabolites suitable as biomarkers of drug intake in urine using high-resolution mass spectrometry, authentic urine samples, and different model systems including human liver microsomes, cryopreserved hepatocytes, and a mice model. The main metabolites of meclonazepam found in human urine were amino-meclonazepam and acetamido-meclonazepam; also, minor peaks for meclonazepam were observed in three of four urine samples. These observations are consistent with meclonazepam having a metabolism similar to that of other nitro containing benzodiazepines such as clonazepam, flunitrazepam, and nitrazepam. Both metabolites were produced by the hepatocytes and in the mice model, but the human liver microsomes were only capable of producing minor amounts of the amino metabolite. However, under nitrogen, the amount of amino-meclonazepam produced increased 140 times. This study comprehensively elucidated meclonazepam metabolism and also illustrates that careful selection of *in vitro* model systems for drug metabolism is needed, always taking into account the expected metabolism of the tested drug.

KEY WORDS: benzodiazepine; *in vitro* metabolism; LC-MS/MS; new psychoactive substance; toxicokinetics.

INTRODUCTION

Meclonazepam, also known as (S)-3-methylclonazepam, RO 11-3128, or RO 11-3624, is a benzodiazepine developed and patented by Hoffman-La Roche in 1977 (1) and recently introduced as a drug of abuse (2). Benzodiazepines are of forensic interest for several reasons. Many are widely prescribed drugs used for legit purposes, but benzodiazepines are also encountered in drug facilitated sexual assaults (3), over-dose cases, and lethal intoxications, in the latter often together with opioids (4).

The pharmacology of meclonazepam has been investigated in clinical trials as an anxiolytic (5) and, interestingly, as a schistosomicidal drug to treat the parasitic worms *Schistosoma haematobium* and *Schistosoma Mansori* (6,7). Meclonazepam can successfully cure parasitic infections with a single dose of at least 0.3 mg/kg, but clinical tolerance was limited by severe adverse drug effects including drowsiness, dizziness, slurred speech, ataxia, muscle weakness, reduced mental alertness, and lateral nystagmus (7). The effects are dose dependent with a narrow therapeutic window; a dose of 0.1 mg/kg result in no detectable adverse drug effects, 0.3 mg/kg in mild to moderate adverse drug effects, and doses above 0.4 mg/kg give severe adverse drug effects (7). Boyle *et al.* (8) reported that meclonazepam showed adverse drug effects typical of benzodiazepines, such as clumsiness, feebleness, drowsiness, and mental slowness, with the most pronounced effects within 3 h of oral dosing >1 mg as well as amnesia after a 4-mg dose. The adverse drug effects can be reversed by co-administration with flumazenil (9). As an anxiolytic, Ansseau *et al.* (5) report a potency three times that of

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Electronic supplementary material The online version of this article (doi:10.1208/s12248-016-0040-x) contains supplementary material, which is available to authorized users.

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diazepam in the conflict test in rats as well as potency similar to flunitrazepam in the *in vivo* benzodiazepine binding assay.

In a forensic setting, most drug tests are conducted using urine as this is a less intrusive matrix not requiring a medical professional for sampling with an often longer window of detection than in blood (10). The latter seems to hold true for nitro-containing benzodiazepines as illustrated for flunitrazepam by Forsman *et al.* (11). While flunitrazepam was undetectable in urine 12 h after a 0.5-mg dose, at least one metabolite was detected in every case 120 h post-dosing (11), indicating a 10 times longer detection window of metabolites in urine.

There is limited data on the pharmacokinetics of meclonazepam. Based on data from a single patient published by Coassolo *et al.* (12), the bioavailability can be estimated to be close to 100%, volume of distribution to be around 300 L and a plasma half-life approximately 80 h. Ansseau *et al.* (5) report meclonazepam half-life to be 40 h. Coassolo *et al.* (12) claim amino-meclonazepam, 3-methylhydroxy-meclonazepam, and amino-3-methylhydroxy-meclonazepam to be metabolites but provide no data (12). This is in agreement with Huppertz *et al.* (2) who described two metabolites generated in human liver microsome experiments, amino-meclonazepam, and a hydroxylated metabolite. Meyer *et al.* (13) analyzed urine samples of persons with suspected meclonazepam intake and found amino-meclonazepam and acetamido-meclonazepam but did not mention hydroxylated metabolites. This is in agreement with the metabolism of the unmethylated analog of meclonazepam, clonazepam, which is mainly metabolized by CYP3A4 to amino-clonazepam and then further by N acetyl transferase 2 (NAT2) into acetamido-clonazepam (14–16). This metabolic pathway has also been described for other nitro-containing benzodiazepines including flunitrazepam (11) and nitrazepam (15).

In summary, there are three papers (2,12,13) providing possible metabolites of meclonazepam. However, their study designs are neither comparable nor complete and therefore the results are not consistent.

The aim of this study was to conduct a complete and comprehensive metabolite investigation to answer the questions about meclonazepam metabolism using authentic urine samples as well as verifying identified metabolites in an *in vivo* mice model and *in vitro* using human liver microsomes (HLM) and cryopreserved hepatocytes.

MATERIAL AND METHODS

Four authentic urine samples and a blank urine were analyzed by liquid chromatography–quadrupole time of flight mass spectrometry (LC-QTOF-MS). The analysis was repeated after hydrolysis with β-glucuronidase and aryl-sulfatase. The origin of identified metabolites was verified by collection of urine over 24 h from two mice exposed as well as in HLMs and hepatocytes incubated with meclonazepam. All samples were analyzed using the same methodology as the human urine samples.

Analytes and Reagents

Meclonazepam was obtained from Chiron A/S (Trondheim, Norway). Liquid chromatography–mass spectrometry (LC-MS) grade acetonitrile (ACN), formic acid, and methanol were purchased from Fisher Scientific (Gothenburg, Sweden). Ammonium formate was obtained from Fluka (Sigma–Aldrich, Stockholm, Sweden) and β-glucuronidase/arylsulfatase (*Helix pomatia*) from Roche (Mannheim, Germany). Saline (9 mg/mL sodium chloride) was from Braun (Melsungen, Germany) and 99.5% ethanol from Kemetyl (Haninge, Sweden). HLM (UltraPool™, 150-donor-pool) and NADPH regenerating system solution A and B were purchased from Corning (Corning, NY, US), cryopreserved human hepatocytes (LiverPool™, 10-donor-pool), InVitro Gro HT, and InVitro Gro KHB media from BioreclamationIVT (Baltimore, MD, USA). High-purity water was produced in-house in a MilliQ Gradient 10 production unit from Millipore (Billerica, MA, USA).

Human Urine Samples

Four authentic human urine samples from case work at the National Board of Forensic Medicine were analyzed in this study. Two of them were driving under the influence of drugs (DUID) cases (H1, H2), and the other two were from cases involving violent crimes (H3, H4). Cases were identified by an accompanying whole blood sample positive for meclonazepam by LC-MS-QTOF (qualitatively verified, unpublished data).

Aliquots of 100 μL of urine were diluted with 110 μL of mobile phase A (see below) either directly or after hydrolysis with 10 μL beta-glucuronidase/arylsulfatase for 1 h at 40 °C. In the non-hydrolyzed set, 10 μL water was added.

Mice Experiments

Animal studies were performed at the Center of Biomedical Resources at Linköping University. Male C57BL/6N mice of age 7–12 weeks weighing approximately 25 g were obtained from Charles River Laboratories (Sulzfeld, Germany) and were housed 4 per cage in an environmentally controlled room with a 12-h light/dark cycle. The mice had free access to water, containing 25 g/L dextrosol to increase liquid consumption, and food. The protocol was approved by the Swedish Board of Agriculture's Animal Ethics Committee in Linköping, Sweden (Dnr 49–14).

After i.p. injection of 1 mg/kg of meclonazepam, urine was separately collected from two mice in metabolic cages for 24 h (Tecniplast, Buguggiate, Italy) in which urine is separated from feces. The sample volumes at 24 h were 3–4 mL.

In Vitro Incubations

Meclonazepam of 10 μM was incubated with 0.5 mg/mL HLM for up to 90 min using an NADPH regenerating system in potassium phosphate buffer (0.1 M, pH 7.4) at 37 °C. The reaction was stopped by addition of ice-cold acetonitrile (100 to 50 μL of incubation mixture) and the precipitate removed by centrifugation. The incubations with the HLM were also repeated under nitrogen flow. The reaction mixture (containing NADPH regenerating system, HLM, and potassium phosphate buffer) was purged with nitrogen gas for 20 min prior to starting the reaction by addition of meclonazepam.

Under continuous flow of nitrogen, the reaction was carried out for 60 min.

Meclonazepam was also incubated with cryopreserved hepatocytes (1 × 10^6 cells/mL) for up to 5 h in InVitro Gro KHB media at 37 °C. The reaction was stopped by addition of ice-cold acetonitrile (500- to 500-μL sample). After centrifugation, the supernatant was diluted 1:4 with 0.05% formic acid and 5 mM ammonium formate in 50% acetonitrile.

LC-QTOF-MS Analysis

The metabolites of meclonazepam were separated using an LC-QTOF-MS system consisting of an Agilent 1290 Infinity LC instrument and an Agilent 6550 quadrupole time-of-flight mass spectrometer with a JetStream interface (both from Agilent, Kista, Sweden). Gradient elution with 0.05% formic acid in 10 mM ammonium formate (A) and 0.05% formic acid in acetonitrile (B) with a flow of 0.5 mL/min was used for the separation on an Acquity HSS T3 column (150 mm × 2.1 mm, 1.8 μm, Waters, Sollentuna, Sweden). After an initial period of 1% B for 0.6 min, a linear gradient from 5 to 50% B (0.7–8.0) was followed by another linear gradient from 50 to 95% B (8–10 min, held until 11 min) and re-equilibration of the column (1% B, 11.1–12.0 min).

MS-data was collected in positive electrospray mode (gas temperature, 150 °C; gas flow, 18 L/min; nebulizer, 50 psi; sheath gas temperature, 375 °C; sheath gas flow, 11 L/min) using auto-MS/MS, i.e., a data-dependent acquisition mode (*m/z* 100–950, 5 candidates selected for MS/MS in each cycle based on intensity). In a second run, a targeted approach based on a preferred list was used for possible meclonazepam metabolites to obtain higher-quality MS-spectra.

Data Analysis

Peaks were extracted using a Personal Compound Database and Library (PCDL) of possible metabolites based on the following biotransformations: nitro reduction, acetylation of a primary amine, mono-, di-, and trihydroxylations, dehydrogenation, dihydrodiol formation, oxidative dechlorination, glucuronidation, and aryl-sulfatation as well as combinations thereof (including carboxylic acid formation and ketone formation). Ammonium, sodium, and potassium adducts as well as the neutral loss of water or formyl groups were considered as well.

Peaks were filtered based on accurate mass, intensity, and in a second step average mass and presence in unrelated samples. Metabolites only found in the model systems, i.e., mice, HLM, and/or hepatocytes were excluded from the final results.

The resulting metabolite candidates were individually evaluated based on chromatographic peak quality, MS/MS spectra (were available), possibility of suggested biotransformation, and in source fragmentation. Seventeen possible meclonazepam metabolites (including the neutral loss of formyl for 12 of them and the neutral loss of water for one of them) were added to a preferred list for the second LC-QTOF run.

RESULTS

From the raw data, 22,760 possible peaks were identified in the authentic urine samples. After filtering, 28 metabolite candidates remained, which after manual review were reduced to 13 as shown in supplemental Table I. Human metabolites of meclonazepam identified in at least one human urine and by at least one model system, either mice, HLM, and/or hepatocytes, are shown in Table I and Fig. 1.

Small amounts of parent compound were identified in three out of four human urines. Mass spectra were only obtained from the parent, Fig. 2, and the major metabolites amino-meclonazepam (M9, Fig. 3) and acetamido-meclonazepam (M11, Fig. 4). Apart from one dihydrodiol (M1) and one dihydrodiol acetamido metabolite (M6), all minor metabolites were second- or third-generation metabolites of amino-meclonazepam (M9). M9 could be further hydroxylated to yield M2, M3, M5, M7, and M13; two of these metabolites underwent dehydration to generate M8 and M12. We also found a signal for a dechlorinated and sulfated amino metabolite in two human urine samples, although this compound might originate from synthesis impurities.

Of the 13 metabolites identified in the human urine samples, three (M6, M9, and M11) were confirmed in mice urine, only one by HLM (M9), and two by hepatocytes (M9,

Table I. Metabolites Identified in Cases and Confirmed by Human Liver Microsomes, Hepatocytes, and/or the Mouse Model

#	RT	Biotransformation formula	HYD	Peak area (×10^3 counts)						Mass (Da)	m/z [a]	HLM
	(min)		+/−	H1	H2	H3	H4	M1	M2	Diff (ppm)	Adduct	Hep
M6	5.20	Acetamido + monohydroxy	−		542			139	92	357.1	358.1	
		C18 H16 Cl N3 O3	+		203			133	58	−2.0	H+	
M9	5.98	Amino	−	410	28,472	1980	3944	1616	1252	299.1	300.1	HLM
		C16 H14 Cl N3 O	+	341	17,799	2544	3973	1630	1311	−0.6	H+	Hep
M11	6.18	Acetamido	−	510	15,707	561	708	903	514	341.1	342.1	
		C18 H16 Cl N3 O2	+	411	8429	995	950	1089	684	−1.4	H+	Hep
P	8.81	Parent	−		270		124	75	103	329.1	330.1	HLM
		C16 H12 Cl N3 O3	+		128	51	122	75	119	−1.6	H+	Hep
Sum of signals in hydrolyzed sample				751	26,559	3590	5045	2927	2172			

Diff difference between measured and theoretical mass, *Hep* cryopreserved hepatocytes, *HLM* human liver microsomes, *HYD* hydrolysis with glucuronidase, *ppm* parts per million, *RT* average retention time

[a] m/z values and adduct given for most prominent ion species

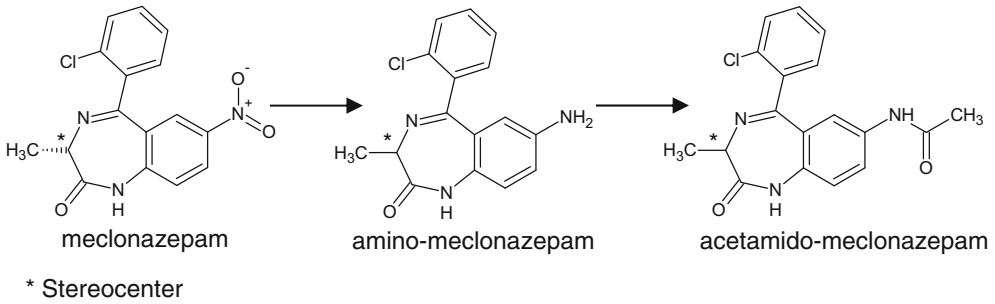

Fig. 1. Main metabolic pathway of meclonazepam

M11). The remaining nine metabolites were only observed in the human urines, supplemental Table I.

The fragmentation pattern for meclonazepam, as seen in Fig. 2, includes loss of the nitro group (*m/z* 284) followed by contraction of the 7-membered ring to a 6-, 5-, or 4-membered ring, corresponding to the fragment ions at *m/z* 255, 238/239, and 214, respectively. The base peak at *m/z* 204 as well as the fragment ion at *m/z* 179 are produced by the loss of chloride from *m/z* 238 and 214, respectively. The fragmentation of amino- and acetamido-meclonazepam (Figs. 3 and 4) show similar fragments as seen for meclonazepam with the exception that the amino- and acetamido-groups were more likely to be retained during fragmentation than the nitro-group.

DISCUSSION

In general, the metabolism of meclonazepam, Fig. 1, was found to be in accordance with the transformations previously

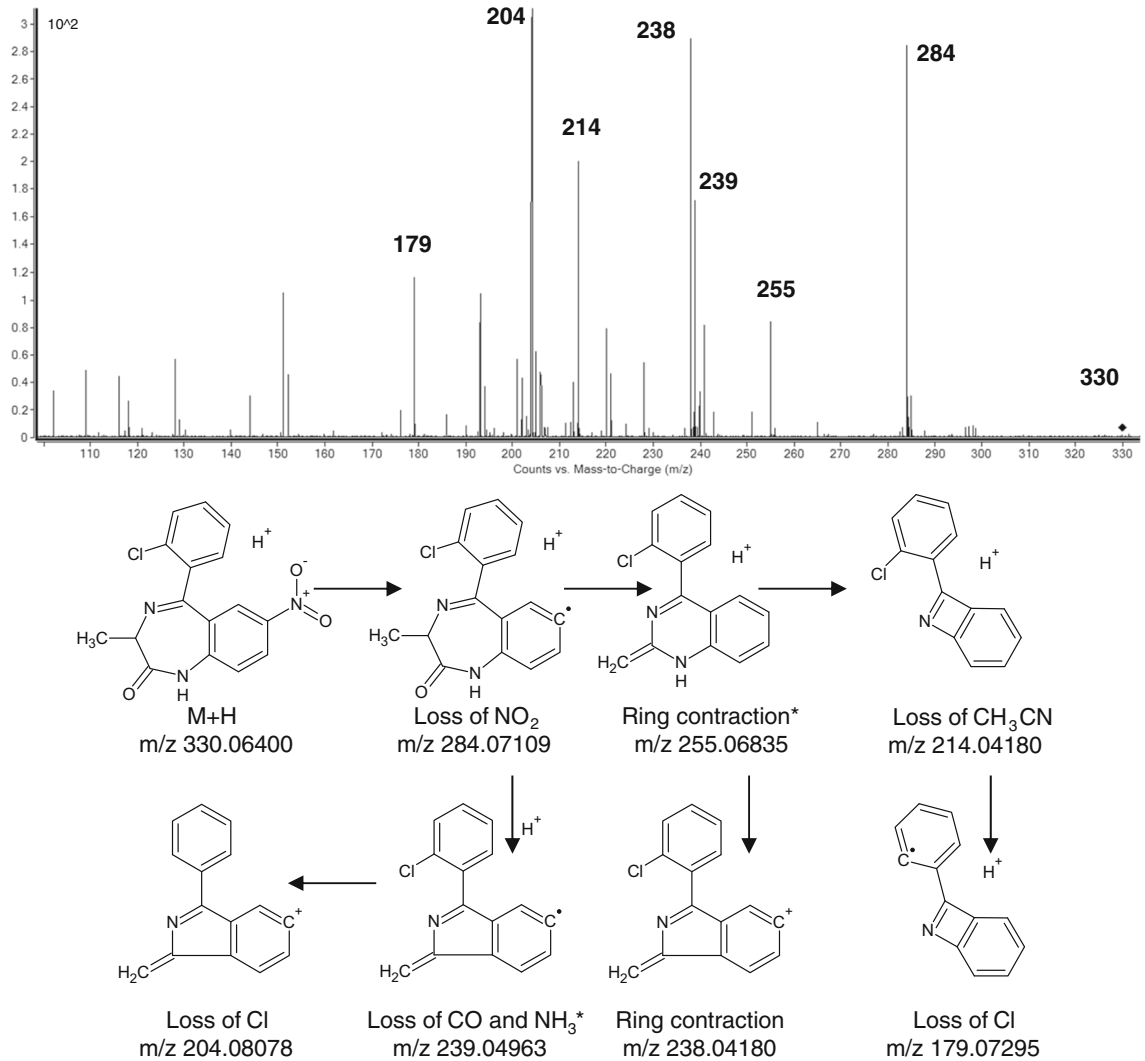

Fig. 2. Mass spectra and fragmentation pattern of meclonazepam. *Asterisk* several different configurations are possible

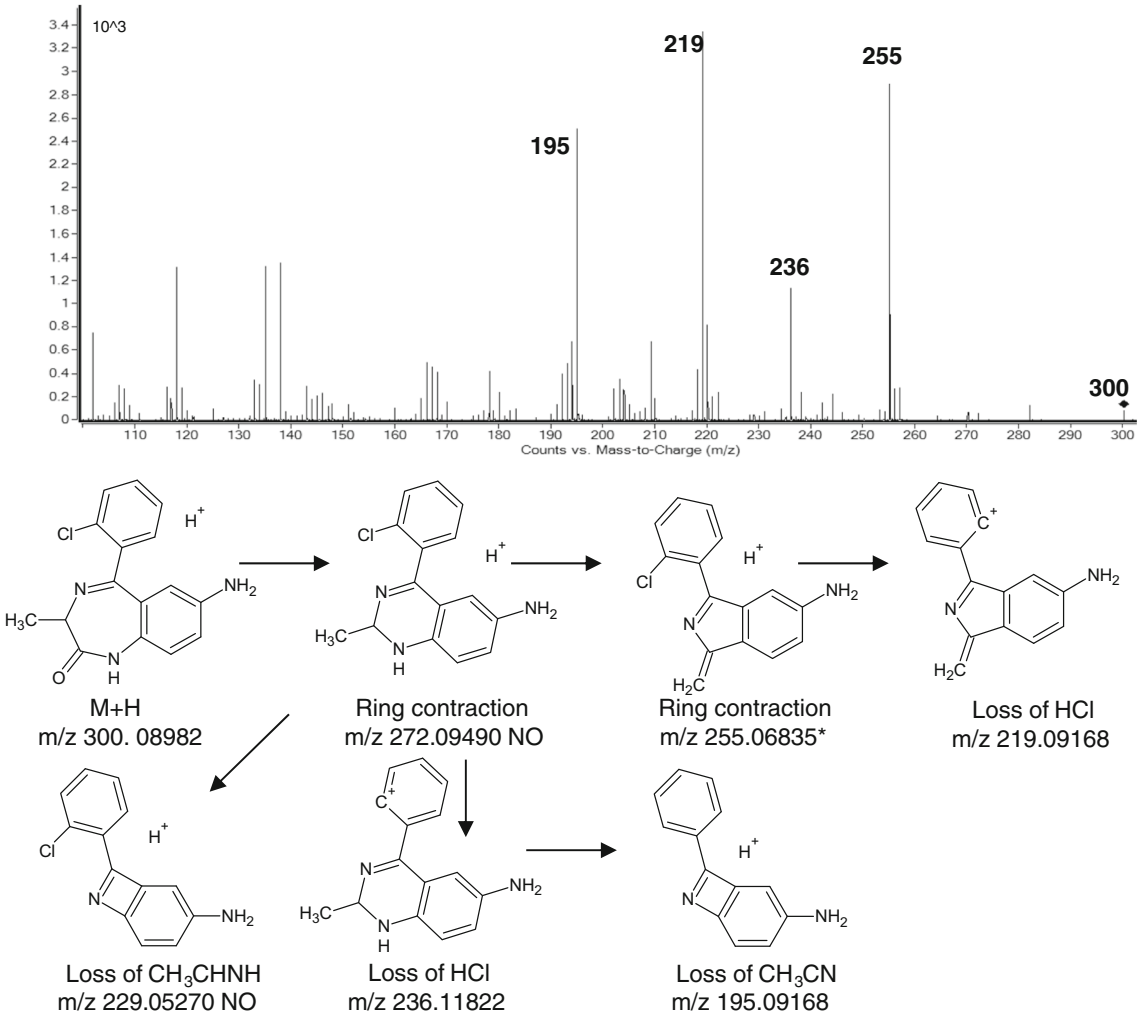

Fig. 3. Mass spectra and fragmentation pattern of amino-meclonazepam (M9). NO, fragment not observed. *Asterisk* several different configurations are possible

described for meclonazepam (2,13) and other nitro-containing benzodiazepines such as clonazepam (14–16), flunitrazepam (11), and nitrazepam (15). However, the hydroxylated metabolites reported by Coassolo *et al.* (12) and Huppertz *et al.* (2) were not confirmed.

The main metabolites clearly are amino-meclonazepam and acetamido-meclonazepam, which were most abundant in all human urine samples. Both are also formed in the mice model and by the hepatocytes. Notably, in HLM incubated under standard condition, only minimal amounts of the amino metabolite and none of the acetamido metabolite were identified. However, when meclonazepam was incubated with HLM under nitrogen, the amount of amino metabolite increases 140 times.

It is unknown which enzyme catalyzes the reduction of the nitro to the amino group in meclonazepam metabolism, but it has been shown that the conversion of clonazepam to amino-clonazepam is catalyzed by CYP3A4 (17). CYP3A4 can catalyze both oxidative and reductive biotransformations. However, the reductive capabilities are inhibited in the presence of excess oxygen (18). The fact that using the HLM under nitrogen dramatically increased the formation of the amino metabolite strongly indicates a similar mechanism for meclonazepam. When using HLM for metabolite identification studies, this effect should be considered for all substrates where a reduction is possible. A similar effect was not observed for the hepatocytes in this study, presumably as the intact cell ensures that the micro-environment at the endoplasmatic reticulum is similar to that *in vivo*.

In clonazepam metabolism, the acetamido metabolite is formed by NAT 2 (19) which is a cytosolic enzyme not present in HLM. Acetamido-meclonazepam is therefore not expected to be formed by HLM (under any conditions), but should be found in mice urine and hepatocyte incubates. The results of this study are in agreement with the results reported by Huppertz *et al.* (2) and Meyer *et al.* (13).

NAT 2 is also a polymorphic enzyme, and around half of a Caucasian population have a marked reduction in NAT 2 activity (20). In our study, ratios between the acetamido metabolite and the amino metabolite (in the hydrolyzed samples) were 0.47, 0.39, and 0.24 in cases H2, H3, and H4 while in case H1, the ratio was 1.21 which could possibly be caused by a higher NAT 2 activity in this case.

The fragmentation observed in our study differs from the fragments and spectra provided by Meyer *et al.* and Huppertz *et al.* Compared to the earlier studies, a more pronounced fragmentation

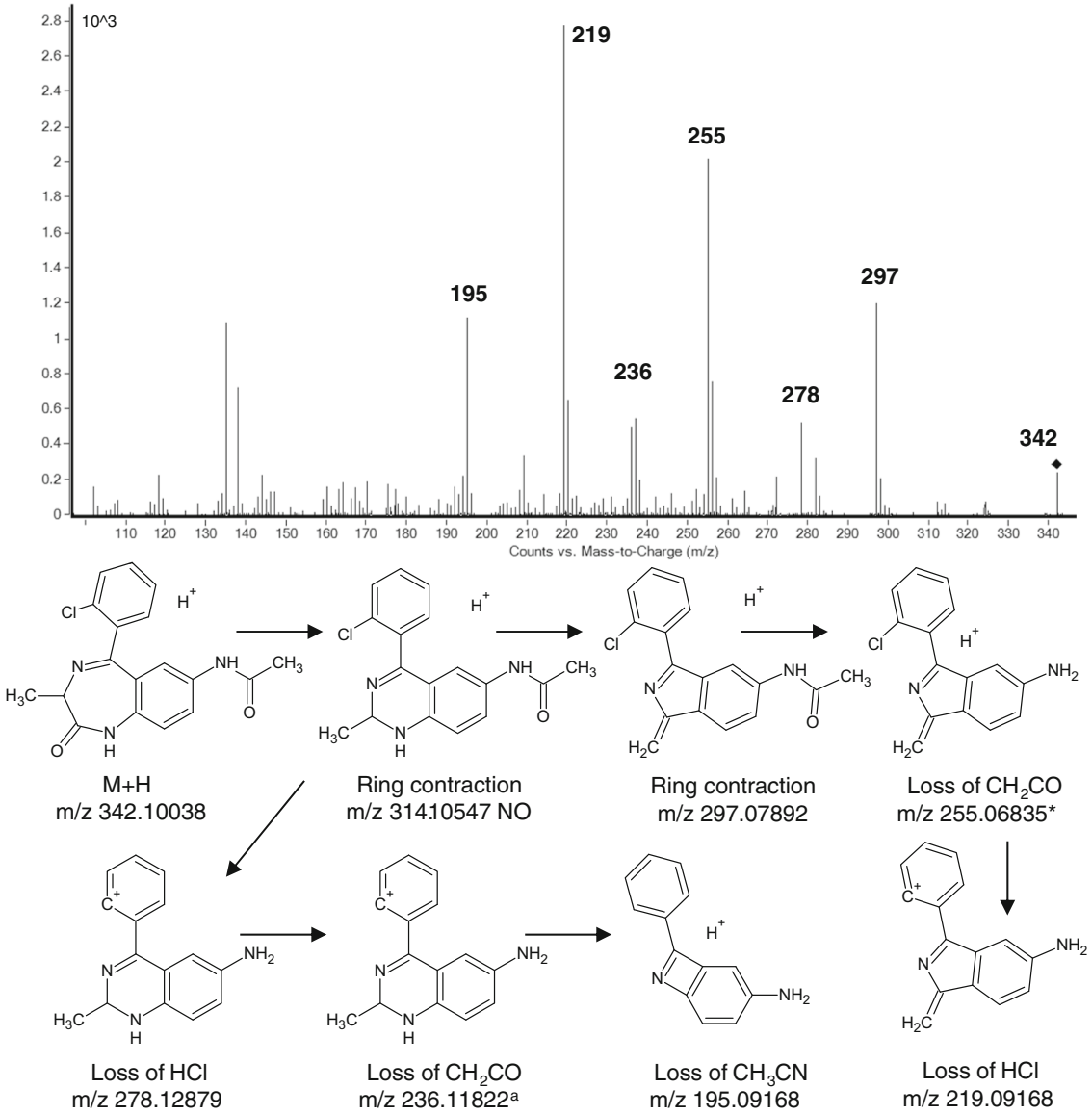

Fig. 4. Mass spectra and fragmentation pattern of acetamido-meclonazepam (M11) NO, fragment not observed. *Asterisk* several different configurations are possible. [a]*m/z* could theoretically also be formed from *m/z* 314 by loss of CH2CO followed by loss of HCl

was observed showing only traces of the protonated molecule. A likely explanation for this is that a higher collision energy was used in this study. However, as different instruments have different collison cells, direct comparisons are difficult.

Furthermore, the structural suggestions for the fragments provided by Meyer *et al.* (13) are far from the only ones possible. Specifically, we postulate contractions of the diazepine ring to a 5-membered or 4-membered ring, as in fragments 255 and 219 for amino-meclonazepam whereas Meyer *et al.* suggest the loss of the amino group followed by the formation of a triple bond within the benzene ring. A similar difference exists in the interpretation of the corresponding fragments of acetamido-meclonazepam as well as for fragment 195 of acetamido-meclonazepam. We postulate contraction of the diazepine ring to a 4-membered ring, whereas Meyer *et al.* suggest contraction of the benzene ring. The contraction of the diazepine ring to 5- and/or 4-membered rings has also been described for several other benzodiazepines, such as diazepam (21,22), flurazepam (23), flunitrazepam (21,23), and 3-hydroxy-flunitrazepam (23) and seems more plausible than the reactions suggested by Meyer *et al.*

In Table I, only metabolites also confirmed in at least one model system were included as this is a good indicator that the metabolite is generated from the parent drug. The first positive urine samples for a novel psychoactive substance (NPS), such as meclonazepam, that comes in to the lab are often are related in time and/or origin and might actually come from the same batch of drugs. As NPS products are often contaminated by synthesis by-products and/or mixtures of several NPS, there is a risk that an analyte is falsely attributed as a metabolite when in fact it is related to either a different drug or a synthesis by-product.

We suggest that amino- (M9) and acetamido (M11) - meclonazepam are the best candidates for identification of meclonazepam intake. Furthermore, the parent drug is a valuable target as it was observed in three out of four human urines.

In this study, the concentration of parent in the samples might have been underestimated due to pre-analytical factors

as there is evidence of poor stability under certain conditions. While no studies of meclonazepam stability in urine are known to us, Zaitsu *et al.* (24) reported that amino-flunitrazepam was degraded both by bacteria during storage at 4 °C and by adhesion to vessel surface. On a similar note, Coassolo *et al.* (12) reported that meclonazepam at 1 ng/mL in plasma was stable at −20 °C for 3 months in polypropylene tubes but not in glass tubes nor at room temperature for 24 h.

In this study, samples were analyzed both with and without hydrolysis to estimate the level of conjugation. This approach was used as glucuronides, and other conjugated metabolites are difficult to measure directly as they behave differently from unconjugated metabolites in the chromatographic separation and usually have lower ionization efficiency in positive electrospray ionization mode. While neither meclonazepam nor the major metabolites amino-meclonazepam (M9) and acetamido-meclonazepam (M11) appeared to be conjugated, some minor metabolites were (M8, M10, and M13).

CONCLUSIONS

We conclude that the major metabolites suitable as markers of meclonazepam intake are amino-meclonazepam (M9) and acetamido-meclonazepam (M11). These metabolites appear to be unconjugated in urine.

We also underline that a model system for metabolite identification should be carefully selected considering the expected metabolism of the tested drug. Reductive reactions may be prominent but may require anaerobic conditions usually not considered in standard *in vitro* experiment settings. Therefore, we believe that a more complete metabolic system, such as found in hepatocytes and animal models, is imperative to understand the complexity of metabolism of many NPS.

ACKNOWLEDGEMENTS

This study was conducted within the Strategic Research Area in Forensic Science (Strategiområdet forensiska vetenskaper) at Linköping University.

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