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Structural elucidation of *N*-oxidized clemastine metabolites by liquid chromatography/tandem mass spectrometry and the use of *Cunninghamella elegans* to facilitate drug metabolite identification

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***Cunninghamella elegans* is a filamentous fungus that has been shown to biotransform drugs into the same metabolites as mammals. In this paper we describe the use of *C. elegans* to aid the identification of clemastine metabolites since high concentrations of the metabolites were produced and MS^n experiments were facilitated. The combination of liquid chromatography and tandem mass spectrometry with two different ionization techniques and hydrogen/deuterium exchange were used for structural elucidation of the clemastine metabolites. Norclemastine, four isomers of hydroxylated clemastine, and two *N*-oxide metabolites were described for the first time in *C. elegans* incubations. The *N*-oxidations were confirmed by hydrogen/deuterium exchange and deoxygenation (−16 Da) upon atmospheric pressure chemical ionization mass spectrometry. By MS^n fragmentation it was concluded that two of the hydroxylated metabolites were oxidized on the methylpyrridyl moiety, one on the aromatic ring with the chloro substituent, and one on the aromatic ring without the chlorine.**

The antihistamine drug clemastine acts as a competitive inhibitor of histamine 1 receptors. It has been used in human medicine since the 1960s and some of its indications are urticaria and allergic eczema.[1] In a previous study by our group,[2] new clemastine metabolites in urine from humans, horses, and dogs were discovered by the use of high-performance liquid chromatography (HPLC) positive electrospray ionization (ESI) tandem mass spectrometry (MS/MS). Many oxidized metabolites were detected but the positions of the oxidative reactions were not determined closer than on which half of the molecule they had occurred (A or B in Fig. 1). It was possible that the oxidation on the B fragment of the molecule had resulted in *N*-oxidized metabolites[2] as clemastine-*N*-oxide has been previously detected in rat urine.[3] Drugs that contain a tertiary amine function are commonly oxygenated to *N*-oxides by flavin-containing monooxygenase (FMO). Typically, the *N*-oxide is water-soluble and hence easily excreted, and often has lower pharmacological/toxicological potency than the parent drug.[4] However, there are also examples of *N*-oxides that have been suspected to be carcinogenic and thus it is important to identify such metabolites.[5] There are a few different approaches to differentiate *N*-oxidations from carbon hydroxylations by the use of LC/MS(/MS). The loss of a hydroxyl radical ion (−17 Da) has been reported for several *N*-oxides upon collision-induced dissociation (CID).[6–10] Deuterium oxide can be added to the LC mobile phase, and the number of hydrogen atoms that have been exchanged to deuterium can be determined.[6,10,11] Another procedure is to search for thermally induced deoxygenation (−16 Da) that has been reported for *N*-oxides using atmospheric pressure chemical ionization (APCI).[8,9,12–14]

Cunninghamella elegans is a filamentous fungus that expresses the same enzymes that perform drug metabolism in mammals.[15] According to the literature, it can perform phase I metabolic reactions such as aromatic or aliphatic hydroxylation, oxidation of heteroatoms, and demethylation on nitrogen or oxygen atoms, as well as conjugation reactions such as sulfate, acetyl, glycoside, and glucuronic acid conjugation.[15] *C. elegans* has previously been used as an *in vitro* model of drug metabolism[16,17] and for the production of drug metabolites in amounts sufficient for structural confirmation.[18,19] In a different project by our group,[20] *C. elegans* was used to metabolize the non-steroidal anti-inflammatory drug meloxicam. The metabolites formed by the fungus were identical to those detected in horse urine. However, the concentrations were higher in the microbial samples which facilitated MS^n experiments on the metabolites produced by the fungus, which resulted in valuable structural information.[20]

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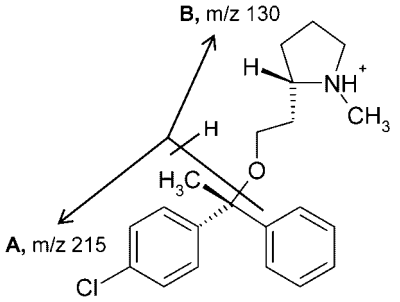

Figure 1. Structure of protonated clemastine, $[M+H]^+$ *m/z* 344, and the definition of the A (*m/z* 215) and B (*m/z* 130) fragments created by collision-induced dissociation.

In this study we wanted to examine whether *C. elegans* could assist in the structural elucidation of the clemastine metabolites earlier discovered in urine samples from humans, horses, and dogs,[2] particularly the ones where *N*-oxidation was suspected but not confirmed. The techniques used were UPLC and HPLC/MS/MS with APCI or ESI, and hydrogen/deuterium (H/D) exchange experiments.

EXPERIMENTAL

Chemicals

Clemastine fumarate, deuterium oxide (99.9 atom% D) and acetic acid-OD (99 atom% D) were purchased from Sigma Aldrich (Steinheim, Germany). The Sabouraud agar plates and the broth to the *C. elegans* experiments were obtained from the National Veterinary Institute (SVA) in Uppsala, Sweden. The water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) and all the chemicals used were of analytical grade or better and used without further purification.

Microbial transformation

Cultures of *Cunninghamella echinulata varians elegans* (American Type Culture Collection 9245, lot no 3656357, purchased from LGC Promochem, Borås, Sweden) were grown on Sabouraud dextrose agar plates (mycological peptone 10 g/L, dextrose 40 g/L, agar 15 g/L) for 5 days at 27°C and were then stored at 4°C. The spores and mycelia from one agar plate were blended with 75 mL of sterile physiological saline solution in a sterile flask. A volume of 5 mL of the mycelia suspension was used to inoculate 30 mL of broth in a 250 mL Erlenmeyer flask. After approximately 48 h at 27°C the drug was added (200 μL of clemastine solution, 1.0–44 mM in methanol/dimethylformamide 1:1). The cultures were incubated for 24–96 h before the experiment was terminated by the addition of 25 mL of methanol. Two blank samples were prepared in the same way at the same time, one with *C. elegans* but without the drug and one with the drug but without the fungus. The samples were stored at −20°C until sample preparation.

In order to find the optimum conditions for clemastine metabolism, four different kinds of broth were compared at pH 5.5 and 7.0; these were yeast extract broth (yeast extract 8 g/L, sodium chloride 4.6 g/L, sucrose 20 g/L, monopotassium phosphate 3.75 g/L, disodium phosphate 7.09 g/L), peptone broth (mycological peptone 8 g/L, sodium chloride 4.6 g/L, sucrose 20 g/L, monopotassium phosphate 3.75 g/L, disodium phosphate 7.09 g/L), and two different Sabouraud dextrose broths (mycological peptone 10 g/L and dextrose 20 g/L or 40 g/L, respectively). The pH of the broth was adjusted from about 5.5 to 7.0 by the addition of 1.0 M phosphate buffer pH 6.9 (10 to 1000 mL of broth) and, when needed, 2.0 M sodium hydroxide.

Sample preparation

Solid-phase extraction of C. elegans samples

HCX cartridges (300 mg C_8/cation-exchange material) were purchased from Sorbent AB (Västra Frölunda, Sweden). The cartridges were conditioned with methanol and Milli-Q water (6 mL, respectively) and equilibrated with 6 mL of sodium acetate buffer (0.10 M, pH 5.0). The *C. elegans* samples (25 mL) were diluted with an equal volume of sodium acetate buffer (0.10 M, pH 5.0) and pH was adjusted to approximately 5.0 before they were applied on the solid-phase exreaction (SPE) cartridge. The cartridges were washed with 12 mL of sodium acetate buffer (0.10 M, pH 5.0) and then dried for 5 min. Methanol (10 mL) with 2% ammonia was used for elution. The eluate was evaporated to dryness under a gentle stream of nitrogen at 65°C. The samples were dissolved in 500 μL of 0.1% acetic acid in Milli-Q water (A)/0.1% acetic acid in acetonitrile (B), 85:15, prior to analysis.

Liquid-liquid extraction of C. elegans samples

The pH of the *C. elegans* samples (volume approximately 60 mL) was adjusted to 10 or higher by the addition of 1.0 M sodium hydroxide and then poured through a filter into a separatory funnel. The liquid phase was extracted twice with ethyl acetate (80 mL) and the organic phases were combined and evaporated with a rotary evaporator. The residue was dissolved in 2 × 10 mL of ethyl acetate and divided into three test tubes. The solvent was evaporated to dryness at 65°C under a gentle stream of nitrogen. Before analysis, the residue was dissolved in 0.50–1.0 mL A/B (85:15).

Liquid-liquid extraction of human, horse, and dog urine samples

Urine (8 mL human and horse, 2 mL dog) was mixed with phosphate buffer (0.10 M and pH 6.5, 2 mL human and horse, 8 mL dog) and pH was adjusted to 10.0 with sodium hydroxide (2.0 M). The samples were extracted with ethyl acetate (10 mL) for 20 min and centrifuged for 10 min (1000 *g*). The organic phase was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen gas at 50°C. The residue was dissolved in 100 μL of A/B (85:15) prior to analysis.

Liquid chromatography (LC)

High-performance liquid chromatography (HPLC)

Solvents in the mobile phase were 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) and mobile phase was delivered at a flow rate of 0.2–0.3 mL/min by a quaternary Surveyor HPLC system (Thermo Electron Corp., San José, CA, USA) or a binary HPLC system of the 1100 series (Agilent Technologies, Waldbronn, Germany). A number of different gradients were used (see Table 1). The

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Table 1. Liquid chromatography gradients

	Time (min)	A (%)	B (%)
Gradient I	0.0	85	15
	5.0	85	15
	95.0	65	35
	96.0	10	90
	98.0	10	90
	98.1	85	15
	102.0	85	15
Gradient II	0.0	85	15
	5.0	85	15
	80.0	68	32
	81.0	10	90
	83.0	10	90
	83.1	85	15
	88.0	85	15
Gradient III	0.00	95	5
	4.99	15	85
	5.46	15	85
	5.48	95	5
	6.22	95	5
Gradient IV	0.0	85	15
	5.0	75	25
	100.0	65	35
	105.0	10	90
	108.0	10	90
	108.1	85	15
	112.0	85	15

A: 0.1% acetic acid in Milli-Q water B: 0.1% acetic acid in acetonitrile Flow rate 0.74 mL/min in gradient III and 0.20 mL/min in the others.

analytical column was a Phenomenex Luna 5 μ C_{18} (2) (150 mm × 2.00 mm length × i.d.) and the guard column was an ODS-C_{18} (4.0 mm × 2.0 mm) both from Scandinaviska Genetec AB (Västra Frölunda, Sweden). The injection volumes used were 10–20 μL.

In the H/D exchange experiments, the mobile phase consisted of (A) 0.1% acetic acid-OD in deuterium oxide (D_2O) and (B) 0.1% acetic acid-OD in acetonitrile.

Ultra-performance liquid chromatography (UPLC)

An Acquity UPLC® system with a binary solvent manager, a sample manager, and a column heater from Waters Corporation (Milford, MA, USA) was used. Solvents in the mobile phase were the same as above and were delivered as gradient III (Table 1) at a flow rate of 0.74 mL/min. The separation was achieved on an Acquity UPLC® BEH C_{18} column with 1.7 μm particles (50 × 2.1 mm; Waters Corp., Milford, MA, USA). The volume injected was 2 μL for the *C. elegans* samples and 15 μL for the human and horse urine samples.

Mass spectrometry (MS)

Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used to create positive ions in this project. The MS parameters on the different instruments were optimized for sensitivity during direct infusion of a clemastine solution that was mixed with the LC flow (A/B, 50:50) through a connecting T. The total flow rate was 0.2–0.3 mL/min in both the ionization techniques in the HPLC systems and 0.74 mL/min for the UPLC system.

Quantum Discovery (QqQ)

The metabolite screening and H/D exchange experiments were performed on a triple quadrupole TSQ Quantum Discovery instrument (Thermo Electron Corp., San José, CA, USA). Either an ESI or an APCI interface was used in the full scan modes of MS, MS/MS and neutral loss (NL) as well as selected reaction monitoring (SRM). Instrument control, data acquisition, and data processing were carried out with the Xcalibur™ software (version 2.0 SR2). When APCI was used for the ionization the nitrogen sheath and auxiliary gas flows were 30 and 5 arbitrary units, the discharge current was 4 μA, and the vaporizer and capillary temperatures were 400 and 150°C, respectively. For ESI the sheath and auxiliary gas (nitrogen) flow rates were 50 and 19 arbitrary units, respectively. The capillary voltage was 4.3 kV and its temperature was 380°C.

LCQ ion trap

MS^n experiments with both clemastine standard solution and *C. elegans* samples were performed with a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corp., San José, CA, USA), equipped with an ESI source. Instrument control, data acquisition, and data processing were carried out with the Xcalibur™ software (version 1.3). A clemastine standard solution (5.4 μM in Milli-Q) was introduced into the mass spectrometer as a constant infusion (20 μL/min) that was mixed with the HPLC mobile phase (A/B, 50:50, 200 μL/min) through a connecting T. The temperature and voltage of the capillary were 275°C and 7 V, respectively. The nitrogen sheath gas flow rate was 90 arbitrary units and the source voltage was 4.5 kV. The ion trap was filled with helium gas in the MS^2, MS^3, and MS^4 scan modes and data was collected for 60 s. The relative collision energy and the activation Q were optimized for each mass transition (see Table 2). The wide band activation parameter was not used in these experiments.

Acquity TQD

In the UPLC experiments, an Acquity TQ (triple quadrupole) detector with a dual orthogonal ZSpray™ API source (Waters Corp., Milford, MA, USA) was used for detection of

Table 2. MS^n experimental settings

MS^n (n =)	*m/z* (collision energy %, activation Q)	Scan window (*m/z*)
2	344 (18, 0.25) → scan	100–400
3	344 (20, 0.25) → 130 (32, 0.25) → scan	50–140
4	344 (20, 0.25) → 130 (32, 0.25) → 112 (30, 0.27) → scan	50–120
3	344 (20, 0.25) → 215 (34, 0.27) → scan	60–230
4	344 (20, 0.25) → 215 (34, 0.25) → 180 (37, 0.25) → scan	50–250
3	346 (20, 0.25) → 217 (34, 0.25) → scan	55–250

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the substances in the urine and *C. elegans* samples. The capillary, cone and extractor voltages were 700, 25, and 3 V, the source and desolvation temperatures 150 and 450°C, and the cone and desolvation gas flows were 50 and 900 L/h, respectively. Eight parallel SRM channels were used to scan for clemastine and its metabolites in the samples; *m/z* 344→215 and 344→130 (clemastine), *m/z* 330→215 and 330→116 (norclemastine), *m/z* 360→231 and 360→130 (hydroxyclemastine, oxidation on fragment A), *m/z* 360→215 and 360→146 (hydroxyclemastine, oxidation on fragment B). The collision gas was argon at a flow rate of 0.1 mL/min, and the collision energy was 25 eV. The instrument control, data acquisition, and data processing were managed by the Masslynx software (version 4.1).

RESULTS AND DISCUSSION

Clemastine metabolites in *C. elegans* incubations and in urine samples from humans, horses, and dogs

In order to investigate whether the fungus *C. elegans* was able to metabolize the antihistamine clemastine, the fungus was incubated with the drug and the samples were cleaned up and concentrated by liquid-liquid extraction prior to HPLC/MS and HPLC/MS/MS analysis with the triple quadrupole mass spectrometers (for details, see the Experimental section). Figure 2 illustrates the extracted ion chromatograms (XICs) corresponding to the parent drug clemastine (*m/z* 344) and the expected metabolites norclemastine (*m/z* 330), hydroxyclemastine (*m/z* 360), and dihydroxyclemastine (*m/z* 376) that were previously reported by our group in human, horse and dog urine.[2] Interestingly, peaks occurred in all the XICs indicating that the metabolites could be formed by *C. elegans* as well. The peak emanating from remaining parent compound was heavily overloaded, as the metabolic yield was low. However, this tailing peak was not considered to be a major analytical interference, as all the metabolites previously found in mammalian urine were also detected in the *C. elegans* samples. Other antihistamine drugs, with structural similarities to clemastine, have been extensively metabolized into *N*-oxidized and *N*-demethylated metabolites by *C. elegans*, e.g. brompheniramin, chlorpheniramine and pheniramine,[21] pyrilamine,[22] and cyproheptadine.[23] In our study, different cultivation media and times for incubation did not significantly affect the yield of metabolites. In the blank samples, prepared without the addition of *C. elegans*, some of the peaks corresponding to clemastine metabolites were found but the intensities were significantly higher with *C. elegans* than without. The finding of the substances in the blank samples could indicate either spontaneous chemical degradation or the presence of synthetic impurities. However, the higher amount detected after incubation with the fungus indicated that metabolism was the major pathway. As the same substances were previously found in human, horse and dog urine, the structural elucidation described in this paper has a biological bearing, no matter how the products were formed. Furthermore, since the intensities of the peaks of the clemastine metabolites were much higher in the fungal incubations, than in the human, horse, and dog samples from our previous study, we decided to carry on with the structural elucidation of the peaks of interest in the microbial transformations.

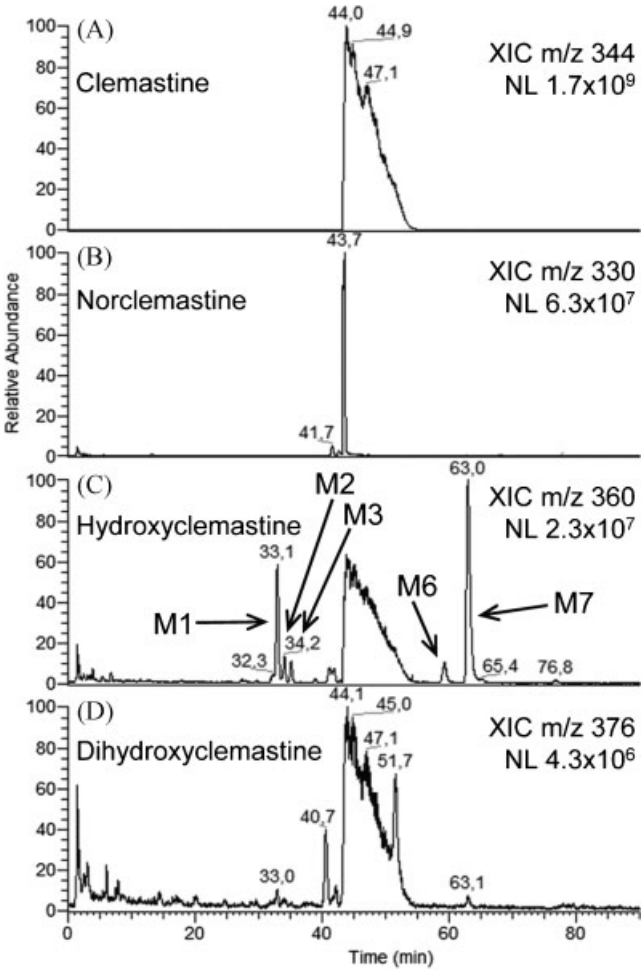

Figure 2. Extracted ion chromatograms for clemastine (A, *m/z* 344) and expected metabolites, norclemastine (B, *m/z* 330), hydroxyclemastine (C, *m/z* 360), and dihydroxyclemastine (D, *m/z* 376) after full scan MS of a *C. elegans* sample. Quantum Discovery triple quadrupole instrument, gradient I. Details can be found in the Experimental section.

A *C. elegans* sample was analyzed with collision-induced dissociation (CID) of *m/z* 360, i.e. $[M+H]^+$ for clemastine plus 16 *m/z* units. The peaks with retention times 48, 50, and 51 min in the chromatograms in Figs. 3(A) and 3(B) resulted in mass spectra with the fragment ions *m/z* 231 and 130 (i.e. + 16 *m/z* units on the A fragment, cf. Fig. 1). Further, the peaks at 55, 57, 74, and 76 min showed that *m/z* 360 could also produce the fragment ions *m/z* 215 (Fig. 3(C)) and 146 (Fig. 3(D), oxidation on the B fragment of clemastine). Thus, the chromatograms indicate that at least seven isomers of monooxidized clemastine can be detected in the *C. elegans* samples using the HPLC/QqQ system. These metabolites will be called M1 to M7 throughout this article, where M1 eluted first and M7 eluted last in the chromatograms.

In order to confirm that the metabolites discovered in the *C. elegans* samples were the same as those detected in the urine samples in a rapid manner, the samples were analyzed on the UPLC/TQD system and the retention times and mass spectrometric data were compared. The UPLC gradient used

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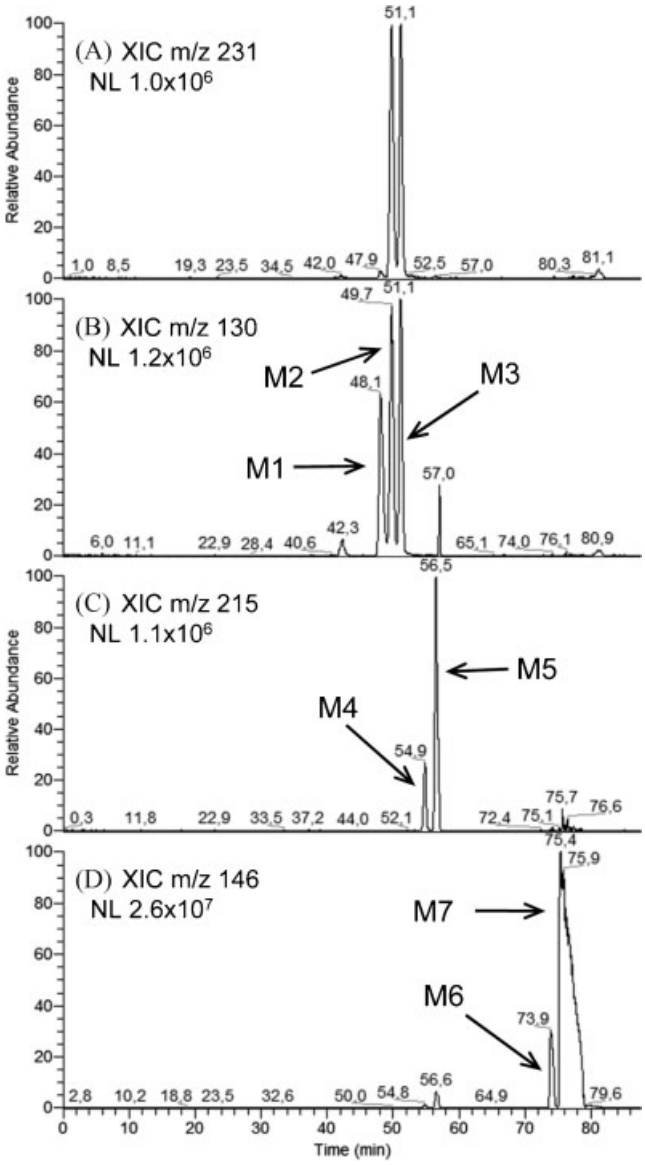

Figure 3. MS/MS of *m/z* 360 corresponding to $[M+H]^+$ of oxidized clemastine metabolites in a *C. elegans* sample. Extracted ion chromatograms for *m/z* 231 (A), *m/z* 130 (B), *m/z* 215 (C), and *m/z* 146 (D). The instrument used was the Quantum Discovery, the collision energy was 20 V, and the chromatographic separation was achieved by gradient II.

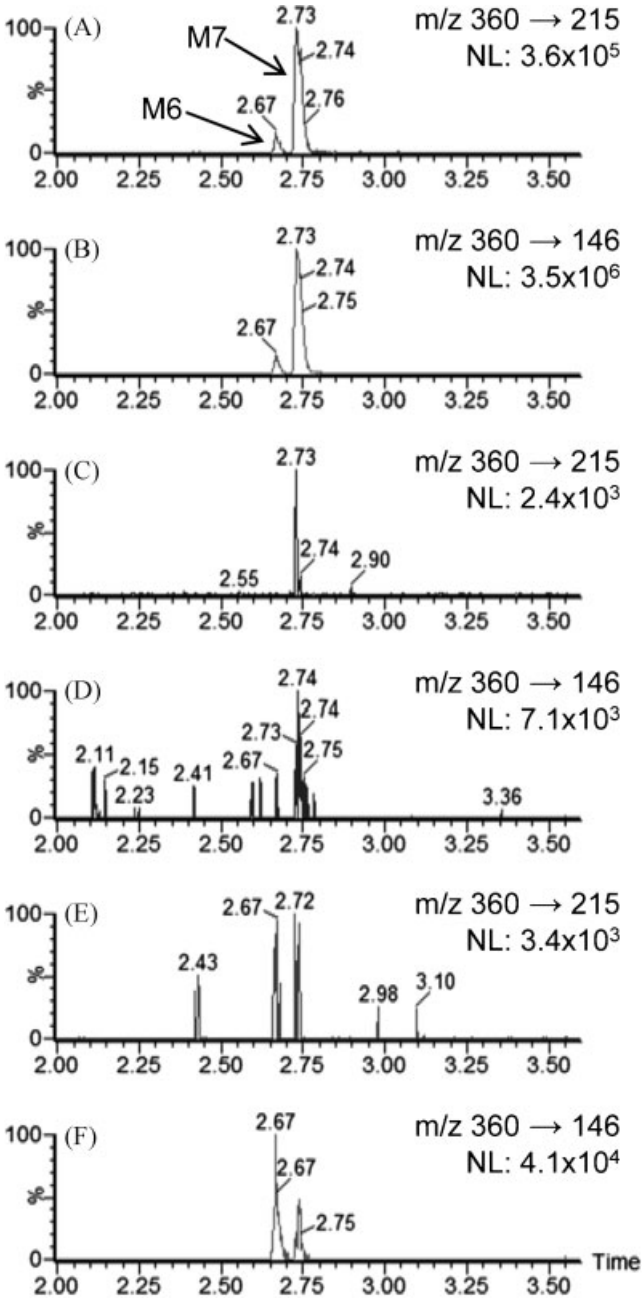

Figure 4. UPLC chromatograms (gradient III) for the SRM transitions of *m/z* 360→215 and *m/z* 360→146 for a *C. elegans* sample (A, B), a horse urine sample (C, D), and a human urine sample (E, F). For details, see the Experimental section.

was only 6.22 min long compared to the HPLC gradients of 88 and 102 min, respectively. The SRM channels of *m/z* 360→215 and *m/z* 360→146 (i.e. the B fragment plus 16 *m/z* units) are displayed in Fig. 4. In the *C. elegans* sample (Figs. 4(A) and 4(B)), the M6 and M7 metabolites resulted in high intensity peaks in the chromatogram at 2.67 and 2.73 min, respectively; however, the M7 peak was more intense than the M6 peak. In the horse urine sample (Figs. 4(C) and 4(D)) only the M7 peak could be detected. For the human urine sample (Figs. 4(E) and 4(F)), both peaks were seen in the chromatograms; however, the intensities of the M6 and M7 peaks were similar.

With the UPLC system used, two of the isomers of the metabolite oxidized on the A fragment of clemastine, channels *m/z* 360→130 and 360→231, were discovered in the SRM chromatograms from *C. elegans* (results not shown). Those corresponded to M2 and M3 which resulted in the highest response in the extracted ion chromatograms in Figs. 3(A) and 3(B). The dog sample was not analyzed by UPLC but the chromatograms from HPLC/QqQ indicated one isomer of the metabolite resulting in the fragments *m/z* 130 and 231 but which one could not be determined. In the horse urine sample, only the first eluting isomer was present in the chromatograms and in the chromatograms from the human urine sample the retention time was consistent with the peak also found in horse, i.e. M2.

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The peak corresponding to norclemastine could be seen in the chromatograms of the *m/z* 330→215 and 330→116 channels in the human urine and *C. elegans* sample (chromatograms not shown). Data from the HPLC/QqQ mass spectrometer indicated that the norclemastine metabolite was present in dog urine as well.

Hence, by comparison of mass transitions and retention times, the metabolites M2, M3, M6, M7, and norclemastine that were found in the C. *elegans* samples appeared to be identical to the metabolites found in the urine samples, however present at higher concentrations. The further structural elucidation work was therefore performed only on the fungal samples and the results are presented below.

The microbial samples were also analyzed for phase II metabolites with the HPLC/QqQ mass spectrometer. Both directly injected samples and samples extracted with the mixed-mode solid phase were analyzed by MS full scan, MS/MS as well as SRM transitions of *m/z* corresponding to conjugates detected in Tevell *et al.*[2] However, phase II metabolites of clemastine could not be found in the *C. elegans* samples in this study.

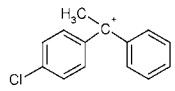
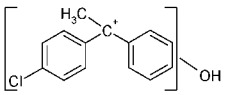
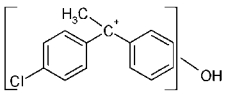
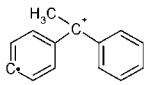
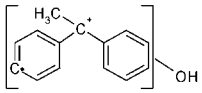
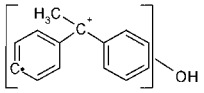
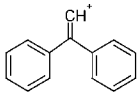
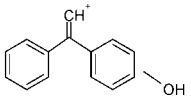
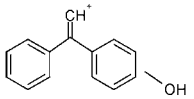
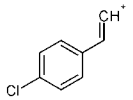
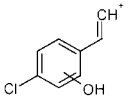
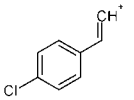
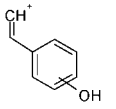
Structural elucidation of oxidized clemastine metabolites using HPLC/MS^n

The CID of protonated clemastine, $[M+H]^+$ *m/z* 344, was thoroughly investigated by HPLC/MS^n, and the results were used for comparison with the product ions formed from the oxidized metabolites. A clemastine standard solution was infused into the ESI source of the ion trap mass spectrometer and MS^2, MS^3, and MS^4 were applied (MS^n details can be found in Table 2 and concentrations, flow rates, and other settings are described in the Experimental section). MS/MS of *m/z* 344 resulted in the two main fragments *m/z* 215 (A) and 130 (B) produced by the breakage of the ether bond (Fig. 1).

The A fragment contains a chlorine atom and, hence, both MS^3 of *m/z* 344→215→scan (the ^{35}Cl isotope) and *m/z* 346→217→scan (the ^{37}Cl isotope) were performed (see Table 3 for proposed structures of the main fragments). The most abundant fragment ions in the spectra from both precursors *m/z* 215 and 217 were *m/z* 180 and 179, a result of the loss of a chlorine radical (−35 or 37 *m/z* units) or hydrochloric acid (−36 or 38 *m/z* units). The two minor fragments in the spectra were *m/z* 103 and 137/139. The *m/z* 103 fragment corresponded to the loss of 112 from *m/z* 215 (or 114 from 217) and could be the chlorobenzene ring (^{35}Cl-benzene equals 112 and ^{37}Cl-benzene equals 114). The *m/z* 137/139 fragment probably corresponded to the loss of the benzene ring without the chloro substituent (215/217 – 78). MS^4 of *m/z* 344→215→180→scan resulted in one major fragment ion, *m/z* 165, i.e. the loss of 15 mass units (presumably -CH_3) from *m/z* 180.

A *C. elegans* sample was injected into the HPLC/MS^n system and the parent compound and metabolites were chromatographically separated. In the MS^3 spectra of *m/z* 360→231→scan and *m/z* 362→233→scan (i.e. the chlorine-37 isotope) for the metabolites called M2 and M3 the fragments *m/z* 195 and 196 were the most abundant (i.e. 179/180 plus 16 *m/z* units). However, the intensity between them differed; *m/z* 195 was slightly more intense than *m/z* 196 in metabolite M2 while the *m/z* 195 fragment was approximately 40% of the *m/z* 196 peak in the spectra of M3. In the spectra of M3 the *m/z* 137/139 peaks were found as in clemastine standard but, in M2, *m/z* 153/155 (i.e. 137/139 plus 16 *m/z* units) were present. Further, the *m/z* 103 fragment (that can be seen in the clemastine spectrum) was found in the M2 spectra but not in the spectra of M3, where the fragment *m/z* 119 (*m/z* 103 plus 16 *m/z* units) was found instead. Hence, the hydroxyl group seemed to be situated

Table 3. Proposed fragmentation of the A fragment of clemastine and the oxidized metabolites M2 and M3

Clemastine standard, Proposed fragment	*m/z* (Intensity)	M2, Proposed fragment	*m/z* (Intensity)	M3, Proposed fragment	*m/z* (Intensity)
	215/217 (MS^3 precursor ions)		231/233 (MS^3 precursor ions)		231/233 (MS^3 precursor ions)
	180 (Base peak)		196 (90%)		196 (Base peak)
	179 (70%)		195 (Base peak)		195 (40%)
	137/139 (10%)		153/155 (5%)		137/139 (30%)
	103 (5%)		103 (5%)		119 (10%)

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on the aromatic ring with the chloro substituent in M2 but on the ring without the chlorine in M3.

For the B fragment, the most abundant ion in the MS^3 spectrum of *m/z* 344→130→scan of the infused clemastine standard was *m/z* 112 (*m/z* 130 – H_2O); the proposed structures of the fragments are presented in Table 4. The *m/z* 84 fragment probably corresponds to the loss of the $HOCH_2CH_3$ side chain (*m/z* 130-46). Further fragmentation of *m/z* 112 (MS^4, *m/z* 344→130→112→scan) resulted in the fragments *m/z* 84 (same as above) and 58 (*m/z* 112 – 54) possibly formed by the loss of $HC=CH_2CH=CH_2$.

In the MS^3 spectra of *m/z* 360→146→scan for the M4, M5, M6, and M7 metabolites from a *C. elegans* *m/z* 128 was the most abundant fragment, i.e. *m/z* 146 minus water (or *m/z* 112 plus 16 *m/z* units). It might be the same water loss as could be seen in the spectrum of the clemastine standard, i.e. the aliphatic alcohol. Other major fragments were *m/z* 110 (*m/z* 146 – 36), that probably corresponded to the loss of two molecules of water, and 100 (*m/z* 84 plus 16 *m/z* units or *m/z* 146 – 46), which would be the loss of the $HOCH_2CH_3$ side chain. At least in the spectrum of M5, *m/z* 84 (*m/z* 146-62) was a main fragment, in concordance with the loss of water and $HOCH=CH_2$. In MS^4, *m/z* 360→146→128→scan the *m/z* 110 fragment was formed in both M4 and M5, and *m/z* 84 was found in M5. Thus, M4 and M5 appear to be oxidized on the methylpyrridyl moiety. However, it could not be determined whether the hydroxylations were in different positions on the methylpyrridyl group or if M4 and M5 were diastereomers.

In the M6 and M7 spectra the *m/z* 128 and 129 ions were suspected to be one even-electron ion and one radical ion since they were only 1 *m/z* unit apart, which was consistent with the loss of water (−18 Da) resulting in *m/z* 128 and an OH radical (−17 Da) resulting in *m/z* 129. The loss of 17 Da (a hydroxyl radical) in the positive ion MS/MS spectrum has been previously reported for *N*-oxide metabolites[7–10,24] and a hydroxylamine metabolite,[10] but is unlikely for C-OH metabolites. The *m/z* 84 fragment was the most abundant in both the M6 and M7 spectra and might be the same fragment as in M5 (loss of $HOCH=CH_2$ from *m/z* 128). The *m/z* 100 fragment that was observed in the M4 and M5 spectra was not observed in the spectra of M6 and M7. Oxidative metabolism normally results in decreased retention in reversed-phase HPLC but *N*-oxidation is an exception,[25–27] which was another indication that M6 and M7 were *N*-oxides since the retention times of these metabolites were longer than that of the parent drug. To further explore the possibility of *N*-oxidation, H/D exchange experiments and experiments with APCI were applied.

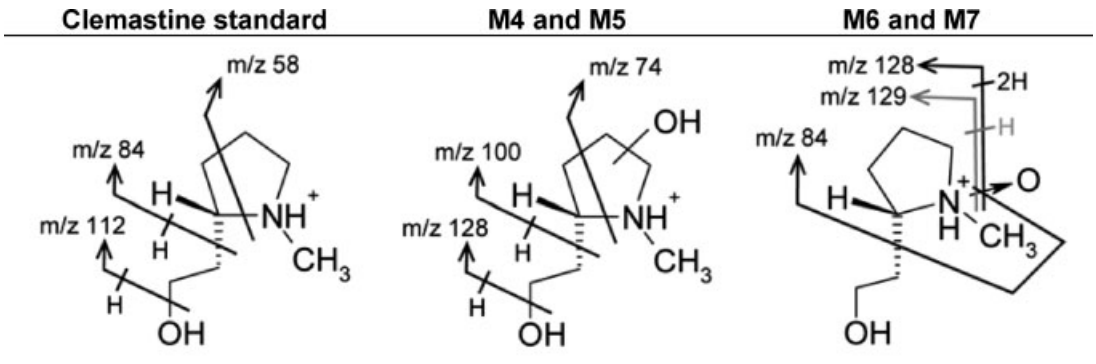
Hydrogen/deuterium exchange HPLC/MS/MS

The water and acetic acid in the HPLC mobile phase was changed to deuterium oxide and acetic acid-OD and MS full scan analysis of a *C. elegans* sample showed peaks with *m/z* 361 and 362, corresponding to one and two exchangeable hydrogen atoms of oxidized clemastine, respectively (chromatogram not shown). Thus, MS/MS of *m/z* 361 and 362 were applied, corresponding to oxidized clemastine with one or two deuterium atoms, respectively. As expected, the *m/z* value of the metabolites M2 and M3 shifted to *m/z* 362 upon H/D exchange and the main fragments were *m/z* 232 and 131, i.e. one deuterium on the hydroxyl group on the A fragment and one deuterium giving the positive charge of the B fragment (spectra not shown). The aliphatic alcohol group on the B fragment, resulting from the breakage of the ether bond upon CID, most likely took its proton from the carbon next to the ether oxygen and hence was not exchanged to deuterium.

M4 and M5 also proved to have two exchangeable hydrogen atoms (retention times 37 and 40 min, respectively; Figs. 5(A)–5(C)). As can be seen in their corresponding spectra (Figs. 5(D) and 5(E)), CID resulted in the fragments *m/z* 215 and 148, i.e. the B fragment in clemastine plus 16 *m/z* units plus two ^2H atoms. Thus these two metabolites appear to be hydroxylated on carbon atoms resulting in two possible places for proton exchange, the OH group and the proton giving the fragment its positive charge.

The M6 and M7 metabolites, eluting at 58 and 62 min, respectively (Figs. 5(F)–5(H)), only exchanged one hydrogen to deuterium resulting in the protonated molecule of *m/z* 361. The main fragments in the spectra presented in Figs. 5(I) and 5(J) were *m/z* 215 and 147, corresponding to the B fragment in clemastine plus 16 *m/z* units plus one ^2H atom. Thus, only one hydrogen could be exchanged in the molecule and for that to be possible the M6 and M7

Table 4. Proposed fragmentation of the B fragment of clemastine and the oxidized metabolites M4, M5, M6, and M7



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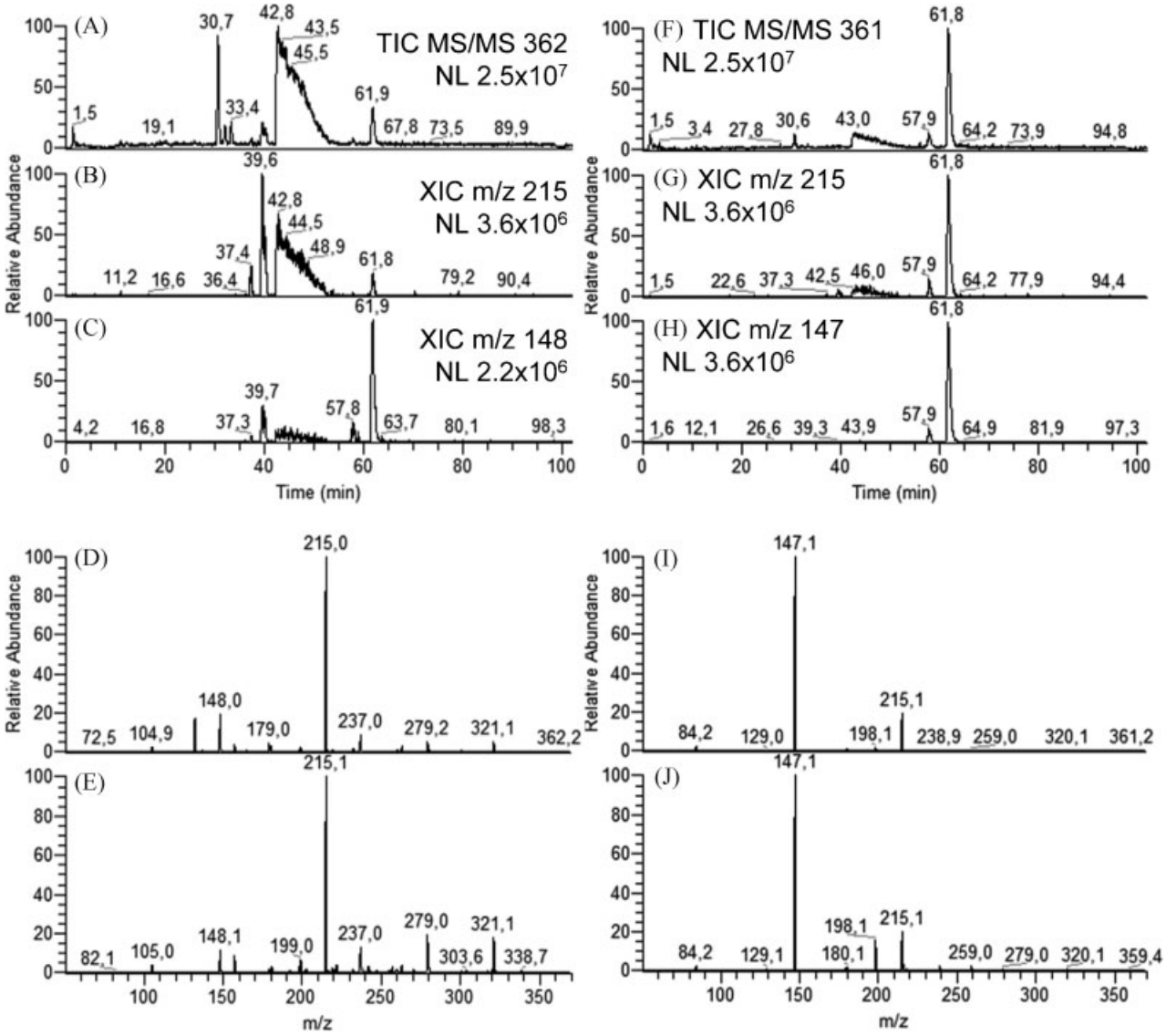

Figure 5. Chromatograms and spectra from the hydrogen/deuterium exchange experiment with a *C. elegans* sample. Left: MS/MS of *m/z* 362, corresponding to two exchangeable hydrogen atoms in the hydroxyl metabolite. Total ion chromatogram (A) and extracted ion chromatograms for *m/z* 215 (B) and *m/z* 148 (C). Spectra of the peaks at 40 min (D) and 37 min (E). Right: MS/MS of *m/z* 361, corresponding to one exchangeable hydrogen in the metabolite. Total ion chromatogram (F) and extracted ion chromatograms for *m/z* 215 (G) and *m/z* 147 (H). Spectra of the peaks at 62 min (I) and 58 min (J). HPLC gradient I was used for the separation and the Quantum Discovery mass spectrometer for the detection.

metabolites were either *N*-oxides (and the deuterium atom resulted in the positive charge of the fragment) or hydroxylamines, where the hydrogen atom in the hydroxyl group was exchanged and the quaternary amine carried a constant positive charge. However, hydroxylamines are normally formed by oxidiation of primary or secondary amine functions[5] and clemastine is a tertiary amine, which indicates that M6 and M7 are two *N*-oxides of clemastine.

Atmospheric pressure chemical ionization MS

N-Oxides can undergo deoxygenation during APCI due to thermal degradation.[8,12–14] This loss of an oxygen atom (16 Da) has been observed in the HPLC/APCI-MS spectra of the piperidyl and pyridyl *N*-oxides of loratadine, but not in the HPLC/APCI-MS/MS, HPLC/ESI-MS, or MS/MS spectra, indicating that the ion was formed by thermal energy activation in the vaporizer of the APCI source prior to the ionization.[12] A *C. elegans* sample was analyzed by HPLC/APCI-MS and -MS/MS, HPLC/ESI-MS and -MS/MS and the resulting mass spectra were compared. Figures 6(A) and 6(B) illustrate the spectra from M6 and M7, respectively, after HPLC/ESI-MS analysis. The most abundant ion in the spectra was *m/z* 360, i.e. $[M+H]^+$ of the metabolites, and no fragmentation occurred. The spectra of M6 and M7 after the HPLC/APCI-MS scan (Figs. 6(C) and 6(D)) show a significant conversion of *m/z* 360 into *m/z* 344, corresponding to the previously reported deoxygenation (−16 Da). The MS/MS spectra from APCI and ESI did not show any formation of the *m/z* 344 fragment (results not shown). The main fragments in APCI-MS/MS were the same as those described

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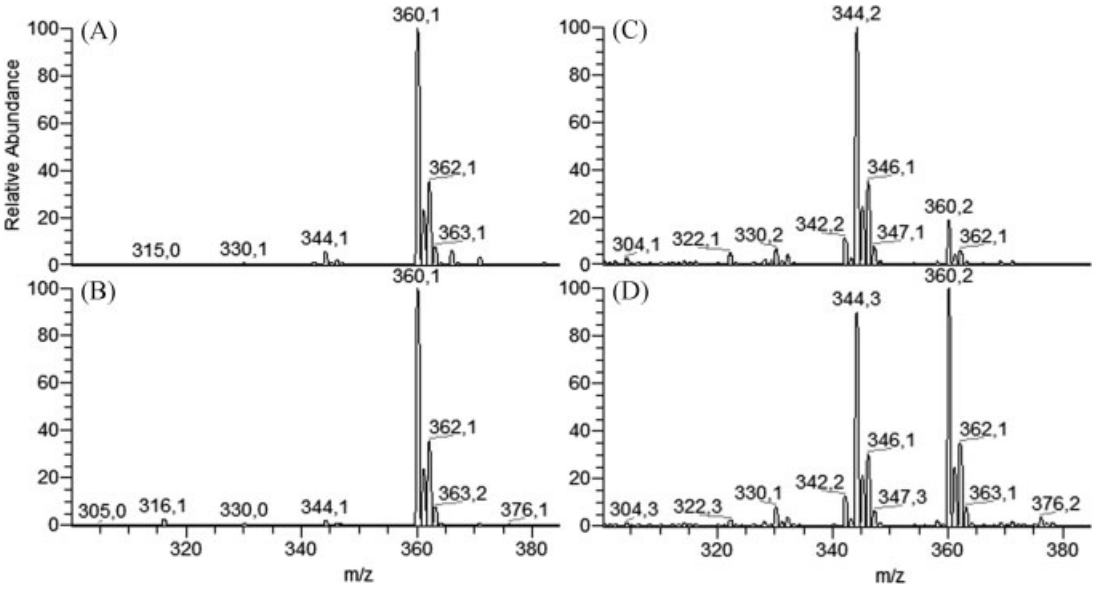

Figure 6. HPLC-ESI-MS spectra of M6 (A) and M7 (B) and HPLC-APCI-MS spectra of M6 (C) and M7 (D). Gradient IV was used for the HLPC separation and the Quantum Discovery mass spectrometer was used for the detection. For details, see the Experimental section.

for ESI-MS/MS but the intensities of the fragments were reversed (the *m*/*z* 215 fragment was more abundant than the *m*/*z* 146 fragment) and the total ion intensity was approximately 100 times lower in APCI compared to ESI.

Hence, the M6 and M7 metabolites of clemastine were both deoxygenated upon APCI-MS in concordance with results for *N*-oxides of other drugs described in the literature; loratadin,[12] clozapine, hyoscyamine, and tribenzylamine.[8] Stereoselective production of *N*-oxides has been reported, e.g. nicotine is metabolized to stable *cis*/*trans* diastereomers of nicotine-*N*′-oxide.[28,29] Thus, since clemastine is a single enantiomer, M6 and M7 probably are diastereomeric clemastine-*N*-oxides.

CONCLUSIONS

In this study, the combination of liquid chromatography and tandem mass spectrometry with two different ionization techniques and H/D exchange proved to be very useful for the structural elucidation of clemastine metabolites. The use of the fungus *C. elegans* facilitated the identification since higher concentrations of the metabolites were produced which enabled MS^3 and MS^4 experiments.

Clemastine has previously been shown to be biotransformed into several oxidized metabolites both in humans, horses, and dogs[2]. Here we describe that norclemastine, four isomers of hydroxyclemastine, and two diastereomer *N*-oxide metabolites of clemastine were formed in *C. elegans* incubations. Two of the hydroxylated metabolites were shown to be oxidized on the methylpyrridyl moiety, one on the aromatic ring with the chloro substituent, and one on the aromatic ring without the chlorine. Both H/D exchange experiments and APCI-MS were used in order to confirm the structures of the two metabolites oxidized on the nitrogen.

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