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Identification of some new clemastine metabolites in dog, horse, and human urine with liquid chromatography/tandem mass spectrometry

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The metabolism of clemastine was studied in dogs, horses, and humans after a single dose of Tavegyl®. The urine collected was extracted by solid-phase extraction or hydrolyzed with β-glucuronidase and then extracted by liquid-liquid extraction, prior to analysis for unchanged drug and phase I and II metabolites by liquid chromatography/tandem mass spectrometry. The metabolites were identified by their molecular mass and interpretation of the product ion spectra, since no standard substances were available. Unchanged drug was recovered in urine samples from dogs and humans, but not from horses. In dogs and humans, the phase I metabolite, norclemastine, was identified, and clemastine metabolites with one and two additional oxygens were found in all three species. In horses and dogs monohydroxylation on one of the aromatic rings or the adjacent methyl group was favored while, in humans, the additional oxygen was positioned on either the aromatic or the aliphatic part of the structure, and the aliphatic reaction seemed to result in at least three isomers. In the metabolites with two additional oxygens, both the oxygens were found on the aliphatic fragment in humans and dogs, whereas they were situated on the aromatic part of the structure in horses. In human patients, glucuronidated monohydroxyclemastine was recovered, and in urine from horses both mono- and dihydroxyclemastine glucuronides were identified, while phase II metabolites could not be recovered from the dog urine. Clemastine metabolism in dogs and horses has, to our knowledge, not been studied before, and new metabolites from humans are presented in this article. Thus, the metabolites described in the present work have not been previously reported in the literature.

Clemastine (Fig. 1) is an antihistamine used for the relief of symptoms associated with allergic rhinitis, eczema, and urticaria in humans.[1] In Sweden, Tavegyl® (clemastine) has been used since the 1960s, and the drug is also used for treatment of allergic reactions in dogs and horses. Recent studies of the pharmacokinetics of clemastine in dogs[2] and horses[3] showed that the bioavailability of the drug was low, dogs 3% and horses 3.4%, indicating that the dose regimens for oral administration of clemastine suggested in the literature are likely to result in a systemic exposure too low to allow effective therapy. After intravenous injection, the terminal half-life of the drug was 3.8 h for the dogs and 5.4 h for the horses, which is why the drug has to be injected at least three times daily to maintain therapeutic plasma concentrations. Thus there are clinically relevant pharmacokinetic differences between these two species and humans where oral administration twice daily has been found adequate.[1]

Despite widespread use of clemastine since its introduction, there are only a few reports on the pharmacokinetics and metabolism of this drug. In a study of the metabolism of clemastine *in vitro* in the presence of radioactive cyanide it was suggested that metabolism to the corresponding amide could take place.[4] The detection of clemastine metabolites in rat,[5–7] as well as one article about the identification of urinary metabolites of clemastine in humans,[8] have been reported. In these studies nothing but aromatic *O*-dealkylated metabolites were structurally determined, i.e. structures formed after cleavage at the ether function. As far as we know, no reports have been published regarding the metabolism of clemastine in horses and dogs.

The aim of the present study was to identify metabolites formed and excreted in urine after administration of Tavegyl® in dogs, horses, and humans, and then compare the results between the species. High-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used, and the metabolites were identified by their

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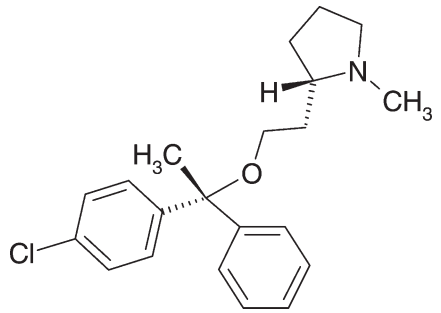


Figure 1. The structure of clemastine, (+)-2-[2-[1-(4-chlorophenyl)-1-phenylethoxy]ethyl]-1-methylpyrrolidine.

molecular mass and interpretation of the product ion spectra, since no standard substances were available.

EXPERIMENTAL

Materials

Clemastine fumarate was obtained from Novartis Sweden AB (Täby, Sweden). β-Glucuronidase from *E. coli* (K12 strain) was purchased from Roche Diagnostics Scandinavia AB (Bromma, Sweden). Methanol, acetonitrile, dichloromethane, 2-propanol, ammonia 25%, hydrochloric acid, acetic acid, potassium dihydrogen phosphate, sodium acetate, sodium carbonate, sodium hydrogen carbonate, and sodium hydroxide were of analytical grade or better, and used without further purification. The water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Isolute™ solid-phase mixed-mode columns (C18 and cation exchanger; 300 mg HCX-3, 3 mL) were a gift from Sorbent AB (Västra Frölunda, Sweden).

Instrumentation

High-performance liquid chromatography was performed with an HP series 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) with a binary pump, degasser and autosampler. The chromatographic columns used were a Phenomenex® Luna 5 μ C18 (2) (size 150 × 2.00 mm, length × i.d., particle size 5 μm), or a Phenomenex® Luna 5 μ C8 (2) (size 50 × 2.00 mm, length × i.d., particle size 5 μm). The mobile phase consisted of 50% methanol, and 50% water containing 0.1% acetic acid, and the flow rate was 0.2–0.3 mL/min. The HPLC column outlet was connected to a Quattro LC (Micromass, Manchester, UK) quadrupole-hexapole-quadrupole mass spectrometer equipped with an electrospray interface (ESI). The instruments were controlled using the MASSLYNX software vers. 3.3. The MS parameters were optimized for sensitivity manually during direct infusion of an aqueous solution of clemastine. During analyses, the ESI parameters were; capillary voltage 1.3 kV, cone 24 V, extractor 3 V, and RF lens 0.90 V. The desolvation temperature was 350°C, and the source block temperature was 120°C. The nebulizer and desolvation gas flows were 105 and 510 L/h, respectively. When running MS/MS, the hexapole collision cell was filled with argon gas at a pressure of 1.2×10^{-3} mbar. Solid-phase extraction (SPE) was performed with a Vac-Master sample processing station (Sorbent AB, Västra Frölunda, Sweden) and pH measurements were made using a Metrohm 744 manual pH-meter (Herisau, Switzerland).

Drug administration

Three horses and three dogs were given clemastine intravenously (Tavegyl® solution for injection 1 mg/mL, Novartis, diluted in 0.9% sodium chloride solution). The doses given were 50 μg/kg body weight for the horses administered as a constant 20-min infusion, and 100 μg/kg body weight for the dogs administered as a 10-min injection. The doses were chosen to give plasma concentrations suitable for pharmacokinetic purposes. The pharmacokinetic studies are presented elsewhere.[2,3] Urine was collected before, as well as 5–7 and 3–5 h after administration, for horses and dogs, respectively. The Ethical Committee for Animal Experiments, Uppsala, Sweden, approved the study protocols. One female and one male patient donated urine when treated with clemastine (Tavegyl®). The dose taken was 2 mg perorally for the female, and 3 mg perorally for the male, and urine was collected for 24 h.

Sample preparation

Hydrolysis with β-glucuronidase

Enzymatic hydrolysis was performed when searching for phase I metabolites. Urine (dog 2.0 mL, horse and human 10.0 mL) was transferred to a test tube. Phosphate buffer pH 6.5 (for dog samples 0.50 mL, horse and human samples 2.0 mL) was added and, if necessary, pH was adjusted with HCl and NaOH. β-Glucuronidase (dog 100 μL, horse and human 200 μL) was added, and the test tubes were incubated in a water bath at 50–60°C for 60 min. The residue was dissolved in 100 μL methanol/water (50:50).

Liquid-liquid extraction

Liquid-liquid extraction (LLE) was used to extract phase I metabolites. The urine, which had been hydrolyzed with β-glucuronidase, was made alkaline with 2 M NaOH (dog 200 μL, horse and human 800 μL). Extraction was performed for 20 min with dichloromethane/2-propanol, 85:15 (dog, horse, and human 10.0 mL). After 10 min of centrifugation the aqueous phase was removed and the organic phase was transferred to a clean test tube. Acetic acid was added (dog, horse, and human 50 μL) and the solvent was evaporated at 50°C and 3 psi. The residue was dissolved in 100 μL methanol/water (50:50).

Solid-phase extraction

Solid-phase extraction (SPE) was used to isolate the phase II metabolites. The pH of the urine (dog 2.0 mL, horse and human 10.0 mL) was adjusted to 5.0 with HCl and NaOH. The SPE columns were conditioned with methanol (5 mL) and 2 M sodium acetate buffer pH 4.9 (5 mL). The urine was added, and the column was then washed with 2 M sodium acetate buffer pH 4.9 (2 mL). To elute the analytes, methanol with 2% ammonia (5 mL) was used. The solvent was evaporated at 50°C and 3 psi.

RESULTS AND DISCUSSION

Method characterization

When analyzing a standard solution of clemastine by LC/ESI-MS scan, the protonated molecular ion ($[M+H]^+$,

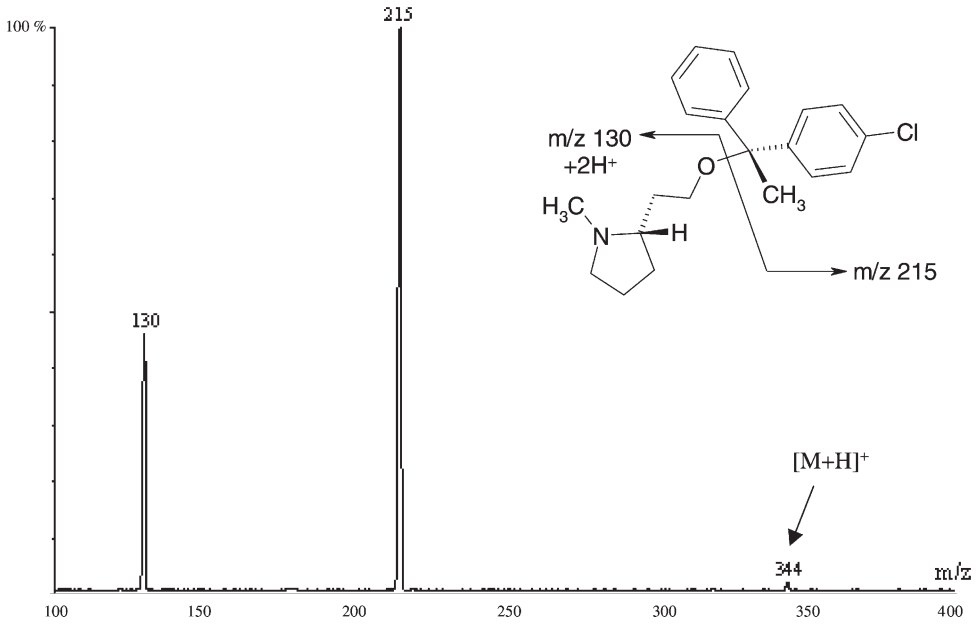


Figure 2. Fragmentation of clemastine. The injected clemastine solution was an aqueous solution with a concentration of 1.42 μg/mL. The column used was a Phenomenex® Luna 5 μ C8 (2) (size 50 × 2.00 mm, length × i.d., particle size 5 μm). The collision energy was 15 eV. Other conditions as described in the Experimental section.

m/z 344) was the main peak in the mass spectrum. The fragmentation of clemastine was obtained by collision-induced dissociation (MS/MS) and resulted in two main fragments, *m/z* 130 and 215, which was interpreted as a result of the cleavage at the ether function (Fig. 2).

The reason for using two different extraction methods, i.e. LLE and SPE, was to try to recover a broad range of metabolites. These methods could not be evaluated concerning precision and sensitivity for the metabolites, since there were no standard substances available for any of them. The SPE was performed with a mixed phase column, C18 and cation exchanger, to obtain both ion exchange and hydrophobic qualities in the solid phase, and thus to improve the extraction of the glucuronides, as the relatively high polarity of these conjugates could give a low retention on pure reversed-phase materials. Despite that, it is reasonable to expect that the recovery of the glucuronides was not complete.

For clemastine, the precision and limit of detection (LOD) were determined. The variation in absolute chromatographic peak area (CV), when liquid-liquid extracting five hydrolyzed urine samples with addition of clemastine standard solution to a concentration of 14.2 ng/mL urine, was 13% for horses and 9.7% for dogs after LC/MS/MS analysis. In human urine, CV for four hydrolyzed and liquid-liquid extracted samples (clemastine concentration 16.6 ng/mL urine) was 21%. Being a qualitative study, the precision was thought acceptable for its purpose. The LOD, corresponding to a signal-to-noise ratio of three from the mass chromatogram of *m/z* 215 after full product ion scan, was estimated to 0.6 ng/mL urine for all three species.

Unchanged drug and phase I metabolites

In a previous study of clemastine metabolism in humans, performed with gas chromatography/mass spectrometry,[8] unchanged drug could not be identified, but in this study clemastine was recovered by LLE and identified by LC/MS/MS in humans and dogs.

In urine from dogs, peaks corresponding to *m/z* 376, 360, 344, and 330 were found after hydrolysis and LLE. After fragmentation of *m/z* 376, corresponding to $[M+H]^+$ of clemastine with the addition of two oxygens, the fragment *m/z* 215 was found just like in clemastine, but the other main fragment was *m/z* 162 (+32 mass units, Fig. 3(A), compared to *m/z* 130, Fig. 2), indicating that both the oxygen atoms were located on the aliphatic part of the molecule. However, it could not be determined whether there were two hydroxyl groups or one *N*-oxide and one hydroxyl group. Fragmentation of *m/z* 360 resulted in the fragments *m/z* 130, as in clemastine, and *m/z* 231 (+16 mass units, Fig. 3(B), cf. Fig. 2) indicating that this was monohydroxylated clemastine. Unchanged drug (Fig. 3(C)), and a metabolite with $[M+H]^+$ at *m/z* 330 corresponding to norclemastine, where the amine has been demethylated leaving a fragment of *m/z* 116 (–14 mass units) compared to *m/z* 130 in clemastine, were also recovered (Fig. 3(D)).

After hydrolysis and LLE of horse urine, chromatographic peaks corresponding to the addition of one and two oxygen atoms to clemastine, $[M+H]^+$ *m/z* 360 and 376, were observed using MS scan. The locations of the two oxygens were examined by MS/MS. The fragment *m/z* 130 existed in both the metabolites, i.e. the one that was also found in clemastine, but the fragment corresponding to *m/z* 215 in clemastine was shifted to 231 (+16 mass units) and 247 (+32 mass units), respectively. The mass spectral results thus indicated that both the substituents were positioned on the aromatic part of the structure or on the adjacent methyl group, and the mass-to-charge ratio was in accordance with one and two hydroxyl groups. It could not be determined where the hydroxyl groups were positioned.

RCM

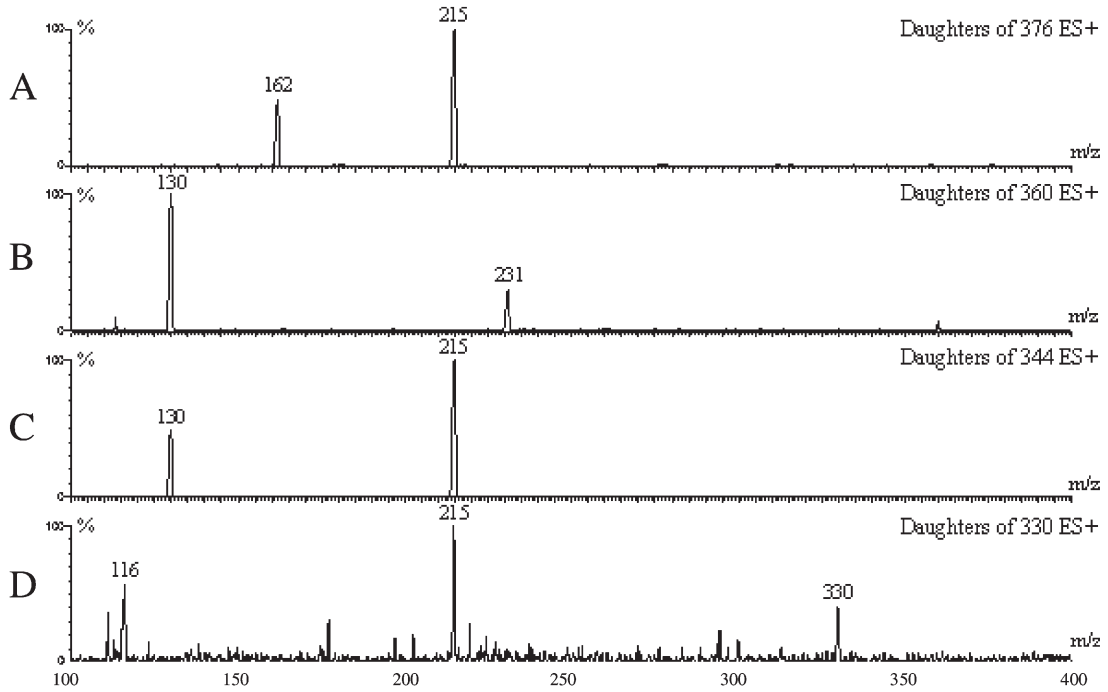


Figure 3. Spectra of metabolites found in dog urine. (A) +32 mass units on the aliphatic fragment; (B) +16 mass units on the aromatic fragment; (C) unchanged clemastine with the two main fragments at m/z 130 and 215; and (D) −14 mass units on the aliphatic fragment. The column used was a Phenomenex® Luna 5 μ C8 (2) (size 50 × 2.00 mm, length × i.d., particle size 5 μm), and the collision energy was 15 eV. Other conditions as described in the Experimental section.

In urine samples from the human patients, unchanged drug was identified together with norclemastine. Fragments at *m/z* 130 and 231 occurred after MS/MS of *m/z* 360, proposing hydroxylation of any of the aromatic rings or the adjacent methyl group, and interestingly, there was more than one chromatographic peak in the chromatogram at *m/z* 146 (+16 mass units) and 215, indicating at least three different isomers of the aliphatic addition of oxygen (Fig. 4, cf.

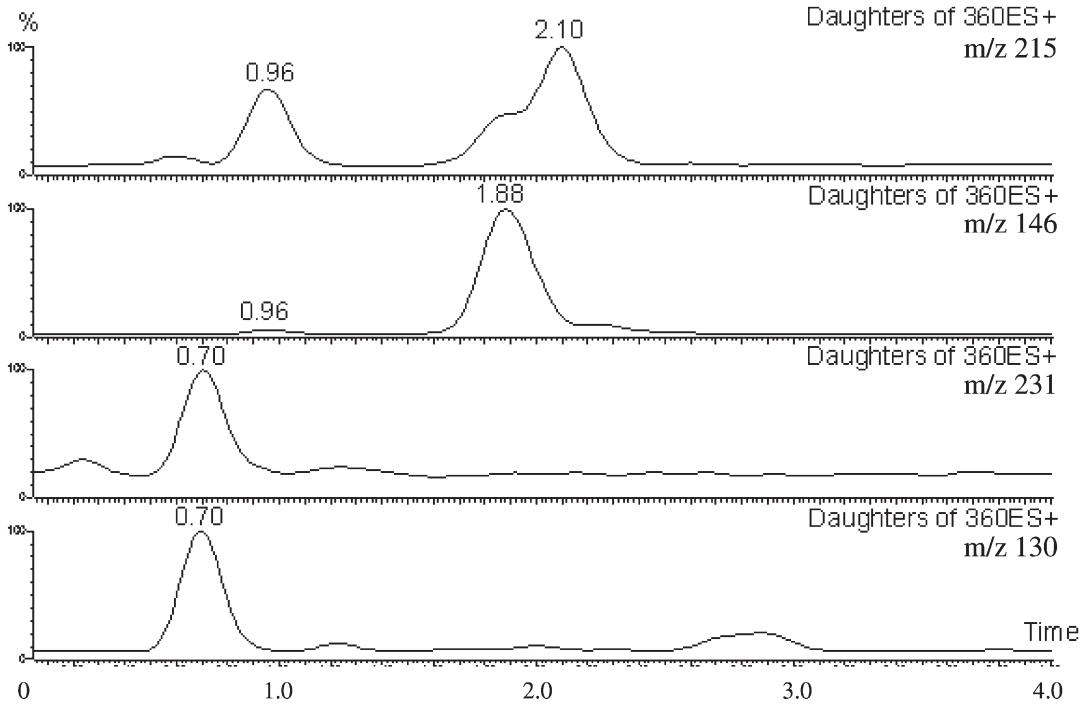


Figure 4. Extracted ion chromatograms for the product ions (daughters) of *m/z* 360. The metabolites were recovered from human urine. The column used was a Phenomenex® Luna 5 μ C8 (2) (size 50 × 2.00 mm, length × i.d., particle size 5 μm), and the collision energy was 15 eV. Other conditions as described in the Experimental section.

Table 1. Mass spectral data from metabolites recovered in urine collected from dogs, horses and humans

Precursor ion [M+H$^+$]	Main product ions Dogs (*m/z*)	Main product ions Horses (*m/z*)	Main product ions Humans (*m/z*)
344 (clemastine)	130, 215	130, 215	130, 215
330 (−14, demethylation)	116, 215	116, 215	116, 215
360 (+16, one additional oxygen)	130, 231	130, 231	130, 231*
360 (+16, one additional oxygen)	—	—	146, 215**
376 (+32, two additional oxygens)	162, 215	130, 247	162, 215
536 (glucuronidation of *m/z* 360)	—	130, 231	130, 231
552 (glucuronidation of *m/z* 376)	—	130, 247	—

*In one chromatographic peak.
**In at least three chromatographic peaks.

Fig. 2). Fragments from *m/z* 376, corresponding to the addition of two oxygens, were *m/z* 162 and 215, and gave only one chromatographic peak, indicating that both the hydroxyl groups were aliphatic. It was never determined if any of the metabolites were *N*-oxides. For a summary of the metabolites found in dogs, horses and humans, see Table 1.

Fragmentation at higher collision energies did not result in any additional structural information for any of the species, why possible *N*-oxides could not be confirmed. The metabolites that could not be identified in this study might have been lost in the extraction, existed in lower concentrations than could be detected, or were not formed during the period of sample collection. The possibility of stereoselective metabolism was not studied.

Phase II metabolites

In horses, glucuronidation seemed to be an extensive way of drug metabolism. Chromatographic peaks for [M+H]$^+$, *m/z* 536 and 552, were observed using MS scan, and interpreted as the glucuronidation of hydroxy- and dihydroxyclemastine, respectively. When performing MS/MS of *m/z* 536 at different collision energies (Fig. 5), fragments from monohydroxyclemastine (*m/z* 130 and 231) were observed, but also *m/z* 343 (−193 mass units), remaining after the loss of glucuronic acid and the hydroxy oxygen. The fragment *m/z* 407 (−129 mass units, loss of the aliphatic fragment) is unusual since the glucuronide bond was intact, and the presence of this fragment indicated that this metabolite was an *O*-glucuronide. For a summary of the main fragments of hydroxyglucuronylclemastine, see Fig. 6. After analyzing urine from horses, there was one peak in the chromatogram for glucuronidated dihydroxyclemastine at *m/z* 552. The main fragments from LC/MS/MS were the ones from dihydroxyclemastine (*m/z* 130, 247), results not shown. There were no indications of structural isomers of any of the conjugates.

In urine from the human patients, the only conjugated metabolite found was [M+H]$^+$ at *m/z* 536, the metabolite identified as glucuronidated monohydroxyclemastine in the

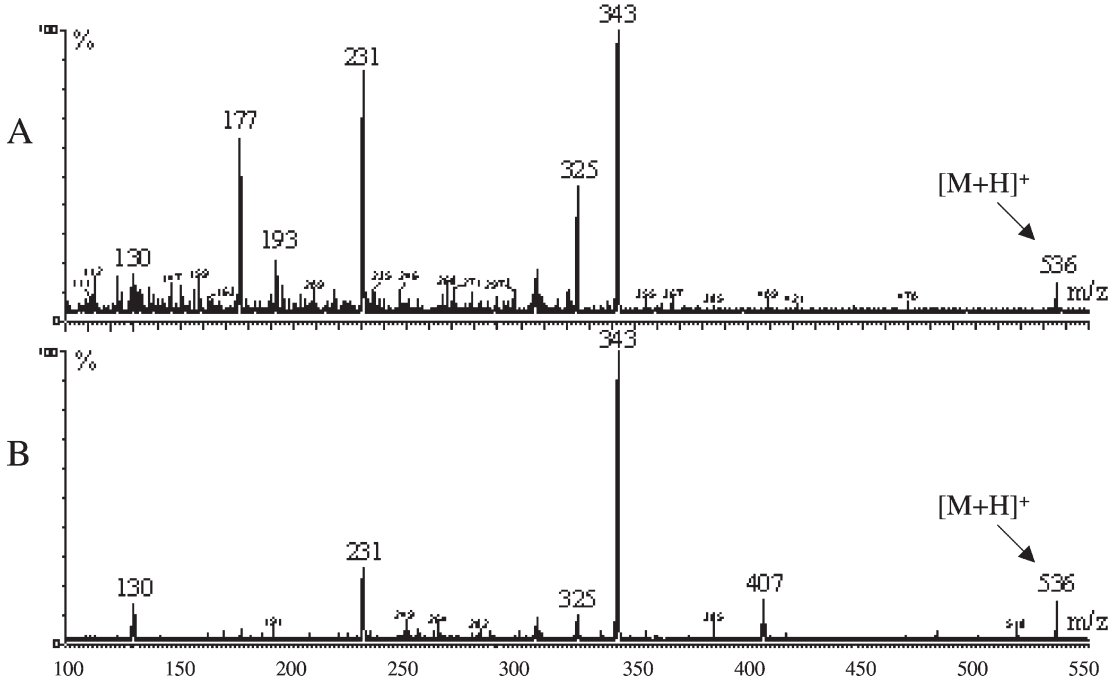
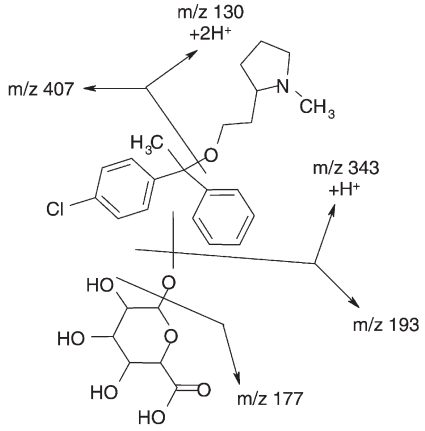


Figure 5. Fragments of hydroxyglucuronylclemastine at different collision energies (A, 15 eV and B, 25 eV). The metabolite was recovered in urine from the horses. The column used was a Phenomenex® Luna 5 μ C18 (2) (size 150 × 2.00 mm, length × i.d., particle size 5 μm). Other conditions as described in the Experimental section.



m/z	**Fragment**
130	Aliphatic fragment
177	Glucuronic acid - OH
231	Aromatic fragment
343	536 - glucuronic acid (*m/z* 193)
407	536 - aliphatic fragment (*m/z* 129)
536	[M+H+] Hydroxyglucuronylclemastine

Figure 6. Proposed fragmentation of glucuronidated monohydroxyclemastine. The metabolite was recovered from horse urine.

horses (results not shown). Product ion spectra indicated that this was a conjugate of a hydroxy metabolite formed after oxidation of any of the aromatic rings or their adjacent methyl group, as the fragments *m/z* 130 and 231 existed (cf. Fig. 3(B)).

No phase II metabolites could be recovered from the dog urine. Maybe dogs do not use glucuronidation to the same extent as horses and humans in the metabolism of clemastine, or maybe they are slower in their phase II metabolism so that the urine samples should have been collected for a longer period of time. No sulfates or *N*-glucuronides were found in any of the species, even though *N*-glucuronidation of other aliphatic tertiary amines has been reported in humans.[9,10] Maybe the extraction procedure was not optimal for extracting these metabolites, or they existed in a concentration that was below the limit of detection, or they were not formed at all.

To the very best of our knowledge, the metabolism of clemastine has not been studied in dogs or horses prior to this work, and, in humans, only phase I metabolites based on cleavage at the ether function have been reported. The metabolites identified here also differ from those reported earlier in rats.[5,6] Thus, none of the metabolites described in this paper have been previously documented.

CONCLUSIONS

This study proposes differences in the drug metabolism of clemastine between dogs, horses, and humans, in both phase I and phase II reactions. The metabolism of clemastine in dogs and horses has, to our knowledge, not been previously reported. In this study, unchanged drug was recovered in urine from dogs but not in that from horses, while phase II metabolites were identified in horses but not in dogs. These results indicate that horses metabolize the drug to a larger extent than dogs. There were also differences in the products formed after phase I metabolism between the species. A previous study of the clemastine metabolism in man suggested only the formation of seven different metabolites, all as a result of *O*-dealkylation followed by different phase I reactions, and unchanged drug was not recovered. In this present work, several new metabolites have been identified as well as unchanged clemastine after oral administration to humans. Our results indicate the formation of different isomers after aliphatic oxidation, and that the drug can be conjugated with glucuronic acid.

Acknowledgements

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