Research Article

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Identification of etamiphylline and metabolites in equine plasma and urine by accurate mass and liquid chromatography/tandem mass spectrometry

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Etamiphylline camsylate (Millophylline V) was administered intravenously to two horses at a dose of 2.8 mg/kg. Urine and blood samples were taken up to 32 h post administration. Unhydrolyzed plasma and urine was extracted using solid phase extraction (SPE). The identity of the parent drug and metabolites was confirmed using a linear ion trap mass spectrometer and accurate mass analysis on an orbitrap mass spectrometer. Desethyletamiphylline (molecular weight 251) was the main metabolite observed in the urine and plasma samples and resulted from the N-deethylation of etamiphylline. The second metabolite detected in urine and plasma resulted from the demethylation of etamiphylline (molecular weight 265). The third minor metabolite detected in urine was proposed to have resulted from a simultaneous N-deethylation and demethylation of etamiphylline (molecular weight 238).

Keywords: etamiphylline; linear ion-trap quadrupole LC-MS/MS; LTQ orbitrap

Introduction

Etamiphylline [7-(N, N-diethylaminoethyl)theophylline] (Figure 1) is a cardiac stimulant that increases cardiac output without an increase in heart rate. It is also a respiratory stimulant prescribed for respiratory failure as it relaxes the smooth muscle of the bronchi and bronchioles and therefore opens up the airways for relief from chronic obstructive pulmonary disease.[1–5]

Horses may be exposed to etamiphylline during legitimate clinic treatment, inadvertently through ingestion of contaminated feed, or as a result of a deliberate attempt to influence race performance. Due to its potential to improve performance, the use of etamiphylline is banned by the British Horseracing Authority during racing.

Previous research has reported the presence of a number of etamiphylline metabolites present in both greyhounds[1,2] and camels[6] following a drug administration. The main metabolites observed were N-desethyletamiphylline, N-desmethyl-etamiphylline and theophylline. Following oral administration of etamiphylline camsylate (Millophylline V) to five greyhounds, urine was collected for up to 24 h post administration and the samples analysed by gas chromatography-mass spectrometry (GC-MS).[2] Etamiphylline was rapidly cleared from the body following oral administration and seven basic and three acidic phase 1 metabolites were identified. Following intra-muscular administration of etamiphylline camsylate to two racing greyhounds at a dose of 10 mg/kg, etamiphylline and desethyletamiphylline were detected in greyhound urine for up to 72 h by GC-MS.[1] Etamiphylline camsylate was intra-muscularly administered to the camels at a dose of 3.5 mg/kg. N-desethyletamiphylline was detected in urine for about 13–14 days. Theophylline and a metabolite possibly resulting from demethylation of etamiphylline were detected in urine for about 5 h after drug administration.[3]

The detection of etamiphylline and its metabolites in equine urine and plasma by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using selected reaction monitoring (SRM) is reported. The samples were also analyzed on an LTQ orbitrap mass spectrometer. The objective of the study was to characterize the metabolites present up to 32 h after intravenous injection of Millophylline V (etamiphylline camsylate). To the best of our knowledge, the metabolism of etamiphylline in equine urine or its detection in blood has not been previously reported.

Experimental

Chemicals and reagents

Etamiphylline camsylate was purchased as Millophylline V from Dales Pharmaceuticals (Skipton, UK). Methanol, ethyl acetate, acetonitrile and ammonia were supplied by Fisher Scientific (Leistershire, UK). Hydrochloric acid was obtained from Preston (Hinckley, UK) and trichloroacetic acid from Sigma (Dorset, UK). All water used was purified by an ElgaStat Option 4 (or 5A) purification unit, which includes deionisation (Elga, High Wycombe, Bucks, UK).

Administration of etamiphylline and sample collection

Millophylline V was administered intravenously to two male thoroughbred horses (ages 6 and 16, body weights 498 and 537 kg). Each horse received a single dose (2.8 mg/kg). Urine and blood samples were collected prior to administration, and

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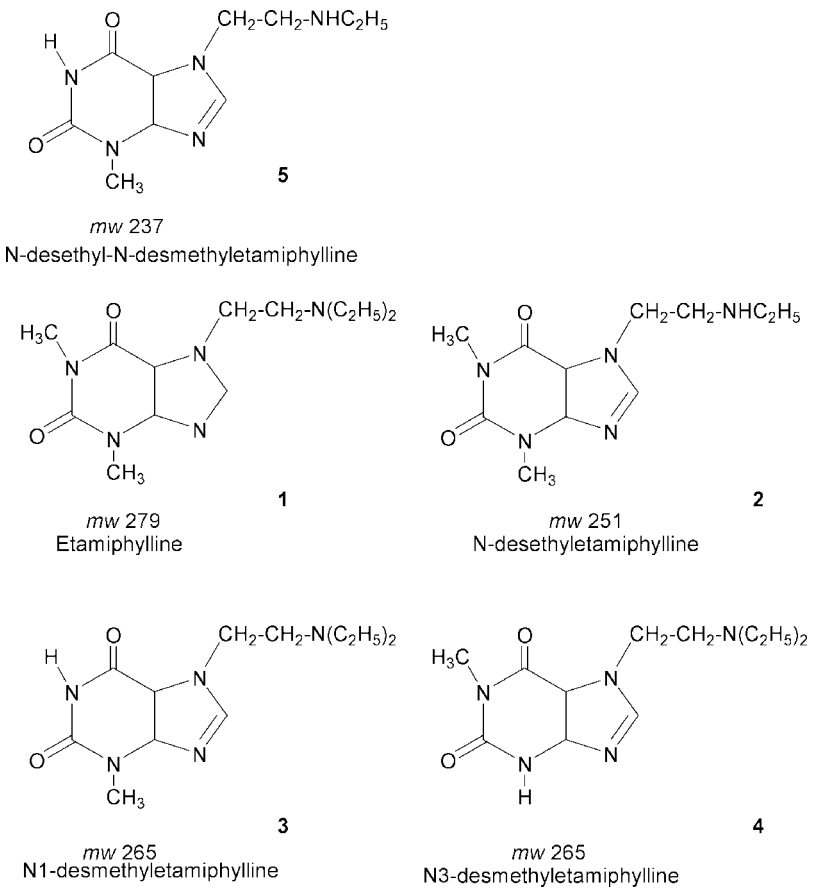

Figure 1. Structures of etamiphylline and proposed metabolites.

post-administration samples were collected for up to 32 h. About 25 mL of blood was collected from each horse at each sampling point and the times recorded. Immediately after collection, the blood was centrifuged, plasma was separated, and aliquots were dispensed into different vials. All urine void times and volumes were also recorded, and samples were stored at −20 °C until required for analysis. Home Office ethical approval was granted prior to administration study commencement by an authorized Animal Welfare Committee.

Plasma sample preparation

Previous experiments indicated that etamiphylline and its major metabolites did not undergo phase II conjugation as little change in recovery was observed in comparison between enzyme hydrolysis and the unhydrolyzed samples. Therefore the samples were not hydrolyzed prior to extraction.

10 ng/mL fenethylline (internal marker) was added to each plasma sample (1 mL). 73 mM trichloroacetic acid (0.5 mL) was added to the plasma, mixed, and left for 5 min at room temperature. Acetonitrile (0.5 mL) was added followed by 1M acetate buffer pH 4.7 (1.5 mL). The mixture was vortexed briefly and centrifuged at 1000 RCF for 10 min.

Urine sample preparation

10 ng/mL fenethylline (internal marker) was added to each urine sample (1 mL). 1M acetate buffer pH 4.7 (1.5 mL) was added and the sample was centrifuged at 1000 RCF for 10 min.

Sample extraction

The samples were extracted by solid phase extraction (SPE) on Strata-X-C 60 mg 3 mL cartridges (Phenomenex, Macclesfield, UK). The cartridges were conditioned with methanol (3 mL) followed by water (3 mL). The prepared sample was applied and the cartridge washed sequentially with 0.1M hydrochloric acid (3 mL), ethyl acetate (3 mL) and methanol (3 mL). The compounds were eluted with 5% ammonia (*v*/*v*), 5% methanol in ethyl acetate (*v*/*v*) (3 mL) followed by 5% ammonia in acetonitrile: methanol (75 : 25, *v*/*v*) (3 mL). The combined elution solvents were evaporated to dryness under nitrogen at ambient temperature and reconstituted in 0.1% acetic acid: acetonitrile (95 : 5, *v*/*v*) (100 µL).

Linear Ion-trap quadrupole liquid chromatography/tandem mass spectrometry

LC-MS/MS analysis was carried out on an API 3200 Qtrap mass spectrometer (ABI, Warrington, UK) interfaced to an HP 1100 series liquid chromatograph (Agilent, West Lothian, UK). Chromatographic separation was achieved using an Atlantis® T3 column (2.1 × 100 mm 3 µm particle size) (Waters, Elstree, UK). The column temperature was maintained at 35 °C. Elution was performed at a flow rate of 300 µL/min with solvents 0.1% acetic acid (aq *v*/*v*; mobile phase A) and acetonitrile (mobile phase B). A gradient was employed starting at 2% B increasing to 7% at 0.2 min, 10% at 1.2 min, 60% at 3.0 min and 100% at 3.5 min. The gradient was maintained at 100% B for 1 min followed by re-equilibration at 2% B for 4.5 min. The ion source was operated

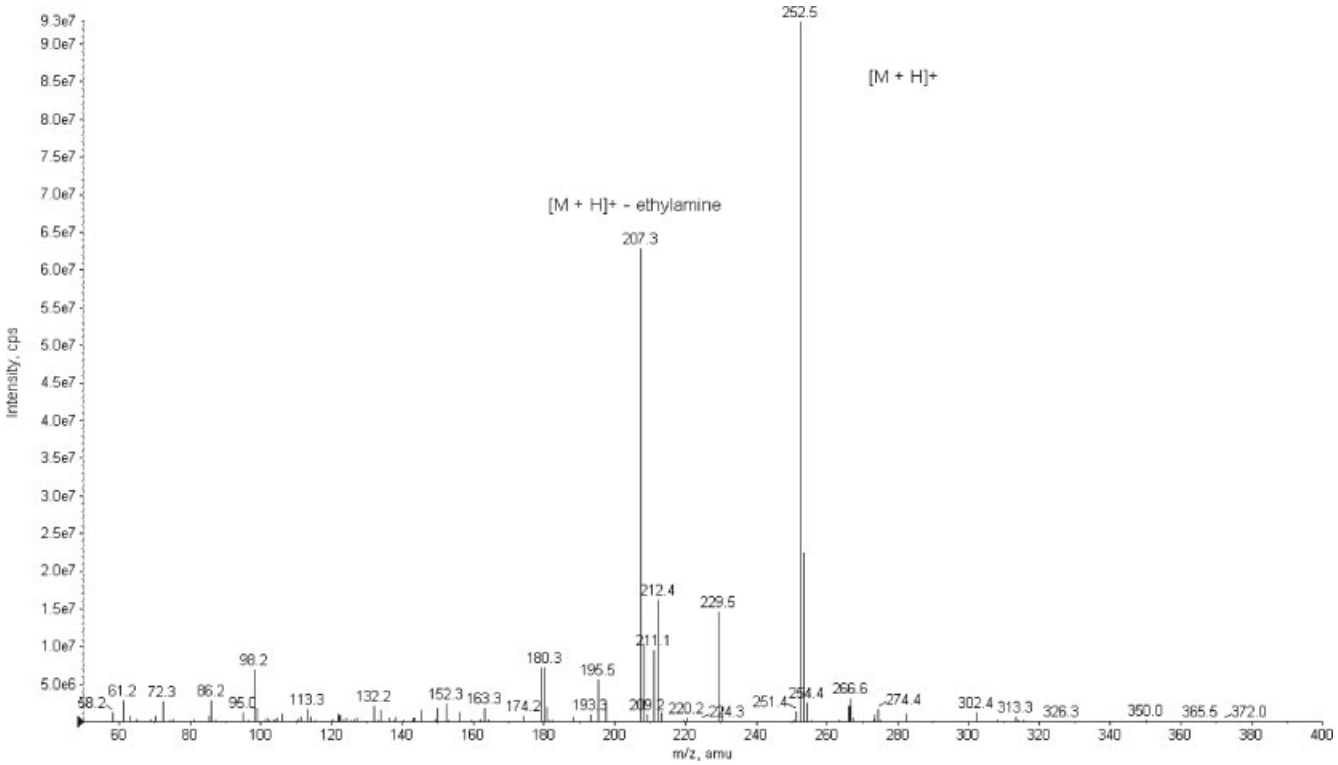

Figure 2. LC-MS/MS full scan analysis of N-desethyletamiphylline in 8.08 hr post administration urine sample. RT = 2.69 min.

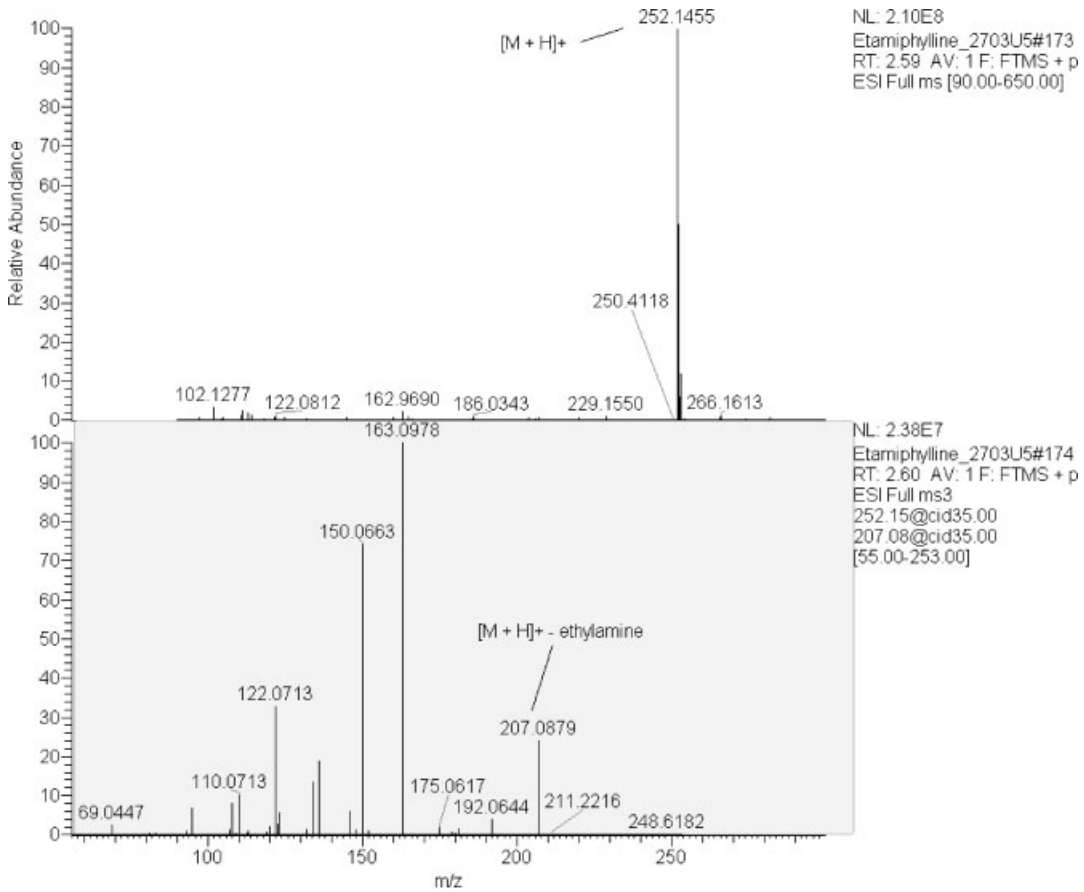

Figure 3. Accurate mass analysis of N-desethyletamiphylline in 8.08 hr post administration urine sample A) full scan B) MS^3. RT = 2.59 min.

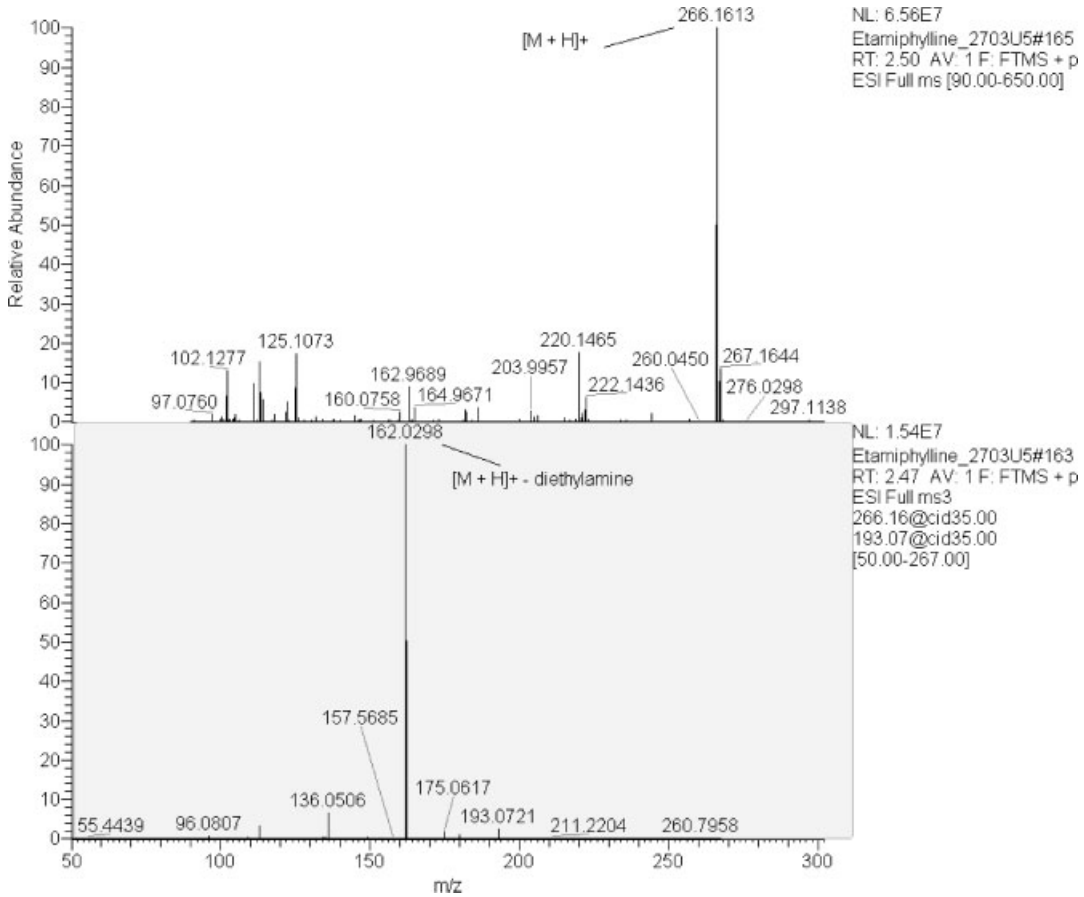

Figure 4. Accurate mass analysis of N1 or N3-desmethyletamiphylline in 8.08 hrs post administration urine sample A) full scan B) MS^3. RT = 2.50 min.

in the electrospray ionisation positive mode at 600 °C. The curtain gas was 15 units, nebuliser gas 75 units, turbo gas 60 units and the ionspray was 5.5 kV. 20 µL of sample was injected onto the linear ion trap LC-MS/MS system.

Data were acquired in the full scan (*m/z* 50–400) and selected reaction monitoring (SRM) modes. The SRM transitions correspond to *m/z* 280/207 for etamiphylline, *m/z* 252/207 for N-desethyletamiphylline, *m/z* 266/193 for N-desmethyletamiphylline and *m/z* 238/193 for N-desethyl-N-desmethyl-etamiphylline.

High resolution accurate mass liquid chromatography mass spectrometry

Analysis was performed on a LTQ orbitrap mass spectrometer (Thermo Scientific, Waltham, UK) at a resolving power of 30,000. Ionization was carried out in the positive mode using an electrospray (ESI) source. The capillary temperature was 200 °C, the sheath gas flow was 30 units, the auxiliary gas flow was 10 units and the ionspray was 4.5 kV.

Chromatographic separation was achieved using an Atlantis® T3 column (2.1 × 100 mm 3 µm particle size) (Waters, Elstree, UK). Elution was performed at a flow rate of 300 µL/min with a gradient of acetonitrile and acetic acid (0.1% aq, *v*/*v*) mixture. A gradient was employed starting at 0% B increasing to 10% at 1.2 min, 35% at 2.0 min, 65% at 3.0 min and 98% at 3.5 min. The gradient was maintained at 98% B for 1 min followed by re-equilibration at 0% B.

Data were acquired in the positive electrospray full scan (*m/z* 90–650) and MS^3 scan modes of product ions 252.15 and 266.1 (*m/z* 55–253). The expected accurate mass values for the parent drug and metabolites were *m/z* 280.1768, *m/z* 252.1455, *m/z* 266.1611 and *m/z* 238.1598 for etamiphylline, N-desethyletamiphylline, N1/N3-desmethyletamiphylline and N-desethyl-N-desmethyletamiphylline, respectively.

Results and Discussion

Previous studies on the identification of etamiphylline and metabolites reported the presence of N-desethyletamiphylline and N-desmethyletamiphylline in greyhound and camel urine and plasma samples.[1–3] A proposed scheme for the metabolism of etamiphylline in equine plasma and urine is illustrated in Figure 1.

Etamiphylline

The presence of etamiphylline (compound **1**) in the plasma and urine samples was confirmed by linear Ion-trap quadrupole liquid chromatography by comparison to a drug standard purchased from Dales Pharmaceuticals (Skipton, UK). Etamiphylline has a nominal mass of 279. An *m/z* 207 ion was detected in both drug standard and post-administration samples with a match in accurate mass and retention time. The *m/z* 207 ion is characteristic fragment due to the loss of N,N-diethylamine.

N-desethyletamiphylline

N-desethyletamiphylline (compound **2**), formed by the N-deethylation of etamiphylline, was the main metabolite observed

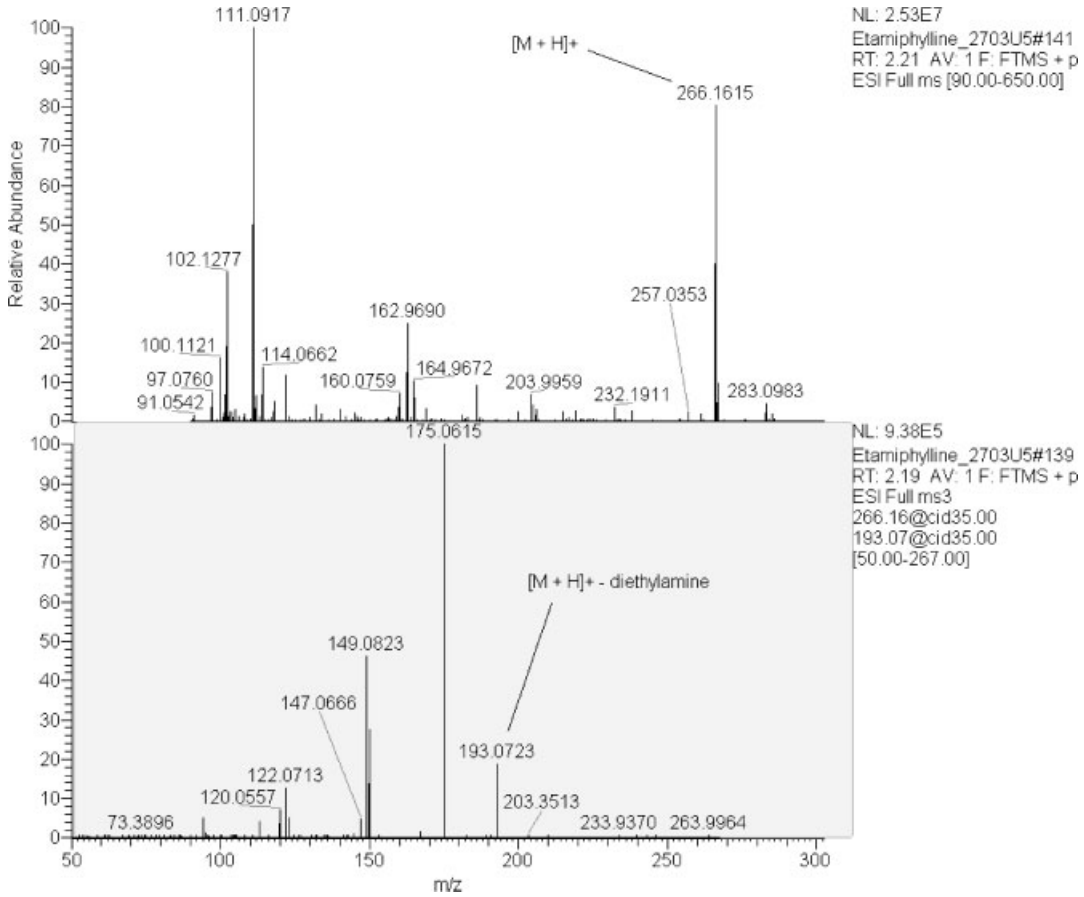

Figure 5. Accurate mass analysis of N1 or N3-desmethyletamiphylline in 8.08 hr post administration urine sample A) full scan B) MS^3. RT = 2.21 min.

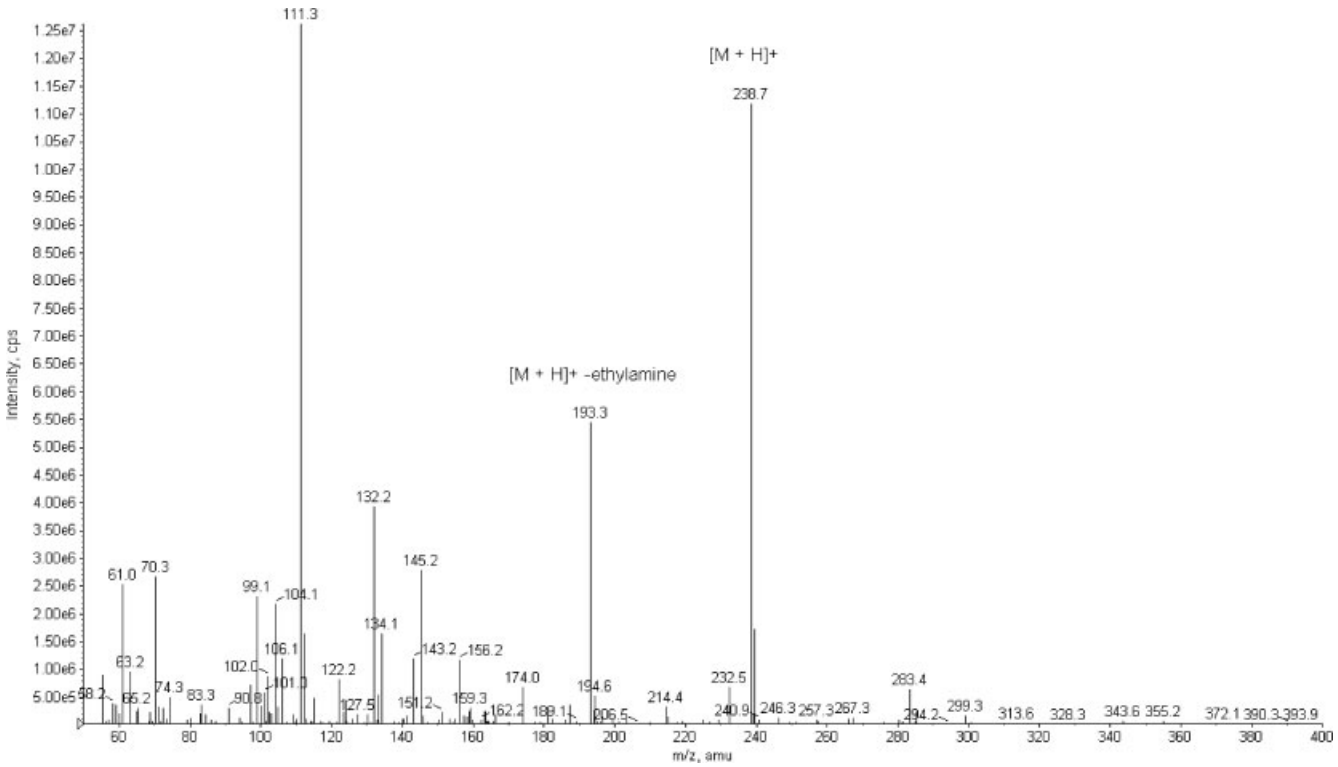

Figure 6. LC-MS/MS full scan analysis of N-desethyl-N-desmethyletamiphylline in 8.08 hr post administration urine sample. RT = 2.29 min.

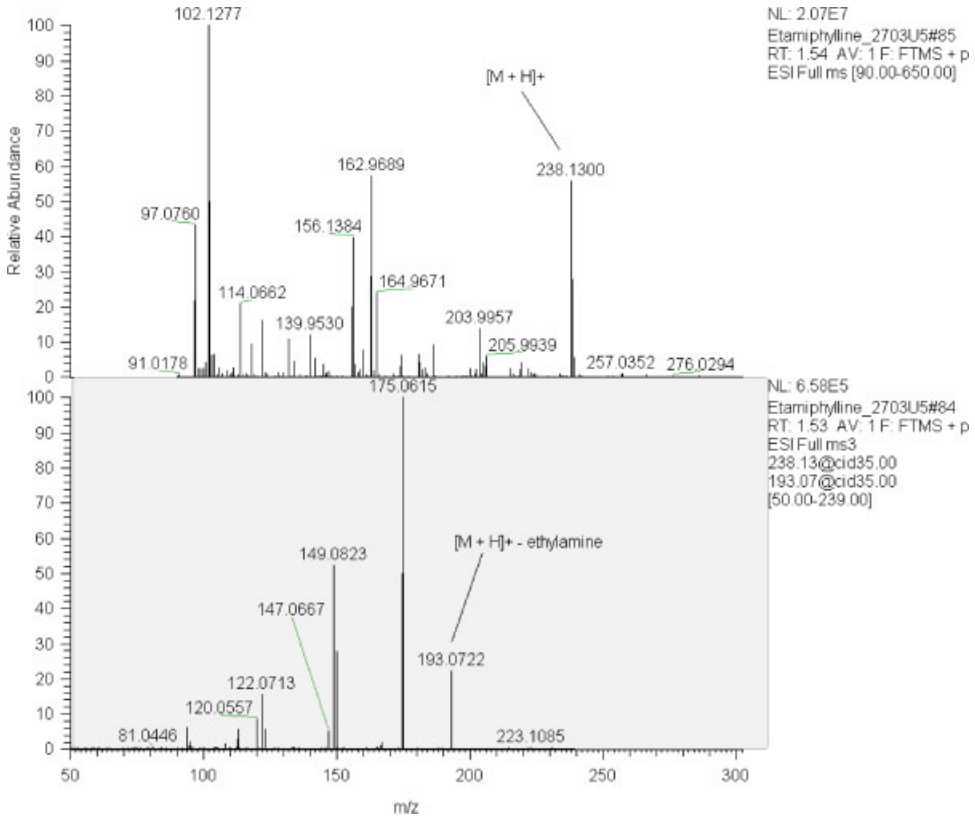

Figure 7. Accurate mass analysis of N-desethyl-N-desmethyletamiphylline in 8.08 hrs post administration urine sample A) full scan B) MS^3. RT = 1.54 min.

in plasma and urine samples. N-desethyletamiphylline has a molecular weight of 251, and was detected up to 32 h post administration. A representative urine sample collected 8.08 h after administration indicated the presence of N-desethyletamiphylline by linear Ion-trap quadrupole liquid chromatography (Figure 2). The *m/z* 207 ion is a diagnostic fragment ion due to the loss of N-ethylamine (45 Da) as described in a previous report.[1] The accurate mass data confirmed the presence of protonated molecular ion ($[M+H]^+$) at *m/z* 252.1455 (full scan) and product ions at *m/z* 207.0879 and *m/z* 163.0979 (MS^3) which are characteristic of N-desethyletamiphylline (Figure 3).

N1/N3 desmethyletamiphylline

The analysis of the post-administration plasma and urine samples indicated the presence of N1 and N3–desmethyletamiphylline (compounds **3** and **4**). After initial protonation of etamiphylline, cleavage of the bond between the nitrogen and the α-carbon atoms of the 2-(diethylamino)ethyl substituent N7 occurs with the simultaneous loss of N,N-diethylamine.[1] Two peaks (RT = 2.17 and 2.50 min) were detected with a molecular ion $[M+H]^+$ at *m/z* 266; it was proposed that each peak was representative of one of the isomers. Further investigation by LC-MS/MS determined the presence of an (*m/z*) 193 fragment in both isomers. The fragments are characteristic of the loss of the N, N-diethylamine (73 Da). Without drug reference standards of the metabolites, the two isomers cannot be distinguished. The accurate mass data confirmed the presence of $[M+H]^+$ at *m/z* 266.1613 (FS), *m/z* 193.0721 (MS^3) and *m/z* 162.0298 (MS^3) ions for one of the isomers (RT = 2.50 min). The other isomer (RT = 2.21 min) established $[M+H]^+$ ion at *m/z* 266.1615 (FS) and product ions at *m/z* 193.0723 (MS^3) and *m/z* 175.0615 (MS^3) (Figures 4 and 5).

N-desethyl-N-desmethyletamiphylline

N-desethyl-N-desmethyletamiphylline (compound **5**) was observed in the post-administration urine and plasma samples. The LC-MS/MS full-scan data of a urine sample collected 8.08 h post administration revealed $[M+H]^+$ ions at *m/z* 238.7 and 193 (Figure 6). The fragment at *m/z* 193.3 indicated a loss of N-ethylamine (45 Da) which was a site specific cleavage for the etamiphylline metabolites. Accurate mass analysis detected $[M+H]^+$ ion at *m/z* 238.1300 and a product ion at *m/z* 193.0722 (Figure 7).

Conclusion

After intravenous administration of Millophylline V (etamiphylline camsylate) to horses, the parent drug and number of metabolites were detected. Desethyletamiphylline was the main metabolite observed in the urine and plasma samples up to 32 h (last available time point) and resulted from the N-deethylation of etamiphylline. N1/N3–desmethyletamiphylline was observed as a minor metabolite in both plasma and urine samples. N-desethyl-N-desmethyletamiphylline was only observed as a minor metabolite in the urine samples. Diagnostic fragments of the corresponding $[M+H]^+$ ions with losses of 73 and 45 Da suggest a site-specific cleavage of the side chain.

The metabolism of etamiphylline appears similar across species. The metabolites observed in horses, greyhounds, and camels were N-desethyletamiphylline and N1/N3 desmethyletamiphylline.

M. Elghazali *et al*. investigated etamiphylline metabolites in camels and identified theophylline as a minor metabolite.[3] Etamiphylline-N-oxide was reported as a metabolite following the administration of etamiphylline in greyhound samples.[1] Theophylline and etamiphylline were not observed in the equine plasma and urine samples. To our knowledge, the results from the study are the first to discuss the presence of N-desethyl-N-desmethyletamiphylline in urine samples following administration.

This study identified the presence of a number of etamiphylline metabolites in horses that were found in plasma and urine following administration. The presence of the metabolites will aid the detection of etamiphylline in the screening of post-race equine samples, and the subsequent confirmation of an etamiphylline administration.

Acknowledgements

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