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Short communication

N-Deethylation and *N*-oxidation of etamiphylline: identification of etamiphylline-*N*-oxide in greyhound urine by high performance liquid chromatography–mass spectrometry

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Abstract

Millophyline-V®, (etamiphylline camsylate) was administered intramuscularly to two racing greyhounds at a dose of 10 mg kg^{-1}. Unhydrolysed pre- and post-administration urine samples were extracted using mixed mode solid phase extraction (SPE) cartridges, the basic isolates derivatised as trimethylsilyl ethers and analysed by positive ion electron ionisation gas chromatography–mass spectrometry (GC/EI+/MS). The parent drug and one metabolite, *N*-desethyletamiphylline, were detected in urine for up to 72 h. For semi-quantification, urine samples were extracted on-line using a Prospekt sample handler. The analytes retained on the C_2 SPE cartridge were eluted by the mobile phase directly on to the analytical high performance liquid chromatography column and analysed by positive ion atmospheric pressure chemical ionisation (LC/APCI+) MS in the multiple selective-ion recording mode. A major peak containing both ions (*m*/*z*) 280 and (*m*/*z*) 252 was observed. Full scan LC/APCI+/MS of the unknown indicated that the ion at (*m*/*z*) 280 was formed by the loss of an oxygen atom [$MH^+ \rightarrow (MH^+ - O)$]. Samples were analysed by positive ion electrospray ionisation LC/MS on two different instruments and the unknown compound was identified as an *N*-oxide of the *tert*. nitrogen atom of the 2-(diethylamino)ethyl substituent on N_7 of the theophylline nucleus. This compound has not been reported previously either as an in vivo or in vitro metabolite of etamiphylline in any species. Thermal decomposition of the *N*-oxide could lead to an increase the detection period of the parent drug during routine GC/MS screening of post-competition greyhound urine samples.

Keywords: Millophyline; Etamiphylline-*N*-oxide; Greyhound urine; Biotransformation; LC/MS; GC/MS

1. Introduction

The synthetic trialkyl xanthine, Millophyline–V® {etamiphylline camsylate; 7-[2-(diethylamino)ethyl]-3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione·7,7-Dimethyl-2-oxobicyclo(2.2.1)heptane-1-methanesulfonic acid}, formulated both as injection solution and tablets, is available in the UK for use in dogs. It is a cardiac stimulant exerting a positive inotropic effect, increasing cardiac output without increase in heart rate. It relaxes the smooth muscle of the bronchi and bronchioles and therefore opens up the airways for relief from chronic obstructive pulmonary disease [1-4].

After oral administration of etamiphylline to the greyhound, the drug was rapidly absorbed with peak urinary levels occurring between 1 and 2 h after administration. Seven basic and three acidic, predominantly unconjugated, phase I metabolites were identified in dog urine [4]. After intramuscular administration of etamiphylline (3 mg kg^{-1}) to camels, the pharmacokinetics and metabolism were determined. The detection period of *N*-desethyletamiphylline, the major unconjugated metabolite in camel urine, was reported to be 2 weeks [5]. After oral and intramuscular administrations of Millophyline–V® at doses between 1 and 3 mg kg^{-1} to thoroughbred horses, the parent drug and six unconjugated basic

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metabolites have been tentatively identified in horse urine (Dumasia, unpublished data).

The present study was undertaken to define appropriate analyte(s) for the detection of etamiphylline in post-race greyhound urine. In unhydrolysed urine from greyhounds administered intramuscularly with Millophyline–V®, etamiphylline and *N*-desethyletamiphylline were detected by GC/MS, further two metabolites etamiphylline-*N*-oxide and *N*-desmethyletamiphylline were detected and tentatively identified by LC/MS. The results of these studies are presented in this paper.

2. Experimental

2.1. Reagents and chemicals

Glass distilled grade organic solvents were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK). Etamiphylline camsylate (pure drug) was obtained from Dales Pharmaceuticals Ltd. (Skipton, North Yorkshire, UK). Millophyline–V® Injection (140 mg/ml; 50 ml) was purchased from Arnolds Veterinary Products Ltd. (Shrewsbury, UK). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK). Worldwide Monitoring XtracT® mixed-mode SPE cartridges (XRDAH506, 500 mg, 6 ml) were obtained from Anachem Ltd. (Luton, Bedfordshire, UK).

2.2. Drug administration to animals, collection and storage of biofluids

Millophyline–V® was administered intramuscularly to two male greyhounds (body weights 36 and 40 kg). Each dog received a single dose (3 ml ≡ 420 mg etamiphylline camsylate or 229 mg free base) as 2 × 1.5 ml injections in each rear catel thigh. Urine samples were collected pre-dose and at 2, 4, 8, 24, 48 and 72 h post-dose. All samples were stored at −20 °C until required for analysis.

2.3. Extraction and analysis of metabolites by GC/MS

For qualitative analysis, aliquots (1 ml) of pre- and sequential post-dose urine samples from both administrations were made up to 4 ml with phosphate buffer (0.1 M, pH 6) and centrifuged at 2000 × *g* for 15 min. The prepared samples were extracted off-line using XtracT® mixed mode SPE columns as described previously [6,7]. The dry residues were derivatised by the addition of MSTFA in toluene (1:1, v/v, 50 μl; 80 °C, 0.5 h), the derivatising reagents removed under nitrogen at 60 °C, re-dissolved in toluene (50 μl) and analysed by GC/EI+/MS.

2.4. Gas chromatography/mass spectrometry

GC/EI+/MS at 70 eV was performed on a Fisons MD800 bench top mass spectrometer interfaced to a Thermoquest Trace 2000 series GC. A SGE BPX5 column (approximately 25 m, 0.22 mm i.d., 0.25 μm film thickness) was used with helium as the carrier gas. The initial column temperature, 90 °C, was maintained for 1 min. The oven temperature was programmed at 15 °C min^{-1} to 320 °C and then maintained at 320 °C for 6 min. The injector, transfer line and the ion source temperatures were 260, 280 and 150 °C, respectively. Aliquots (1 μl) of the derivatised isolates were injected in the splitless mode (1 min) using an autosampler (CTC model A200S, Zwingen, Switzerland). Full scan EI+ mass spectra were recorded from 8 to 18 min of run time by scanning from 50 to 450 Da. Authentic etamiphylline standard (10 ng μl^{-1}; 1 μg dissolved in 100 μl of derivatisation reagent), was analysed with each batch.

2.5. Preparation of samples for LC/APCI/MS analysis

Early urine samples (2–24 h) from dog A were diluted from 1:1 up to 1:500 and from dog B (2–8 h) were diluted up to 1:10 (v/v) with ammonium acetate buffer (0.1 M, pH 6.0). For the semi-quantification of etamiphylline, propentofylline {3,7-dihydro-3-dimethyl-1-(5-oxo-hexyl)-7-propyl-1H-purine-2,6-dione} was added as the internal standard. Duplicate aliquots of the diluted samples (5 ml) were taken for analysis by on-line SPE/LC/APCI+/MS.

2.6. Automated sample extraction and LC/APCI+/MS analysis

On-line SPE was performed with a Prospekt sample handler (Spark–Holland Instrumenten, Emmen, The Netherlands) using a six-port valve, a cartridge-switching device and a solvent delivery unit as described previously [8]. The prepared samples were placed in a rack on the Gilson 231XL sampling injector. A C_2-ethyl cartridge (2 mm i.d. × 10 mm; International Sorbent Technology Ltd., Mid Glamorgan, UK) was conditioned with MeOH (4 ml min^{-1}, 0.25 min) followed by ammonium acetate buffer (3 ml min^{-1}, 0.75 min). The sample (100 μl) was loaded with an excess volume of buffer (0.5 ml min^{-1}, 1 min) followed by a wash step with 0.1% formic acid (1 ml min^{-1}, 0.5 min). Prior to elution, the cartridge was switched in line with the analytical LC column and MS acquisition initiated.

A second flow stream was used for the elution of the analytes and gradient elution chromatography. The delivery of the elution and mobile phase solvents was under control of a Rheos 2000 quaternary gradient HPLC pump connected to a single quadrupole benchtop mass spectrometer (Micromass Platform LC; Micromass UK Ltd., Manchester, UK). A Waters XTerra™ ODS column (RP_{18} 150 mm × 3 mm internal diameter; 5 μ particle size) fitted with a Haiguard holder and C18B Guard-Disc was used. The column temperature was maintained at 30 °C, the maximum pressure was 400 lb in^{-2} and the run time was 9 min per sample. Elution was performed at a flow rate of 0.7 ml min^{-1} with a gradient of ammonium acetate buffer (0.05 M, pH 5) and methanol mixture (Table 1).

Table 1
HPLC mobile phase gradient elution program

Time (min)	Ammonium acetate	Methanol
0	95	5
3.5	30	70
5.0	30	70
5.5	95	5
9	95	5

Mass spectrometry was performed with an APCI+ source, the cone voltage was set at 20 V, APCI pin at 3.5 kV, source temperature was at 120 °C, the probe heater at 350 °C and the gas flow was 425 l h^{-1}. Data were acquired in the multiple selective ion recording (SIR) mode, acquiring the ions (*m*/*z*) 252.2 (*N*-desethyletamiphylline, analyte; RT, ≈3.77 min), (*m*/*z*) 280.2 (etamiphylline, analyte; RT, ≈3.93 min) and (*m*/*z*) 307.2 (propentofylline, IS; RT, ≈5.93 min). A major substrate-related compound (RT, ≈ 4.18 min; see Fig. 3) was detected in the post-administration urine samples.

2.7. Identification of the unknown metabolite by LC/MS

For the identification of the unknown urinary metabolite, full-scan LC/APCI+/MS and LC positive ion electrospray ionisation (ESI+)/MS data were acquired on the Platform instrument under the same LC conditions as above. For ESI+/MS, the cone voltage was set at 30 V, the capillary at 3.5 kV, the source temperature at 130 °C, the multiplier at 650 V and gas flow was 220 l h^{-1}. For APCI+/MS, the cone voltage was set at 20 V, multiplier at 650 V, APCI pin at 4.2 kV, source temperature at 120 °C, the probe heater at 430 °C and the gas flow was 375 l h^{-1}. Data were acquired from 50 to 350 Da. The samples were also analysed on a Finnigan MAT TSQ 70 instrument upgraded with TSQ 700 software. LC/ESI+/MS was performed at a capillary temperature of 280 °C, spray voltage of 4.5 kV, capillary 8.3 V and the sheath gas flow 450 l h^{-1}. Data were acquired in the full-scan mode (scan range 50–350 Da) using the same HPLC conditions.

3. Results

3.1. Qualitative GC/MS analysis

Etamiphylline and its metabolites have no derivatisable functional groups. Derivatisation of the isolates was performed to silylate the co-extracted endogenous compounds to aid chromatographic resolution of the analytes. The TIC, mass chromatograms of the α-cleavage ions and the full scan EI+ mass spectra of both etamiphylline and *N*-desethyletamiphylline (M1) obtained by GC/MS analysis of a post-administration urine sample are shown in Figs. 1 and 2. Similar full scan GC/MS data (not shown) were obtained from both animals for 72 h. No other metabolites were detected in urine.

3.2. LC/MS detection and identification of the unknown metabolite

Typical SIR/LC/APCI+/MS data from the analysis of an unhydrolysed post-administration urine sample is shown in Fig. 3. In addition to etamiphylline (peak 1) and M1 (peak 2), a major metabolite (M2, peak 3) was observed at a retention time of about 4.2 min. The full-scan ESI+ mass spectrum of M2 (Fig. 4; spectrum 1) showed the $[M+H]^+$ ion at (*m*/*z*) 296 with little fragmentation. The full-scan APCI+ spectrum of M2 (Fig. 4, spectrum 2) showed extensive fragmentation with a weak $[M+H]^+$ ion at (*m*/*z*) 296, a major ion at (*m*/*z*) 280 $[(MH)^+–O]$, (*m*/*z*) 278 $[(MH)^+–H_2O]$, (*m*/*z*) 268 (loss of C_2H_4) and the base peak at (*m*/*z*) 252 $\{(MH)^+ - (16+28)\}$. The loss of an oxygen atom (16 amu) is consistent with the thermal "deoxygenation" of *N*-oxides in APCI/MS [9]. This compound was therefore tentatively identified as an *N*-oxide of etamiphylline.

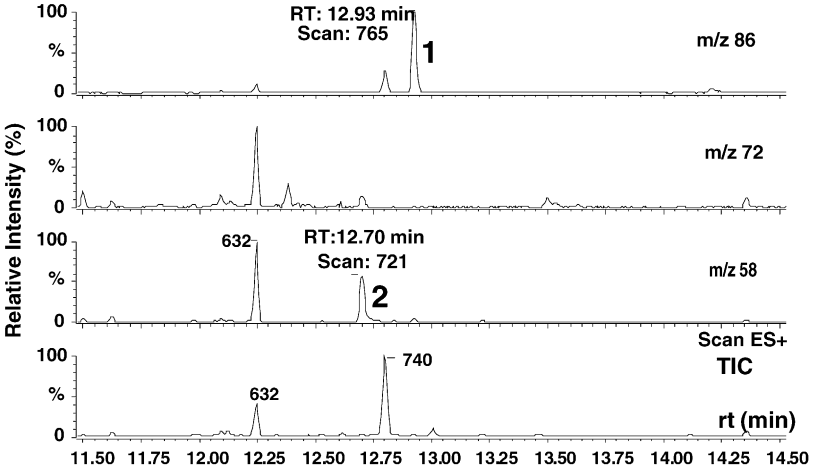

Fig. 1. The TIC and mass chromatograms of the α-cleavage ions (*m*/*z*) 58 and 86 obtained from GC/EI+/MS analysis of a TMS-derivatised isolate of a 72 h post-administration greyhound urine sample showing the presence of etamiphylline (peak 1) and *N*-desethyletamiphylline (peak 2).

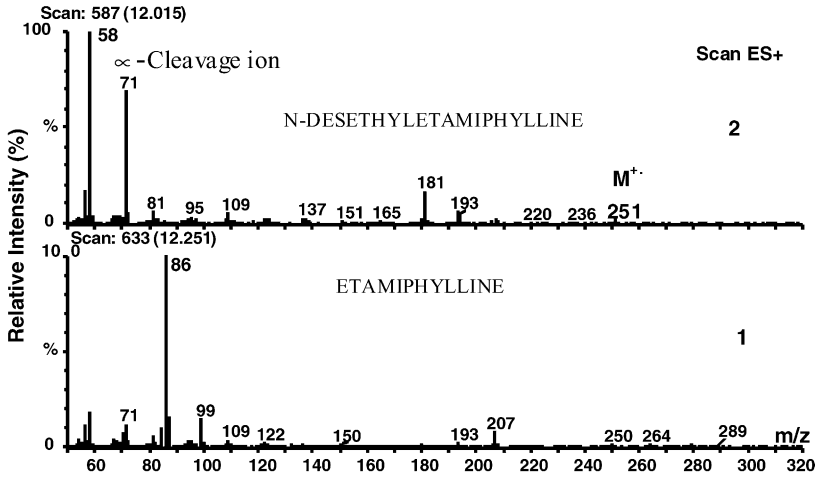

Fig. 2. Representative full scan EI+ mass spectra of (1) etamiphylline and (2) *N*-desethyletamiphylline obtained from a72 h post-administration urine sample.

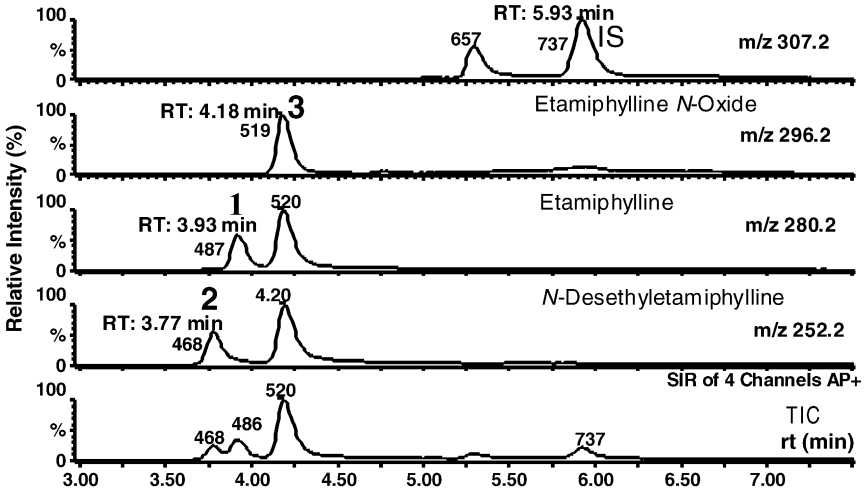

Fig. 3. SIR/LC/APCI+/MS data obtained from the analysis of a post-administration greyhound urine sample showing the presence of etamiphylline (peak 1), *N*-desethyletamiphylline (peak 2) and the unknown major metabolite (peak 3).

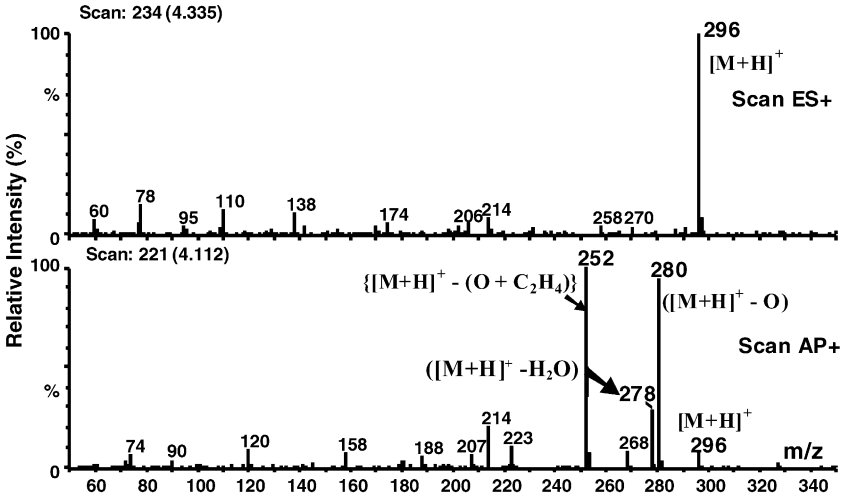

Fig. 4. Full-scan ESI+ (1) and APCI+ (2) mass spectra of etamiphylline-*N*-oxide (M2) obtained by LC/MS analysis of a post-administration urine sample, showing the characteristic thermal deoxygenation ion at (*m*/*z*) 280 [(*M*H)$^+$—O)] in APCI mode.

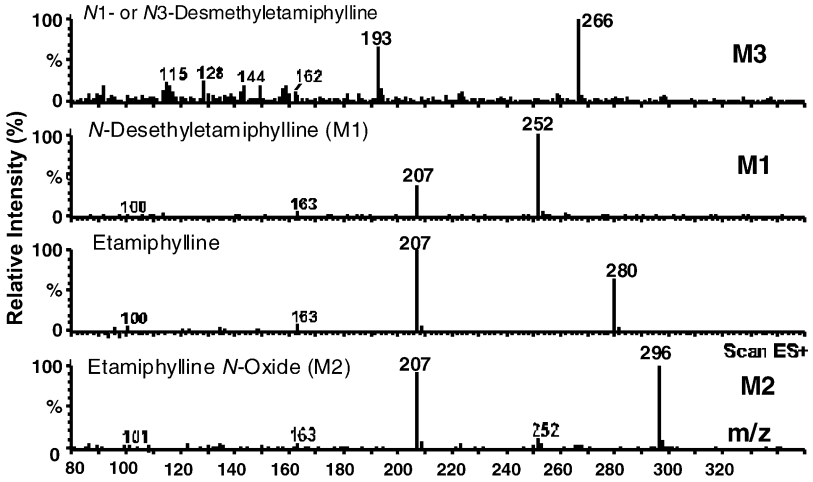

Fig. 5. Full-scan ESI+ mass spectra of etamiphylline, *N*-desethyletamiphylline, etamiphylline *N*-oxide and *N*-desmethyletamiphylline using TSQ 70 instrument.

The full-scan ESI+ mass spectra of etamiphylline and its metabolites obtained by LC/MS analysis of the same samples on the Finnigan TSQ 70 instrument are shown in Fig. 5. Etamiphylline, M1 and M2 show a diagnostic fragment ion at (*m*/*z*) 207 and losses of 73, 45 and 89 Da from the respective $[M+H]^+$ ions suggesting a site specific cleavage for all three compounds. In addition, a third metabolite (M3) having a $[M+H]^+$ ion at (*m*/*z*) 266 and showing the loss of 73 Da (*m*/*z* 193) was also observed. The data indicate that after initial protonation of etamiplylline, cleavage of the bond between the nitrogen and the α-carbon atoms of the 2-(diethylamino)ethyl substituent N_7 occurs with the concomitant loss of *N*,*N*-diethylamine. A similar fragmentation pathway for the *N*-desethyl metabolite (M1) is indicated by the loss of *N*-ethylamine. For M2, initial oxidation of the tertiary nitrogen to form etamiphylline-*N*-oxide occurs in vivo. The compound is then protonated and cleaved at the α-carbon atom to give the loss of *N*-hydroxy-*N*,*N*-diethylamine. The loss of 73 Da (*N*,*N*-diethylamine) for M3 indicates the presence of the intact side chain. Metabolite M3 was tentatively identified as *N*-desmethyletamiphylline; the site of *N*-demethylation at either N_1 or N_3 could not be determined by mass spectrometry.

4. Discussion

Previous studies on the in vivo biotransformation of etamiphylline in the greyhound, camel and horse have reported no phase II conjugation of the drug and its metabolites [4,5]. In the present study, only phase I metabolites were extracted from unhydrolysed urine. In common with most basic compounds containing the *tert*-*N*,*N*-dialkylaminoalkyl and *N*-alkylaminoalkyl secondary amine functionalities, the EI+ mass spectra of etamiphylline and its *N*-deethylated metabolite are dominated by the diagnostic charge-retained α-cleavage ions at (*m*/*z*) 86 and (*m*/*z*) 58. Both, etamiphylline and *N*-desethyletamiphylline (M1) were detected by full-scan GC/EI+/MS for up to 72 h in greyhound urine.

During semi-quantification of etamiphylline by SIM-LC/APCI+/MS, a major peak was observed in the isolates. Using the Micromass Platform instrument, full scan ESI+ mass spectrum of the unknown showed a protonated $(MH)^+$ ion at (*m*/*z*) 296 indicating the addition of an oxygen atom to etamiphylline, whereas in the APCI+ mode, deoxygenation was indicated by the prominent ion at (*m*/*z*) 280 $[MH^+ \rightarrow (MH^+ - O)]$ and other diagnostic fragment ions. From these data, it was concluded that this compound was probably a stable *N*-oxide of etamiphylline. Using the Finnigan TSQ 70 instrument, full scan ESI+ spectra of the metabolites showed a common site-specific fragmentation. Following protonation, neutral losses of 73, 45 and 89 Da from the respective $[MH]^+$ ions indicated that for all three compounds, cleavage occurs between the nitrogen and the α-carbon atoms of the 2-(diethylamino)ethyl substituent at N_7. For metabolite M2, loss of *N*-hydroxy-*N*,*N*-diethylamine (89 Da) can only occur if the tertiary *N* atom of the (diethylamino)ethyl moiety was the site of in vivo *N*-oxidation. A very minor metabolite, also detected by the Finnigan TSQ and showing a neutral loss of 73 Da, was identified a *N*-desmethyletamiphylline (M3).

Identification of *N*-oxides of drugs using solid inlet [10], thermospray MS [11], fast atom bombardment (FAB) and FAB-tandem MS [12] and LC/APCI+ and LC/ESI+/MS [9,13] has been reported. In all these studies, a prominent ion at $[(MH)^+ - 16]$ has been attributed to the loss of elemental oxygen from the protonated *N*-oxide. For FAB/MS and FAB-tandem MS this loss was reported to be matrix dependent [12]. In APCI+, the $[M+H]^+$ ions of *N*-oxides are reported to undergo thermal deoxygenation at elevated temperatures. Since this loss is unique to *N*-oxides, it can also be used to differentiate *N*-oxides from other hydroxylated metabolites [9,13]. Metabolite (M2) was not detected by GC/MS. The thermal instability of *N*-oxides and their deoxygenation back to the parent compound in both GC and LC analysis has been reported [14,15]. In light of this observation, the concentration and detection time of etamiphylline

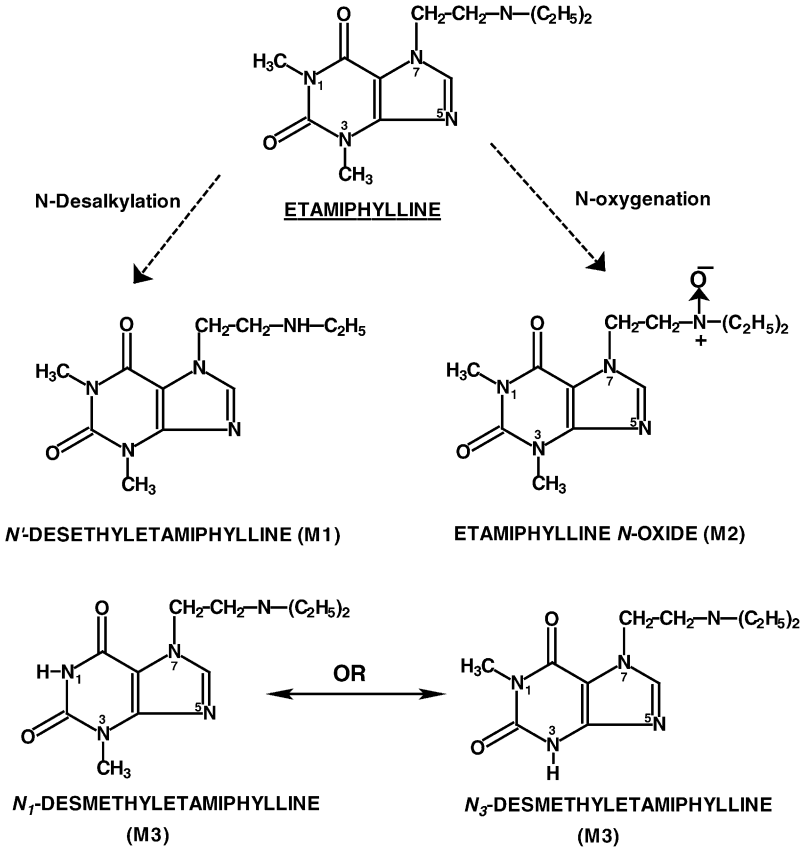

Fig. 6. Structures of etamiphylline and its in vivo metabolites detected in greyhound urine.

in post-dose greyhound urine could be falsely increased by GC/MS analysis.

Metabolism of xenobiotics containing a tertiary amine group may result in the formation of *N*-dealkylated and/or *N*-oxygenated products [16,17]. The formation of an *N*-oxide and *N*-dealkylation of a tertiary alkylamine does not necessarily imply that the two processes are linked. The relative occurrences of these two transformations have been analysed using structure-activity relationships [18]. The extent of *N*-oxide formation in vivo may be influenced by both the percentage of the overall metabolism and the presence of *N*-oxide reducing enzymes [19,20]. In this study, the metabolism of etamiphylline in the greyhound has been shown to proceed via *N*-deethylation and *N*-oxidation, both reactions occurring at the *N*-heteroatom of the 2-diethylaminoethyl substituent. The structures of etamiphylline and the three metabolites are shown in Fig. 6. Other minor metabolites of etamiphylline previously reported in greyhound urine by Wynne et al. [4] were not detected.

5. Conclusions

After intramuscular administration of Millophylline® to greyhounds, phase I metabolism of etamiphylline proceeds mainly by oxidative transformations of the 2-(diethylamino) ethyl substituent at N_7 leading to the formation of the two major metabolites excreted unconjugated in greyhound urine. Etamiphylline and desethyletamiphylline (M1) were detected in urine for up to 72 h by GC/EI+/MS and both compounds could be used as target analytes for routine GC/MS screening for etamiphylline administration to racing greyhounds. However, decomposition of the *N*-oxide metabolite (M2), detected only in the urine by LC/MS, could significantly increase the detection time of the parent drug during routine GC/MS screening of post-competition greyhound urine samples.

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