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In vitro metabolism of tiletamine, zolazepam and nonbenzodiazepine sedatives: Identification of target metabolites for equine doping control

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Within horseracing, the detection of prohibited substance doping often requires urine analysis; hence, it is necessary to understand the metabolism of the drugs in question. Here, the previously unknown equine metabolism of eight sedatives is reported in order to provide information on target metabolites for use in doping control. Phase I metabolite information was provided by incubation with equine liver S9 fraction. *In vitro* techniques were chosen in order to reduce the ethical and financial issues surrounding the study of so many compounds, none of which are licensed for use in horses in the UK.

Several metabolites of each drug were identified using liquid chromatography-high resolution mass spectrometric (LC-HRMS) analysis on an LTQ-Orbitrap. Further structural information was obtained by tandem mass spectrometry (MS/MS) analysis; allowing postulation of the structure of some of the most abundant *in vitro* metabolites. The most abundant metabolites of alpidem, etifoxine, indiplon, tiletamine, zaleplon, zolazepam, zolpidem, and zopiclone related to hydroxylation/*N*-oxidation, deethylation, demethylation, deethylation, hydroxylation/*N*-oxidation, demethylation, hydroxylation/*N*-oxidation and hydroxylation/*N*-oxidation, respectively. In many cases, further work would be required to fully elucidate the precise positioning of the functional groups involved.

The results of this study provide metabolite information that can be used to enhance equine anti-doping screening methods. However, the *in vitro* metabolites identified are at present only a prediction of those that may occur *in vivo*. In the future, any positive findings of these drugs and/or their metabolites in horse urine samples could help validate these findings and/or refine the choice of target metabolites.

Keywords: *in vitro*; metabolism; equine; sedative; LC-MS

Introduction

The use of drugs with sedative properties during competition is prohibited in British horseracing, according to Schedule 3 of the official Rules of Racing.[1] Such sedative drugs include opioid analgesics, tranquilizers, anxiolytics, and hypnotics and can be used to affect performance by 'calming' a horse prior to the race or by deliberately slowing it down. Although some jurisdictions target the abuse of such compounds by monitoring 'parent' drugs in blood, many laboratories base their post-race testing procedures on urine. Since drugs with basic moieties are typically extensively biotransformed by the equine, urinary testing for sedatives requires an understanding of the metabolism in order to target appropriate metabolites.[2]

A number of sedative drugs, relatively new to the market, have recently been identified for which no published information regarding their metabolism in the horse is available. These include the nonbenzodiazepine hypnotics indiplon, zaleplon, zopiclone and zolpidem; the nonbenzodiazepine anxiolytics etifoxine and alpidem; and the tranquilizers zolazepam and tiletamine (Figure 1). As well as being prohibited in competition samples under the British Rules of Racing, a number of these compounds are listed as 'banned' substances by the *Federation Equestre Internationale*[3] and as 'class two' agents by the Association of Racing Commissioners International.[4] The objective of this study was, therefore, to perform metabolism studies on these eight compounds in order to identify target metabolites for use in equine doping control procedures.

Ideally, *in vivo* drug metabolism studies offer the most complete and quantitatively accurate account of the metabolic processes.[2] However, the large number of compounds involved and the fact that none of these products are licensed for use in horses in the UK (and are therefore toxicologically untested) evoke a number of ethical issues, not to mention large investments in time and money, encumbering their administration to live animals. An alternative to the use of *in vivo* administration is the application of *in vitro* models based on equine liver preparations. Two recent equine publications from our laboratories, for example, have shown that for both steroidal[5] and non-steroidal drugs (including five sedatives),[6] all major Phase I *in vivo* metabolites were also detected following *in vitro* incubation. For these reasons, an *in vitro* approach, whereby prepared tissue fractions are incubated with the drug under study, was chosen here so as to permit the rapid (and ethical) acquisition of qualitative drug metabolite information. Liver S9 fraction was used since the liver is the primary

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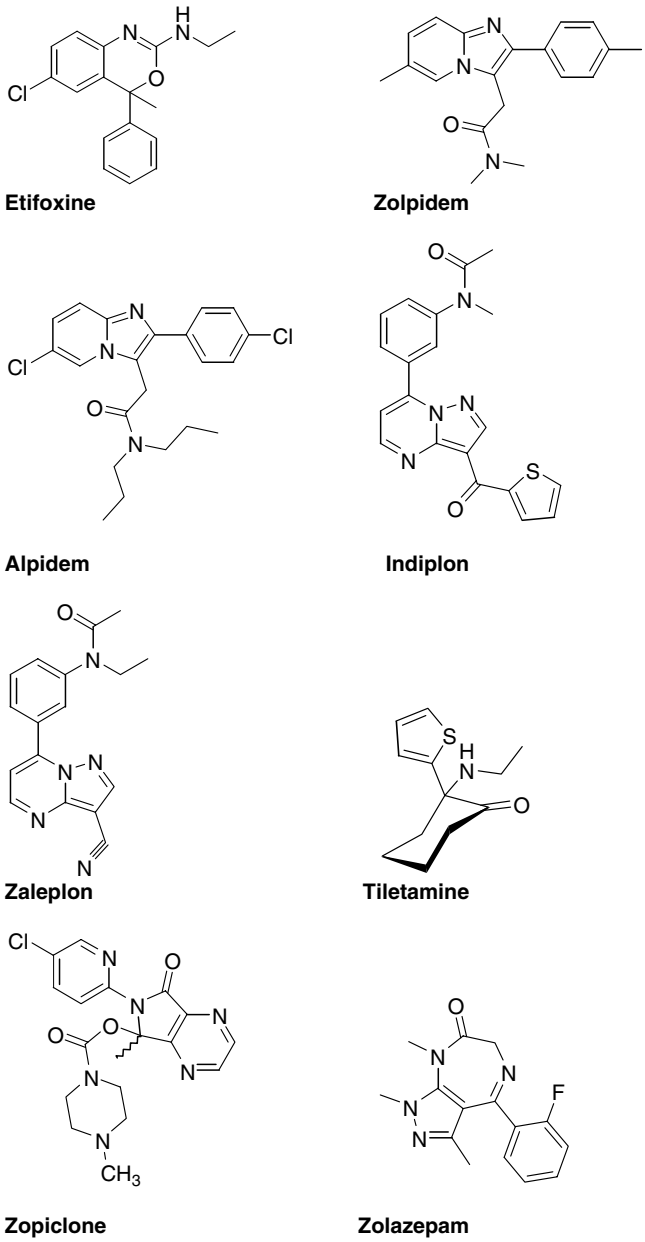


Figure 1. Molecular structures of the studied sedatives.

site of metabolism for many compounds. S9 itself contains a wider range of enzymes than the microsomes and, therefore, typically produces a larger array of metabolites.[6]

An LTQ-Orbitrap (Thermo Fisher Scientific Inc.) was employed for analysis of the pre- and post-incubation samples, primarily because it offers high resolution (30 000 full-width at half maximum, FWHM) accurate mass (<2 ppm) measurement in full-scan mode. The use of liquid chromatography-high resolution mass spectrometry (LC-HRMS) theoretically permits an unlimited number of *m/z* values to be monitored for each injection, which is important when it is not known which of a large number of potential analytes will be present. LC-HRMS is also increasingly used in equine urine screening procedures,[7–9] thus allowing retrospective sample analysis to be carried out once information regarding a new drug and/or its metabolites becomes available.

Experimental

Chemicals and reagents

UPLC-grade water was prepared using a Triple Red Ultra Pure Water System (Triple Red Ltd, Buckinghamshire, UK) while ice was produced by a Porkka KF75 ice flaker (Porkka (UK) Ltd, Watford, UK). Trizma HCl, trizma base, uracil, NAD, zaleplon and zolpidem were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). Acetonitrile, propan-2-ol, acetic acid and methanol were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). NADPH was from BDH (VWR International Ltd, Lutterworth, UK). D_3-stanozolol was sourced from QMX Labs Ltd. (Thaxted, UK), Tiletamine and Zolazepam from USP (Rockville, MD, USA), Alpidem and Etifoxine from Toronto Research Chemicals Inc. (Ontario, Canada), Indiplon from Tocris Bioscience (Ellisville, MO, USA), Zopiclone from Cerillient (Round Rock, TX, USA) and benzyldimethyl phenyl ammonium chloride maleate was from Acros Organics (Geel, Belgium). Equine liver fraction S9 (at 20 mg/ml) was produced from hepatic tissue collected from a Thoroughbred colt (euthanized for reasons not related to the current study) following the acquisition of appropriate ethical consent.

In vitro experiments

Each sedative drug was incubated in duplicate with equine liver S9 fraction. A negative control sample (no drug), to prove that the detected metabolites arose from the parent drug, and a positive control sample (stanozolol), to demonstrate that the system was performing as expected, were also analyzed. It was not necessary to include a 'no tissue' negative control to demonstrate that the metabolites were enzymatically rather than chemically produced, since the mechanism of formation was not considered relevant to the current study. Benzyldimethyl phenyl ammonium (BDPA) was added to each aliquot following incubation to account for any variability at injection.

In vitro experiments were carried out in a 334 µl volume of 48.5 mM pH 7.4 Tris buffer containing NAD and NADPH cofactors (0.62 mM), equine liver S9 (500 µg/ml protein) and 30 µM of drug (or substituted with acetonitrile for the negative control). In order to minimize possible effects of the added organic solvent on enzyme activity, its volume in relation to the overall reaction mixture was kept as low as possible (less than 0.6%). Samples were mixed via a bench top vortexer (Fisher Scientific UK Ltd, Loughborough, UK) and incubated at 37 °C in a shaking water bath (Jeio Tech Co. Ltd, Oxfordshire, UK) for 2 h. Aliquots (50 µl) were removed at 0 ('time-zero') and 120 ('post-incubation') min and the reaction quenched by addition of 75 µl ice-cold acetonitrile with vortexing.

Each quenched aliquot was then diluted with 1 ml of a solution of 1 µg/ml BDPA in 0.1% (*v/v*) acetic acid in water. Finally, these samples were centrifuged at 1500 g for 5 min at room temperature in a microcentrifuge (Eppendorf UK Ltd, Cambridge, UK) prior to transfer to glass vials for LC-MS analysis.

LTQ-Orbitrap LC-MS analyses

Samples were injected (20 µl) onto an LTQ-Orbitrap Discovery interfaced with an Accela autosampler and HPLC system (Thermo Fisher Scientific Inc, Waltham, MA, USA) operating at a flow rate of 400 µl/min. An Atlantis T3 2.1 × 100 mm, 3 µm column (Waters Ltd, Hertfordshire, UK) was installed with SecurityGuard C_{18} guard cartridge (Phenomenex Ltd, Macclesfield, Cheshire, UK) and held

at 35 °C. Mobile phases were 300 ng/ml uracil in 0.1% (*v/v*) acetic acid in water (A) and 300 ng/mL uracil in 0.1% (*v/v*) acetic acid in acetonitrile (B). A gradient was performed over the first 3 min so that the percentage of B increased from 0 to 65. The column was then washed at 98% B for 1 min before starting conditions were resumed for 1 min. Positive mode electrospray ionization was executed with a 4.5 kV voltage applied. The capillary temperature was set to 200 °C and the sheath and auxiliary gas flow rates were 30 and 10 units, respectively. Full scan data was collected over the range 90–650 *m/z* at a resolution of 30,000 FWHM. Data was acquired and analyzed using Xcalibur software (version 2.0.7; Thermo Fisher Scientific Inc, Waltham, MA, USA).

Likely metabolites of the eight sedative drugs were predicted and the calculated accurate masses associated with these were searched for, both in time-zero and post-incubation extracted ion chromatograms, with a 5 ppm mass tolerance. These predictions included structures resulting from single and multiple occurrences of oxygenation, dehydrogenation, reduction, dealkylation, carboxylation, and hydrolysis. Compounds detected at higher abundances post-incubation (and absent from the negative control incubate) were considered to be potential metabolites. In addition, data was analyzed pair wise by time point using Compair software (version 1.01; Thermo Fisher Scientific Inc., Waltham, MA, USA) in an attempt to identify peaks with differing abundances between 0 and 120 min.

LTQ-Orbitrap LC-MS/MS analyses

The accurate masses of those drug metabolites of sufficient abundance were subsequently included in targeted tandem mass spectrometry (MS/MS) product ion scans on the LTQ-Orbitrap in order to allow for a more detailed elucidation of the structure of some of the most abundant *in vitro* metabolites. Precursor ions were first isolated and fragmented with collision energy of 35 eV in the linear ion trap region, before being analyzed by the Orbitrap in order to gain full-scan fragmentation spectra. Where precursor ion abundance had been low by liquid chromatography-mass spectrometry (LC-MS), the linear iontrap was employed alone (without transfer to the Orbitrap) in order to maximize sensitivity.

Results and discussion

All of the studied sedatives were found to be metabolized *in vitro*. While the formation of sodiated adducts was noted in some cases, the protonated ions always offered greater sensitivity and as such, only these species are discussed herein.

The main focus of this work was to use established *in vitro* techniques [2,5,6,23] to predict the possible phase I metabolic pathways of the eight sedative drugs in the equine, with the aim of enhancing current anti-doping screens based on the LC-MS analysis of urine. Recently revised ILAC-G7 guidelines for horseracing laboratories[24] now state that an acceptable reference material for the identification of the presence of a prohibited substance may include an isolate from an *in vitro* incubation, providing the analytical data from it are sufficient to fully justify its identity as a metabolite of the substance incubated. In light of these regulatory guidelines, it was not considered essential to fully rationalize the structures of identified metabolites beyond specifying their empirical formulae and demonstrating that they were derived from the parent drug. However, in order to provide useful reference information for testing laboratories, details of the MS/MS product ion spectra for each compound are tabulated. Also, the pathways of fragmentation of each parent drug and its most abundant metabolite are scrutinized in more detail. In some cases it was not possible (at least within the limits of the current study) to ascertain whether an oxidative biotransformation related to hydroxylation or *N*/*S* oxidation. In these situations, the identified species are simply referred to as 'mono-oxygenated' metabolites and further work, perhaps involving LC-MS^3 analyses or experiments with deuterated solvents, would be necessary to better elucidate the positioning of certain functional groups. However, it is possible that even these experiments may not locate the position of every added functional group and bulk synthesis and purification of metabolites prior to NMR analyses may provide more definitive data.

In the following discussions, unless stated otherwise, the accurate masses of the MS/MS product ions (to 5 ppm) were consistent with the proposed structures of the observed fragments.

Etifoxine

A summary of the results for etifoxine and its identified metabolites is presented in Table 1.

The metabolite producing the largest response (M5) was proposed to be *N*-desethyletifoxine. Four separate mono-oxygenated isomers (M1 to M4) were also produced as well as a de-ethylated and mono-oxygenated metabolite (M6). However, due to the nature of the MS/MS fragments produced, it was not possible to rationalize the precise positions of these oxygenations.

The MS/MS fragmentation of etifoxine and *N*-desethyl-etifoxine is rationalized in Figure 2. Only one significant peak (above 10% of base peak abundance) at *m/z* 230.0731 was observed in the parent drug spectrum and is proposed to relate to the loss of the ethylamino side chain and the adjacent oxygen. A peak at this *m/z* (to 5 ppm) was also observed in the *N*-desethyl-etifoxine spectrum, in addition to a fragment at *m/z* 256.0526 that was proposed to derive from the loss of ammonia. This confirmed the detected metabolite to be identical to the parent sedative other than loss of the ethyl moiety.

No reports on the metabolism of etifoxine in other species could be located.

Zolpidem

A summary of the results for zolpidem and its identified metabolites is presented in Table 2.

The metabolite producing the largest response (M1) was proposed to be a mono-oxygenated-zolpidem isomer. Three further mono-oxygenated products (M2 to M4) and a carboxylic acid derivative (M5) were also identified. Product ion spectra were obtained for all metabolites, with the exception of M4, for which sufficient MS/MS sensitivity could not be obtained.

The MS/MS fragmentation of zolpidem and its most abundant *in vitro* mono-oxygenated metabolite (M1) are rationalized in Figure 3. Major peaks in both spectra related to neutral losses from the amide containing side-chain, which is consistent with the previously reported MS/MS spectra of zolpidem.[10] The same neutral MS/MS losses were also identified in the spectra of the carboxy (M5) and in two of the remaining mono-oxygenated metabolites (M2 and M3), suggesting that each of these oxidations occurred on the aromatic rings rather than the amide-containing side chain.

For mono-oxygenated-zolpidem isomers M1 and M2, the loss of CH_2O from the pseudo-molecular ions to produce a

Table 1. Summary of LC-MS metabolite detection and MS/MS fragmentation following incubation of etifoxine (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Etifoxine	0	3.71	301.1102	N/A	230.0731 (100)
M1	+16	3.46	317.1051	1.83	228.0577(100), 246.0683(21)
M2	+16	3.56	317.1051	5.53	230.0733(100), 299.0948(82), 228.0577(13)
M3	+16	3.80	317.1051	1.83	246.0683(100)
M4	+16	3.85	317.1051	0.30	Insufficient sensitivity
M5	−28	3.53	273.0789	100.00	230.0732(100), 256.0526(42)
M6	−12	3.47	289.0738	6.38	246.0682(100), 272.0475(25), 228.0577(25)

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.

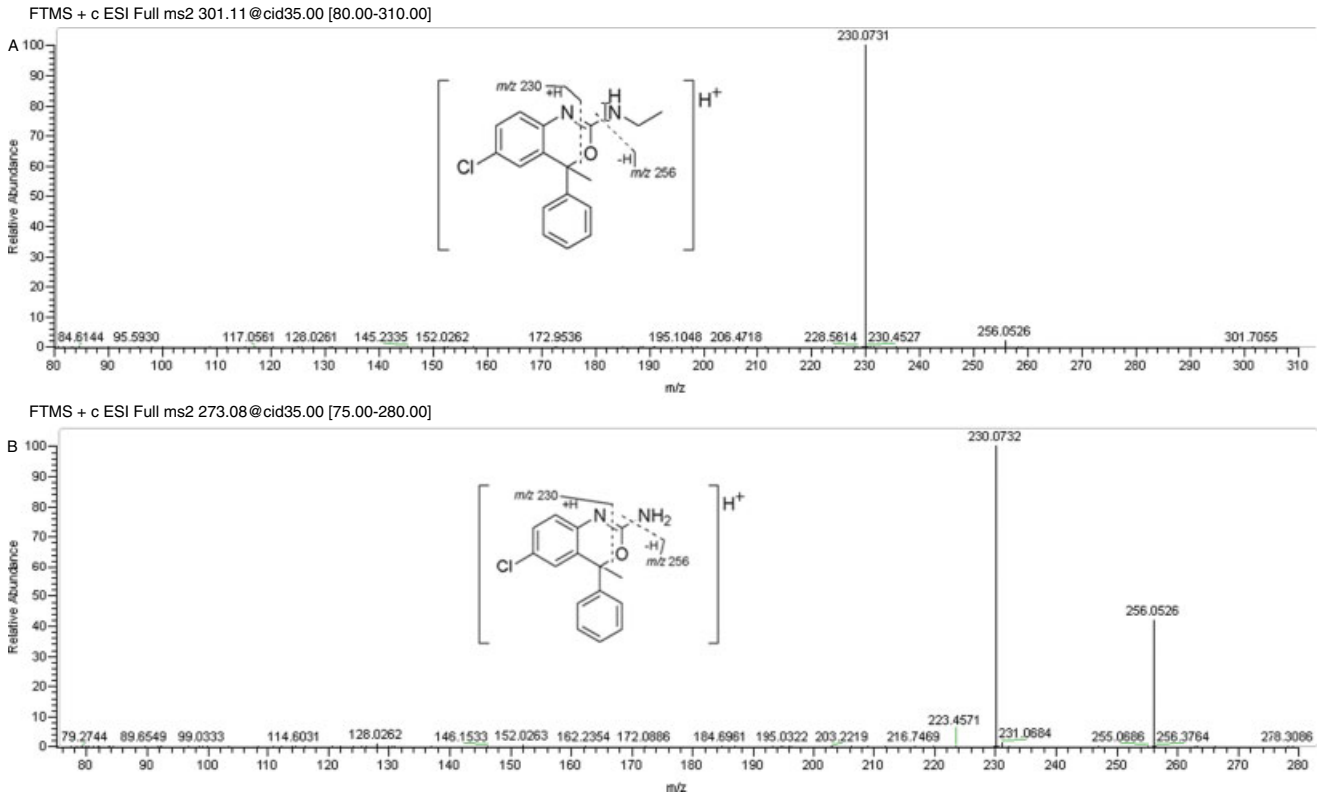


Figure 2. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) etifoxine (3.71 min) and (B) desethylated metabolite M5 (3.53 min).

Table 2. Summary of metabolite detection and fragmentation by LC-MS following incubation of zolpidem (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected m/z (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Zolpidem	0	3.31	308.1757	N/A	263.1178(100), 235.1231(72), 236.1308(22)
M1	+16	3.06	324.1707	100.00	279.1129(100), 251.1181(28), 234.1154(25), 252.1259(22), 306.1603(13), 221.1075(11), 294.1603(2)
M2	+16	3.22	324.1707	9.17	294.1603(100), 279.1131(67), 251.1181(29), 252.1260(14)
M3	+16	3.35	324.1707	20.83	279.1130(100), 251.1181(81), 252.1259(32)
M4	+16	3.41	324.1707	0.55	Insufficient sensitivity
M5	+30	3.15	338.1499	11.67	293.0923(100), 265.0974(47), 266.1052(17), 221.1074 (2)

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed unless of particularly useful diagnostic relevance.

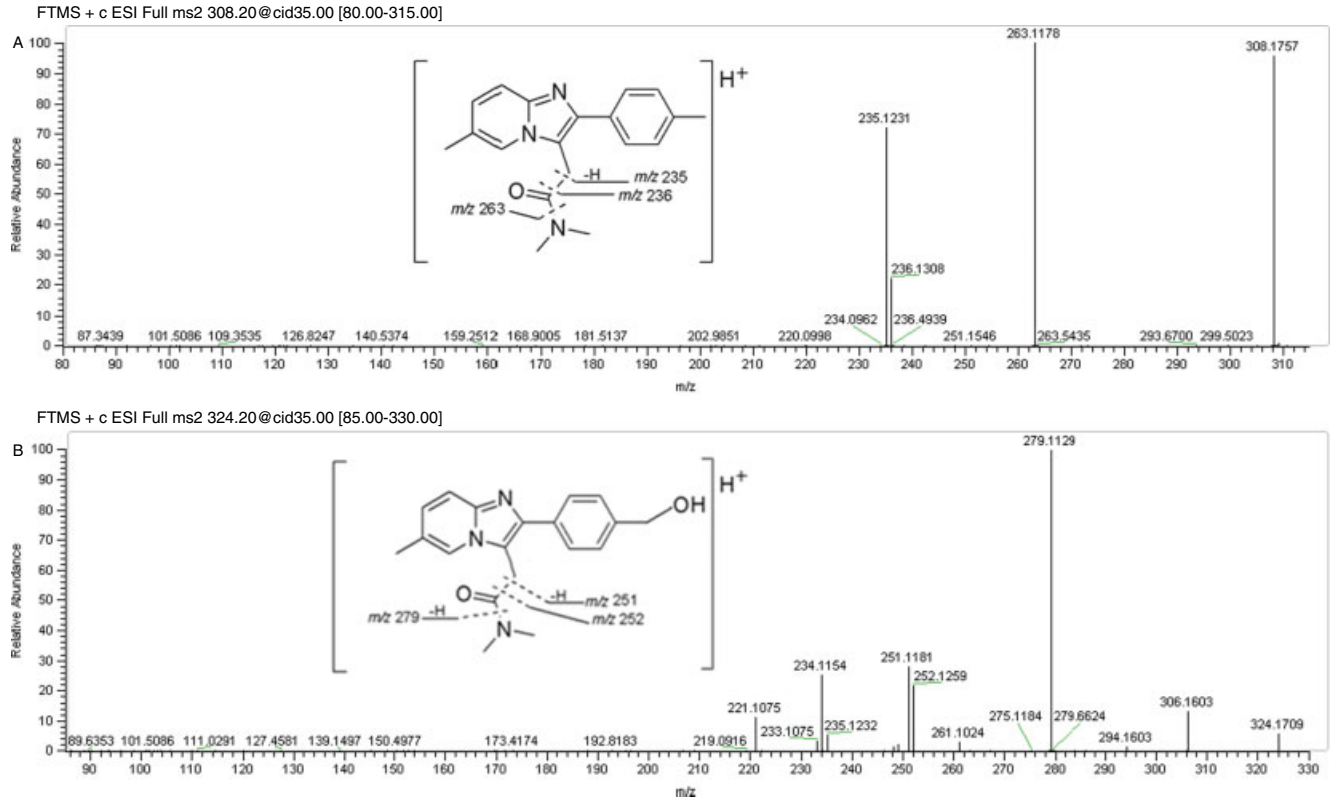


Figure 3. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) zolpidem (3.31 min) and (B) mono-oxygenated-zolpidem isomer M1 (3.06 min).

fragment at *m/z* 294.1603 indicated that each of these metabolites contained a hydroxyl group on one of the two primary carbon substituents of the aromatic rings. Furthermore, the MS/MS spectrum of carboxyzolpidem (M5) contained product ions at *m/z* 221.1074, 265.0974, and 293.0923, which is consistent with the previously reported[11] MS/MS spectrum of *N*,*N*,6-Trimethyl-2-(4-carboxyphenyl)-imidazo[1,2-*a*]pyridine-3-acetamide (zolpidem with a methyl group on the phenyl ring replaced by a carboxy function). These authors also previously reported the MS/MS spectrum of *N*,*N*,dimethyl-6-carboxy-2-(4-methylphenyl)-imidazo[1,2-*a*]pyridine-3-acetamide (zolpidem with a methyl group on the imidazo[1,2-*a*]pyridine ring replaced by a carboxy function). In contrast to the former carboxyzolpidem isomer, this second previously reported isomer lacked a product ion at *m/z* 221 and instead produced a fragment at *m/z* 219.[11] Taken together from the results of the current study, the presence of *m/z* 221.1075 in the spectrum of mono-oxygenated-zolpidem isomer M1 and its absence from that of M2, indicate that the positions of hydroxylation in these two isomers are on the primary methyl groups attached to the phenyl and imidazo[1,2-*a*]pyridine rings respectively. Furthermore, fragmentation of mono-oxygenated-zolpidem isomer M3 indicated that the oxygenation occurred at a site distinct from the amide-containing side chain.

Although the equine metabolism of zolpidem has not previously been described, the drug is reported to be rapidly and extensively metabolized in human and the rat.[12–14] In the former, bioconversion is predominantly into the *N*,*N*,6-Trimethyl-2-(4-carboxyphenyl)-imidazo[1,2-*a*]pyridine-3-acetamide derivative, via a hydroxyl intermediate. Alternatively, carboxylation at the imidazopyridine methyl moiety, again via a hydroxylation event, is seen with approximately 10% of the administered compound; minor metabolites are formed by hydroxylation of either the imidazopyridine structure or the amide-containing side chain.[12,14]

Work undertaken by Pichard *et al*.[15] demonstrated the *in vitro* production of the hydroxymethyl derivatives, intermediate in the aforementioned carboxylation pathways (and not seen in the urinary metabolome), by incubation of zolpidem with human liver microsomes. Conversely, generation of the major carboxyzolpidem metabolite was observed upon incubation of the sedative in hepatocyte cell culture.

Alpidem

A summary of the results for alpidem and its identified metabolites is presented in Table 3.

The metabolite producing the largest response (M3) was proposed to be a mono-oxygenated-alpidem isomer. Additionally, two further mono-oxygenated-alpidem isomers (M2 and M4), a despropylated metabolite (M5), a despropylated and mono-oxygenated-alpidem isomer (M7) and a mono-oxygenated and dehydrogenated product proposed to be keto-alpidem (M6), were also observed in the post-incubation sample.

It was possible to acquire good quality MS/MS data for despropylalpidem (M5) and the three most abundant mono-oxygenated-alpidem isomers (M2 to M4). However, low sensitivity precluded further analysis of the remaining metabolites. The MS/MS spectra of alpidem and the most abundant *in vitro* mono-oxygenated-alpidem isomer (M3) are rationalized in Figure 4. The most significant peaks in the parent drug spectrum corresponded to losses from the amide containing side-chain with cleavage on either side

Table 3. Summary of metabolite detection and fragmentation by LC-MS following incubation of alpidem (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Alpidem	0	4.97	404.1291	N/A	303.0088(100), 275.0139(59), 276.0217(10)
M1	+16	4.11	420.1240	48.48	319.0037(100), 291.0089(53), 292.0167(16)
M2	+16	4.28	420.1240	11.52	275.0140(100), 303.0089(32), 402.1139(15)
M3	+16	4.38	420.1240	100.00	275.0139(100), 303.0088(38), 402.1136(33), 321.0194(23)
M4	+16	4.58	420.1240	1.58	Insufficient sensitivity
M5	−42	4.40	362.0821	39.39	275.0140(100), 303.0089(93), 276.0217(15), 234.0682(10)
M6	+14	4.48	418.1084	1.52	Insufficient sensitivity
M7	−26	3.71	378.0771	4.55	Insufficient sensitivity

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.

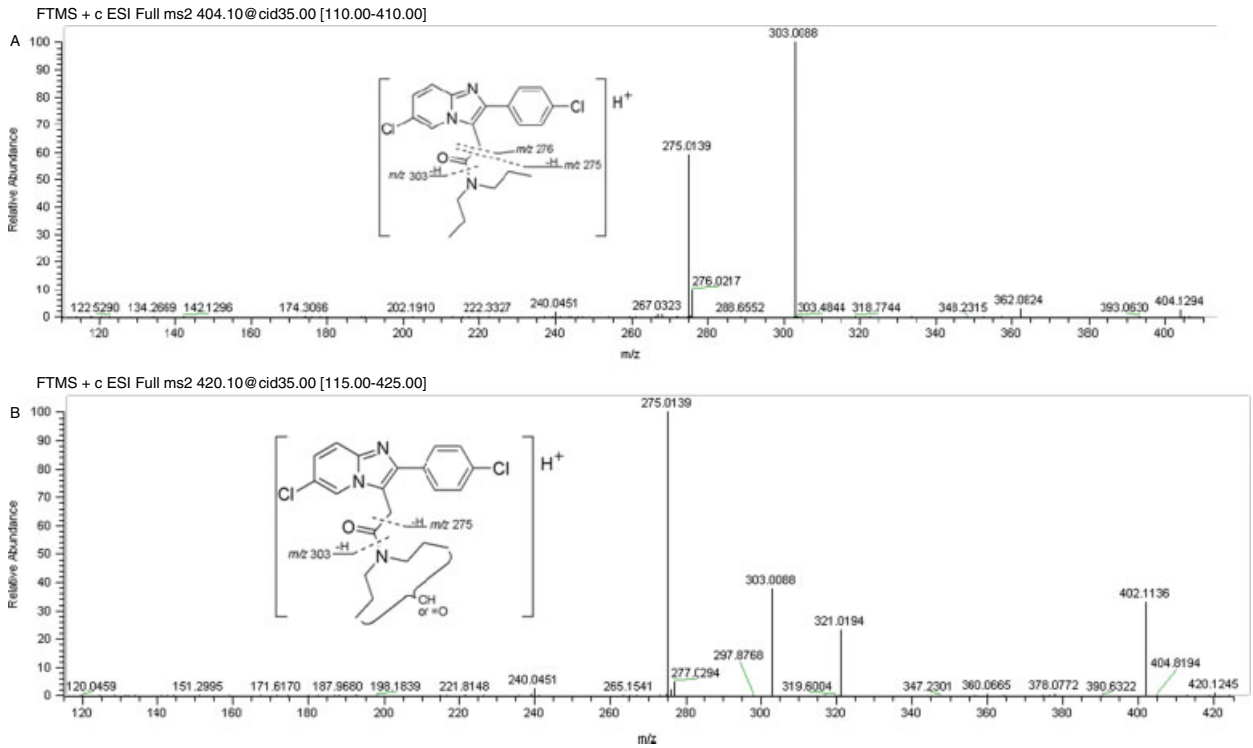


Figure 4. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) alpidem (4.97 min) and (B) mono-oxygenated-alpidem isomer M3 (4.38 min).

of the carbonyl group to form *m/z* 275.0139 and 303.0088. The same product ions were also detected in the spectrum of M3, suggesting that oxygenation occurred on the (di-propyl)amine moiety. Furthermore, these peaks were also observed (matching at 5 ppm) in the spectrum of mono-oxygenated-alpidem isomer M2; again suggesting that oxygenation occurred on the (dipropyl)amine moiety. Further observation of these same product ions for metabolite M5 suggested that this compound was despropylalpidem. On the other hand, the spectrum obtained for the mono-oxygenated-alpidem isomer M1 exhibited analogous fragment masses 16 amu higher than those seen for the parent drug; suggesting that oxidation occurred on one of the aromatic rings.

While the biotransformation of alpidem in horse has not previously been studied, in the rat and human it has been reported to be metabolized by *N*-dealkylation, by oxidation of either the imidazopyridine ring or amide side-chain, or by a combination of these.[16]

Indiplon

A summary of the results for indiplon and its identified metabolites is presented in Table 4.

The metabolite producing the largest response (M1) was proposed to be desmethylindiplon. A proposed desethanoyl metabolite (M2) was also indentified.

Good quality MS/MS spectra were obtained for both indiplon metabolites. The MS/MS spectrum of indiplon and its most abundant *in vitro* metabolite desmethylindiplon (M1) are rationalized in Figure 5. The product ion spectrum associated with the parent drug produce a major fragment at *m/z* 293.1032 relating to the neutral loss of the thiophenyl ring. The same neutral loss (84 amu) was observed in the proposed desmethylindiplon (M1) and desethanoylindiplon (M2) spectra; except that the peaks of interest were shifted by −14 and −52 amu due to the absence of the methyl and ethanoyl groups, respectively.

Table 4. Summary of metabolite detection and fragmentation by LC-MS following incubation of indiplon (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Indiplon	0	4.14	377.1067	N/A	293.1032(100), 311.1139(22)
M1	−14	4.11	363.0910	100.00	279.0879(100), 297.0985 (17)
M2	−42	4.46	335.0961	19.47	251.0931(100), 252.9732(75)

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.

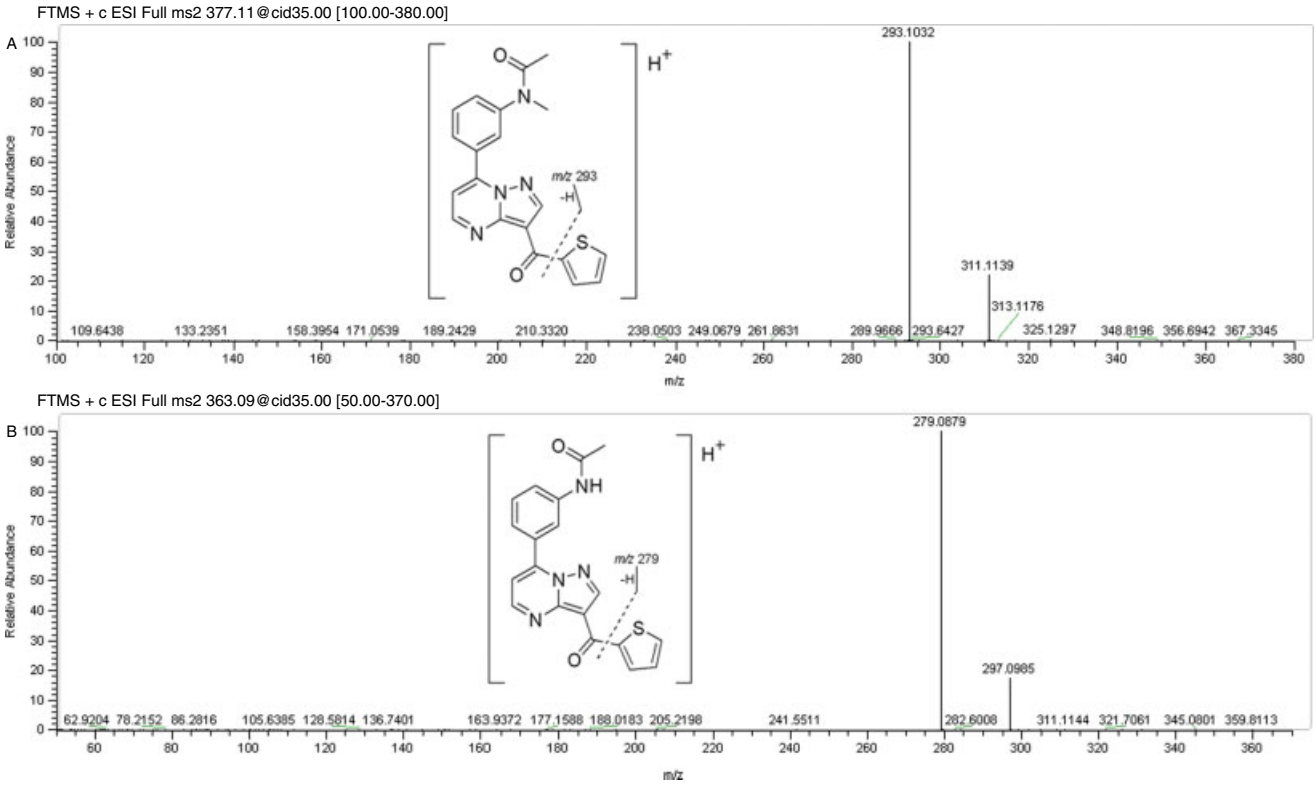


Figure 5. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) indiplon (4.14 min) and (B) desmethylindiplon (M1) (4.11 min).

Although the metabolism of indiplon in the equine has not previously been described, a human *in vitro* study by Madan *et al*. has been completed.[17] In this study, indiplon was modified to *N*-desmethyl, *N*-desethanoyl and *N*-desmethyl-*N*-desethanoyl derivatives following incubation with human liver microsomes.

Zaleplon

A summary of the results for zaleplon and its identified metabolites is presented in Table 5.

The metabolite producing the largest response was proposed to be a mono-oxygenated-zaleplon isomer (M1). Minor metabolites identified included two further mono-oxygenated-zaleplon isomers (M2 and M3) and desethylzaleplon (M4).

It was possible to acquire good quality MS/MS data for mono-oxygenated-zaleplon isomer M1 and desethylzaleplon M4. However, low sensitivity precluded further structural analysis of the remaining metabolites M2 and M3. The MS/MS spectra of zaleplon and the most abundant *in vitro* mono-oxygenated-zaleplon isomer M1 are rationalized in Figure 6. Zaleplon generated characteristic fragments relating to losses of the ethanoyl and ethyl side chains to form *m/z* 264.1243 and 260.0936, respectively. The loss of both of these groups was also observed and produced *m/z* 236.0933. The spectrum of mono-oxygenated-zaleplon isomer M1 exhibited the same aforementioned neutral losses as zaleplon, implying that the addition of oxygen occurred at a site distinct from the amide containing side-chain.

While the equine metabolism of zaleplon has not been reported previously, it has been studied in a number of other species. Following oral administration, the major metabolite detected was *N*-desethylzaleplon in dog, rat, and mouse, but 5-oxo-zaleplon in both monkey and human. The latter two species also converted the parent drug to the *N*-desethyl-5-oxo- derivative. With rat and monkey, these findings were also successfully reproduced *in vitro*.[18]

Tiletamine

A summary of the results for tiletamine and its identified metabolites is presented in Table 6.

The metabolite producing the largest response was proposed to relate to desethyltiletamine (M5). Four separate mono-

Table 5. Summary of metabolite detection and fragmentation by LC-MS following incubation of zaleplon (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Zaleplon	0	4.12	306.1349	N/A	264.1243(100), 288.1245(16), 260.0936(13), 236.0933(12)
M1	+16	3.78	322.1299	100.00	280.1195(100), 276.0884(13), 252.0882(11), 304.1196(11)
M2	+16	3.86	322.1299	3.20	Insufficient sensitivity
M3	+16	3.94	322.1299	1.56	Insufficient sensitivity
M4	−28	3.93	278.1036	5.60	260.0934(100)

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.

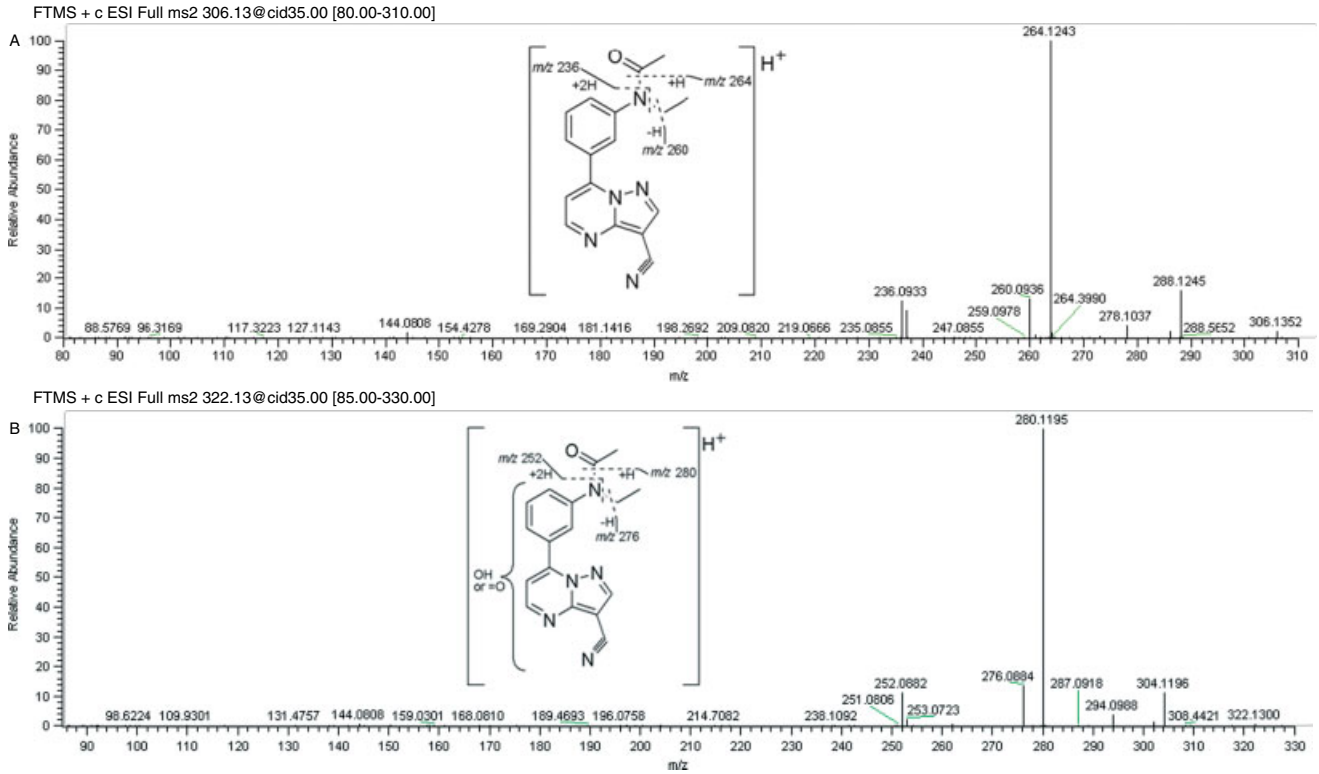


Figure 6. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) zaleplon (4.12 min) and (B) mono-oxygenated-zaleplon isomer M1 (3.78 min).

Table 6. Summary of metabolite detection and fragmentation by LC-MS following incubation of tiletamine (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Tiletamine	0	3.08	224.1104	N/A	179.0525(100), 151.0577(11)
M1	+16	2.61	240.1053	4.55	* 195(100), 222(46), 180(16), 223(13), 208(12), 198(11), 181(11)
M2	+16	2.70	240.1053	0.97	Insufficient sensitivity
M3	+16	2.81	240.1053	3.03	Insufficient sensitivity
M4	+16	2.85	240.1053	10.30	* 195(100), 222(14), 153(12)
M5	−28	2.98	196.0791	100.00	179.0526(100), 151.0578(14)
M6	−2	2.99	222.0947	1.95	Insufficient sensitivity

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed. * MS/MS experiment carried out using linear ion trap alone due to poor sensitivity achieved with Orbitrap.

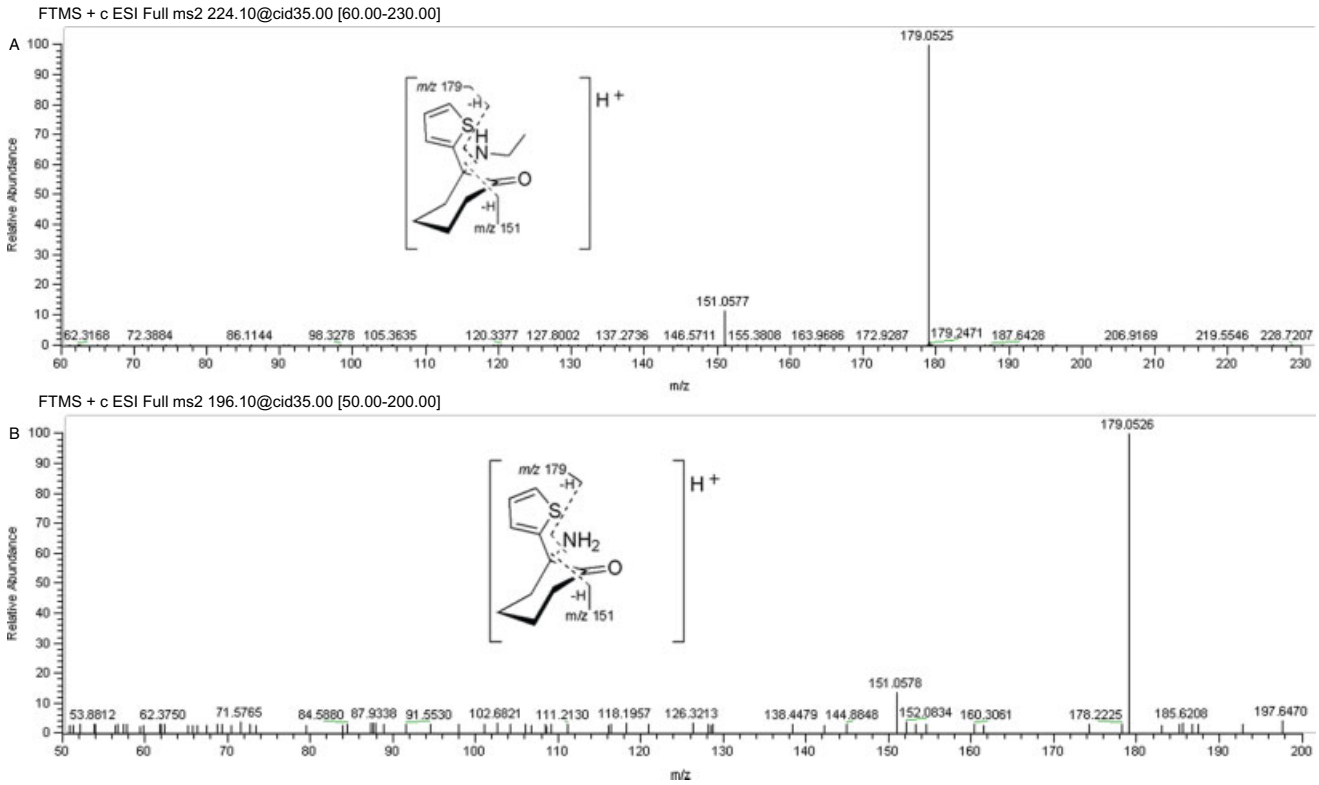


Figure 7. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) tiletamine (3.08 min) and (B) desethyltiletamine (M5) (2.98 min).

Table 7. Summary of metabolite detection and fragmentation by LC-MS following incubation of zopiclone (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Zopiclone	0	3.19	389.1123	N/A	345.1227(100), 245.0226(20)
M1	+16	3.27	405.1073	100.00	261.0178(100), 143.0816(29)
M2	−14	3.17	375.0967	80.00	331.1072(100)

All ions discussed were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.

oxygenated-tiletamine isomers were identified as minor metabolites (M1 to M4) in addition to a dehydrogenated product (M6).

It was possible to acquire good quality MS/MS data for desethyltiletamine (M5) and mono-oxygenated-tiletamine isomers M1 and M4. However, low sensitivity precluded further structural analysis of the remaining mono-oxygenated-tiletamine isomers M2 and M3 or of the dehydrotiletamine metabolite. Also, MS/MS data for M1 and M4 was acquired using the linear ion trap (without the benefit of accurate mass measurement) due to poor sensitivity. The MS/MS spectra of tiletamine and desethyltiletamine are rationalized in Figure 7. The predominant fragment of the parent drug was proposed to correspond to loss of the ethylamine side-chain; forming *m/z* 179.0525. The same fragment (to 5 ppm) was also observed in the desethyltiletamine spectrum; supporting the proposed structure as indicated in Figure 7. An analogous fragment incremented by 16 amu was observed in the ion trap generated spectra of mono-oxygenated-tiletamine isomers M1 and M4, suggesting that the oxidations were not located on the ethylamine side chain and must therefore take the form of hydroxylations or an *S*-oxide elsewhere in the molecule.

No reports on the metabolism of tiletamine in other species could be located.

Zopiclone

A summary of the results for zopiclone and its identified metabolites is presented in Table 7.

The metabolite producing the largest response was proposed to be a mono-oxygenated-zopiclone isomer (M1). A compound proposed to be *N*-desmethylzopiclone (M2) was also identified.

Both metabolites produced good quality MS/MS spectra and the results for zopiclone and the most abundant *in vitro* mono-oxygenated-zopiclone isomer are rationalized in Figure 8. The product ion spectrum associated with zopiclone showed the most predominant fragment to be formed through loss of CO_2 with a subsequent rearrangement (migration of the *N*-methylpiperazine moiety) to form a stable product ion at *m/z* 345.1227. A secondary fragment was proposed to relate to the neutral loss of the *N*-methylpiperazine carboxylate moiety to form *m/z* 245.0226. A peak at *m/z* 143.0816 (very low abundance) was proposed to relate to an equivalent cleavage, but with charge retention

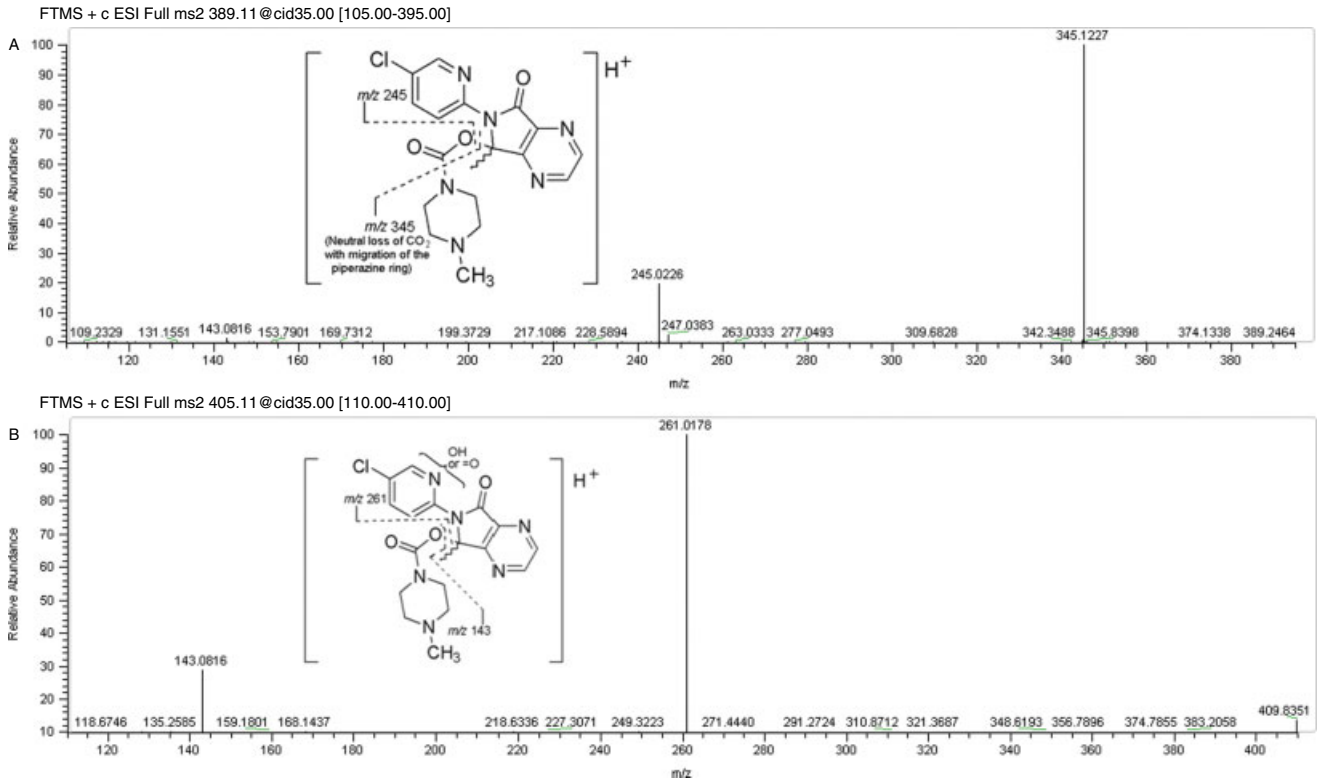


Figure 8. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) zopiclone (3.19 min) and (B) mono-oxygenated-zopiclone isomer M1 (3.27 min).

on the methyl-piperazine carboxylate fragment. The spectrum of mono-oxygenated-zopiclone isomer M1 contained a base peak analogous to the parent zopiclone 245.0226 peak, but at 16 amu higher to form *m/z* 261.0178. This metabolite also produced a similar fragment to parent zopiclone at *m/z* 143.0816, suggesting that the oxidation occurred at a site other than the *N*-methylpiperazine carboxylate moiety.

Although the equine metabolism of zopiclone has not been reported previously, studies in human have shown that as little as 5% of this hypnotic is cleared intact in the urine, with zopiclone-*N*-oxide and *N*-desmethyl-zopiclone the major metabolites observed both *in vivo* and *in vitro*;[19] minor decarboxylation metabolites are excreted, in part, via the lungs as carbon dioxide.[20] In rat and dog, conversely, over half of the administered dose of zopiclone was found to be modified by decarboxylation.[21]

Mistri *et al*. investigated the fragmentation of zopiclone, *N*-desmethylzopiclone and a zopiclone-*N*-oxide derivative by positive ion mode LC-MS/MS.[20] Peaks equivalent to those observed in the current study were present in the product ion spectra for the parent and *N*-demethylated compounds. Furthermore, zopiclone-*N*-oxide (oxygenated on one of the nitrogen atoms on the *N*-methylpiperazine ring) was observed, with a major product ion at *m/z* 245, which differentiates it from the unidentified oxygenated-zopiclone isomer (at *m/z* 261) detected in the current equine study.

Zolazepam

A summary of the results for zolazepam and its identified metabolites is presented in Table 8.

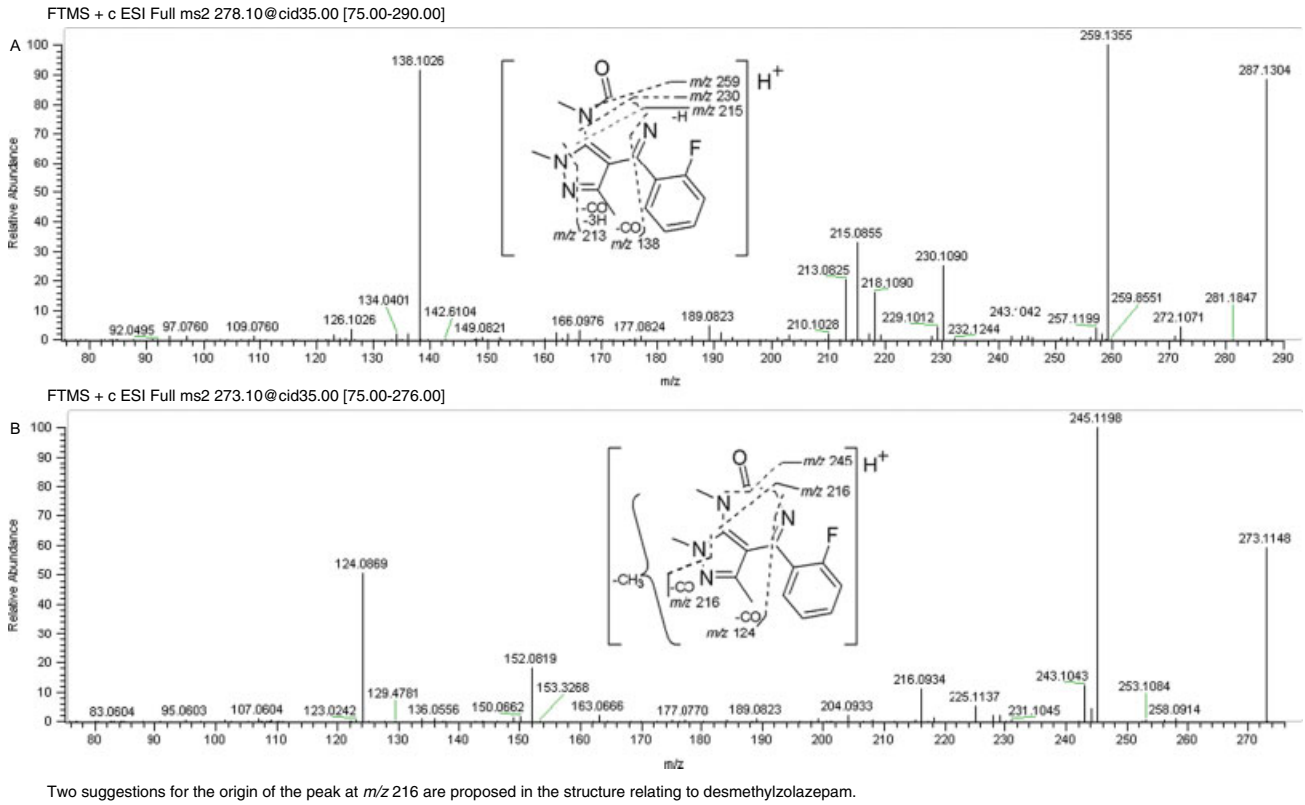
The metabolite producing the largest response was proposed to be desmethylzolazepam (M3). Two mono-oxygenated-zolazepam isomers were identified as minor metabolties (M1 and M2).

It was possible to acquire good quality MS/MS data for each metabolite, with the exception of mono-oxygenated-zolazepam isomer (M1), which was of insufficient abundance. The MS/MS spectra of zolazepam and desmethylzolazepam are rationalized in Figure 9. The major fragments in the parent drug spectrum are proposed to correspond to the neutral loss of CO alone and in combination with loss of the fluorobenzene moiety and part of the attached ring structure of the molecule to form *m/z* 259.1355 and 138.1026, respectively. The same two most abundant peaks were seen in the spectrum of desmethyl-zolazepam but at *m/z* values reduced by 14 amu due to absence of the third methyl group (precise position of metabolism unknown since there are two *N*-methyl groups available for demethylation). Many of the other spectral peaks also related to common losses between the parent and metabolite structures; for example, those seen at *m/z* 230.1090 and 216.0934 in the parent and metabolite spectra, respectively, both indicated a loss of C_2H_3ON. However, the abundant peak in the zolazepam spectrum (*m/z* 215.0855), proposed to have arisen from the loss of C_3H_6ON by cleavage of the diazepine ring, did not have an analogue within the metabolite spectrum (it is possible that *N*-demethylation on this ring led to the proposed cleavage being less favourable). On the other hand, a peak of low abundance (*m/z* 189.0823) present in both spectra and presumably formed through dual losses of CO and much of the pyrazole ring ($C_3H_6N_2$ from the parent and $C_2H_4N_2$ from the metabolite; not indicated in figure) suggested that the diazepine ring remained unchanged between the two compounds and that a methyl group had most likely been removed from the five-member ring.

Table 8. Summary of metabolite detection and fragmentation by LC-MS following incubation of zolazepam (30 μM) with equine liver fraction S9

Compound	ΔM	RT (min)	Expected *m/z* (peak detected at mass tolerance of < 5 ppm)	Relative abundance (%)	MS/MS product ions (% base peak)
Zolazepam	0	3.36	287.1303	N/A	259.1355(100), 138.1026(92), 287.1304(88), 215.0855(33), 230.1090(25), 213.0825(20), 218.1090(16)
M1	+16	3.11	303.1252	0.42	Insufficient sensitivity
M2	+16	3.67	303.1252	5.89	285.1148(100), 164.0819(36)
M3	−14	3.07	273.1146	100.00	245.1198(100), 124.0869(51), 152.0819(18), 243.1043(12), 216.0934(11)

All ions tabulated were singly-charged protonated species. Only MS/MS spectral peaks > 10% of base peak are listed.



Two suggestions for the origin of the peak at *m/z* 216 are proposed in the structure relating to desmethylzolazepam.

Figure 9. Product ion spectrum collected for a 120 min sample following incubation with equine liver fraction S9, corresponding to (A) zolazepam (3.36 min) and (B) desmethylzolazepam (M3) (3.07 min).

While not previously described in the horse, the *in vivo* metabolism of zolazepam has been reported in a number of mammalian species. In the rat, the major metabolite found is an *N*-desmethylhydroxyzolazepam isomer, in the dog, a 1-desmethyl-hydroxyl derivative is the most abundant and in the monkey, an *N*-desmethylzolazepam isomer.[22]

Conclusion

Following *in vitro* incubation with equine liver fraction S9, a number of metabolites were successfully detected for each of the eight sedative drugs by high resolution accurate mass LC-MS. The most abundant metabolites of alpidem, etifoxine, indiplon, tiletamine, zaleplon, zolazepam, zolpidem and zopiclone related to hydroxylation/*N*-oxidation, deethylation, demethylation, deethylation, hydroxylation/*N*-oxidation, demethylation, hydroxylation/*N*-oxidation, and hydroxylation/*N*-oxidation, respectively. In many cases, further work, perhaps involving LC-MS^3 analyses or experiments with deuterated solvents, would be necessary to better elucidate the positioning of certain functional groups. However, it is possible that even these experiments may not locate the position of every added functional group and bulk synthesis and purification of metabolites prior to NMR analyses may provide more definitive data.

As recently highlighted in a report by Taylor *et al.*,[23] metabolites formed *in vitro* can now be used as acceptable reference material, according to the new ILAC-G7 guidelines for horseracing laboratories.[24] This could prove to be extremely useful in future confirmatory analyses since commercial preparations of these metabolites are not as yet available.

The results of this study provide metabolite information that can be used to enhance equine anti-doping screening methods. However, the *in vitro* metabolites identified are at present only a prediction of those that may occur *in vivo*. In the future, any positive findings of these drugs and/or their metabolites in horse urine samples could help validate these findings and/or refine the choice of target metabolites.

Acknowledgements

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References

[1] British Horseracing Authority. *Rules of Racing: Schedule 3 – Prohibited Substances*, **2010**, Available at: http://rules.britishhorseracing.com [20 October 2010].
[2] J. P. Scarth, P. Teale, T. Kuuranne. Drug metabolism in the horse: A review. *Drug Test. Analysis* **2011**, *3*(1), 19.
[3] Federation Equestre Internationale (FEI). *Prohibited Substances List*. **2010**, Available at: www.feicleansport.org/ProhibitedSubstancesList_Jan2010.pdf [12 June 2010].
[4] Association of Racing Commissioners International, Inc. (ARCI). *Uniform Classification Guidelines for Foreign Substances and Recommended Penalties and Model Rule*. **2009**, Available at: www.arci.com/modelrules.html [1 August 2010].
[5] J. P. Scarth, H. A. Spencer, S. C. Hudson, P. Teale, B. P. Gray, L. L. Hillyer. The application of in *vitro* technologies to study the metabolism of the androgenic/anabolic steroid stanozolol in the equine. *Steroids* **2010**, *75*, 57.
[6] J. P. Scarth, H. A. Spencer, S. E. Timbers, S. C. Hudson, L. L. Hillyer. The use of *in vitro* technologies coupled with high resolution accurate mass LC-MS for studying drug metabolism in equine drug surveillance. *Drug Test. Analysis* **2010**, *2*, 1.
[7] S. Hudson, S. Maynard, M. Harrison. Can multiple instrumental analyses be replaced by a single instrument? *Proceedings of the 16th International Conference of Racing Analysts and Veterinarians: Tokyo, Japan*. **2006**, 534.
[8] R. G. Howitt, G. D. Beresford, M. Pelzing, I. Krebs. Routine drug screening by accurate mass using liquid chromatography/time of flight mass spectrometry – part 2. *Proceedings of the 16th International Conference of Racing Analysts and Veterinarians:* Antalya, Turkey. **2008**, 191.
[9] M.-A. Popot, F. Kieken, G. Pinel, J.-P. Antignac, Y. Bonnaire, B. Le Bizec. Urine and plasma metabolomic fingerprints, by LC/HRMS measurements, in order to detect illegal administration of equine growth hormone in horseracing. *Proceedings of the 17th International Conference of Racing Analysts and Veterinarians:* Antalya, Turkey. **2008**, 378.
[10] J. Bhatt, A. Jangid, R. Shetty, B. Shah, S. Kambli, G. Subbaiah, S. Singh. Quantification of zolpidem in human plasma by liquid chromatography-electrospray ionisation tandem mass spectrometry. *Biomed. Chromatog.* **2006**, *20*(8), 736.
[11] F. Klupsch, R. Houssin, L. Humbert, M. Imbenotte, J. Henichart, M. Lhermitte. Major metabolites of zolpidem: Expeditious synthesis and mass spectra. *Chem. Pharmaceut. Bull.* **2006**, *54*(9), 1318.
[12] J. P. Thénot, P. Hermann, A. Durand, J. T. Burke, J. Allen, D. Garrigou, S. Vajta, H. Albin, J. J. Thébault, G. Olive, S. J. Warrington, in *Imidazopyridines in Sleep Disorders*, (Eds: J. P. Sauvanet, S. Z. Langer, P. L. Morselli), Raven Press: New York, **1988**, pp. 139–153.
[13] D. Garrigou-Gadenne, J. T. Burke, A. Durand, H. Depoortere, J. P. Thénot, P. Morselli. Pharmacokinetics, brain distribution and pharmacoelectro-corticographic profile of zolpidem, a new hypnotic, in the rat. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 1283.
[14] G. Gillet, J. P. Thénot, P. L. Morselli. *In vitro* and *in vivo* metabolism of zolpidem in three animal species and in man. *Proceedings of the Third International ISSX Meeting*: Amsterdam. **1991**, 153.
[15] L. Pichard, G. Gillet, C. Bonfils, J. Domergue, J. P. Thénot, P. Maurel. Oxidative metabolism of zolpidem by human liver cytochrome P450S. *Drug Metab. Dispos.* **1995**, *23*(11), 1253.
[16] P. Padovani, P. Guinebault, S. Vajta, A. Durand, J. Allen, J. P. Thenot, P. L. Morselli. A preliminary metabolic study of alpidem in rat and man. *Eur. J. Drug Metab. Ph.* **1987**, *12*(4), 295.
[17] A. Madan, A. Fisher, L. Jin, D. Chapman, H. P. Bozigian. *In vitro* metabolism of indiplon and an assessment of its drug interaction potential. *Xenobiotica* **2007**, *37*(7), 736.
[18] K. Kawashima, K. Hosoi, T. Naruke, T. Shiba, M. Kitamura, T. Watabe. Aldehyde oxidase-dependent marked species difference in hepatic metabolism of the sedative-hypnotic, zaleplon, between monkeys and rats. *Drug Metab. Dispos.* **1999**, *27*(3), 422.
[19] L. Becquemont, S. Mouajjah, O. Escaffre, P. Beaune, C. Funck-Brentano, P. Jaillon. Cytochrome P-450 3A4 and 2C8 are involved in zopiclone metabolism. *Drug Metab. Dispos.* **1999**, *27*(9), 1068.
[20] H. N. Mistri, A. G. Jangid, A. Pudage, P. Shrivastav. HPLC-ESI-MS/MS validated method for simultaneous quantification of zopiclone and its metabolites, N-desmethyl zopiclone and zopiclone-N-oxide in human plasma. *J. Chromatogr. B* **2008**, *864*, 137.
[21] J. Gaillot, D. Heusse, G. W. Houghton, J. Marc Aurele, J. F. Dreyfus. Pharmacokinetics and metabolism of zopiclone. *Int. Pharmacopsychiat.* **1982**, *17*(S2), 76.
[22] J. Baukema, R. A. Okerholm, A. J. Glazko. The comparative metabolism of zolazepam in rat, dog and monkey. *Res. Commun. Chem. Path. Pharm.* **1975**, *10*(2), 227.
[23] P. Taylor, J. P. Scarth, L. L. Hillyer. The use of *in vitro* technologies to study phase II conjugation in equine sports drug surveillance. *Bioanalysis* **2010**, *2*(12), 1971.
[24] International Laboratory Accreditation Cooperation. *ILAC-G7 : 06/2009: Accreditation Requirements and Operating Criteria for Horseracing Laboratories*. **2009**, Available at www.ilac.org/documents [20 October 2010].