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The use of *in vitro* technologies coupled with high resolution accurate mass LC-MS for studying drug metabolism in equine drug surveillance

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The detection of drug abuse in horseracing often requires knowledge of drug metabolism, especially if urine is the matrix of choice. In this study, equine liver/lung microsomes/S9 tissue fractions were used to study the phase I metabolism of eight drugs of relevance to equine drug surveillance (acepromazine, azaperone, celecoxib, fentanyl, fluphenazine, mepivacaine, methylphenidate and tripelennamine). *In vitro* samples were analyzed qualitatively alongside samples originating from *in vivo* administrations using LC-MS on a high resolution accurate mass Thermo Orbitrap Discovery instrument and by LC-MS/MS on an Applied Biosystems Sciex 5500 Q Trap.

Using high resolution accurate mass full-scan analysis on the Orbitrap, the *in vitro* systems were found to generate at least the two most abundant phase I metabolites observed *in vitro* for all eight drugs studied. In the majority of cases, *in vitro* experiments were also able to generate the minor *in vivo* metabolites and sometimes metabolites that were only observed *in vitro*. More detailed analyses of fentanyl incubates using LC-MS/MS showed that it was possible to generate good quality spectra from the metabolites generated *in vitro*. These data support the suggestion of using *in vitro* incubates as metabolite reference material in place of *in vivo* post-administration samples in accordance with new qualitative identification guidelines in the 2009 International Laboratory Accreditation Cooperation-G7 (ILAC-G7) document.

In summary, the *in vitro* and *in vivo* phase I metabolism results reported herein compare well and demonstrate the potential of *in vitro* studies to compliment, refine and reduce the existing equine *in vivo* paradigm.

Keywords: *in vitro*; metabolism; equine; Orbitrap; Q trap; LC-MS

Introduction

The detection of drug abuse in horseracing often requires knowledge of drug metabolism, especially if urine is the matrix of choice. Currently, the majority of drug metabolism experiments are carried out on live animals *in vivo*. This has the advantage that the whole plethora of possible transformations can be considered and allows the most representative picture of the situation for real-life samples. *In vivo* experiments require animal experimentation, however, and hence there are additional ethical considerations and significant resource requirements. Also, the identification of drug metabolites in urine is often complicated by the presence of interferences; the timescales of the experiments are relatively long; drugs without previously defined toxicological profiles such as 'designer' drugs cannot be easily studied for ethical reasons; and it is difficult to carry out mechanistic studies such as the identification of the enzymes responsible for metabolism. On the other hand, *in vitro* methods do not require animal experimentation; can be carried out quickly; produce a 'cleaner' extract for analysis; can be used to study 'designer' drugs; and can be more easily tailored to study mechanistic aspects. Some of the disadvantages of using *in vitro* methods include ethical issues surrounding the supply of tissue; the lack of an intact biological system; and the lack of a guaranteed quantitative *in vivo-in vitro* correlation. It is therefore important to recognize these limitations as well as the advantages.

In vitro methods for studying a number of factors ranging from qualitative metabolite identification to the quantitative assessment of the potential of the drug to interact with other medications are now commonplace in the drug development industry.[1] Despite the many advantages of *in vitro* technologies, we are aware of only one other equine drug surveillance laboratory that routinely use *in vitro* experiments to compliment its *in vivo* studies.[2–5] A large number of laboratories that are engaged in equine drug surveillance around the world use targeted GC- and LC-MS/MS multiple reaction monitoring (MRM) (J. Scarth, personal observation). One consequence is that the number of target analytes needs to be limited to only the most abundant metabolite transitions in order to maximize sensitivity. In the past, a lack of quantitative similarity between the *in vivo* and *in vitro* situations

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has therefore been one of the factors that have limited the use of *in vitro* methods in equine drug surveillance. Alternative mass spectrometric detectors that are based on the acquisition of full-scan data, however, provide an attractive alternative since these do not need to be limited to the transitions of major metabolites only. Recently, robust high-resolution accurate-mass LC-MS (HR-LC-MS) instrumentation has become commercially available. HR-LC-MS therefore provides an opportunity for using *in vitro* methods for studying drug metabolism, where only knowledge of the qualitative profile of metabolites is required in order to insert the relevant masses and retention times into a detection database. Other factors that have in the past limited to use of *in vitro* technologies in equine drug surveillance include the availability of equine tissues and a limited knowledge of the *in vitro* techniques within the individual equine testing laboratories. Equine tissues are now becoming more readily available from specialist suppliers, however, and there is increasing pressure from many angles to reduce the use of animals in experimentation. A major aim of this study was therefore to raise the profile of *in vitro* studies within equine drugs surveillance and to give laboratories the confidence that, given the appropriate technology and expertise, *in vitro* techniques provide a useful adjunct to *in vivo* studies.

Of the small number of previously reported equine *in vitro* studies, liver microsomes have been used to elucidate the phase I metabolism of the androgenic-anabolic steroids clostebol acetate and mesterolone,[2] methenolone acetate,[3] turinabol,[4] a range of designer steroids[5] and the non-steroidal anti-inflammatory drug phenylbutazone.[6] Most recently, two equine cytochrome P450 (CYP) enzyme isoforms, named CYP2D50 and CYP2C92, have for the first time been sequenced and their activity compared to human CYP2D and CYP2C isoforms.[7,8] While the equine and human isoforms shared some substrate specificity, there were significant differences in the enzyme kinetics and the range of metabolites produced in the different species.

In vitro technologies have to date been somewhat more widely applied in the human sports field. For example, hepatocyte preparations have been used to study the phase I and II metabolism of the designer steroid tetrahydrogestrinone (THG).[9] Several authors have also reported the scaling up of *in vitro* conditions to allow the production and purification of mg quantities of human phase I[10,11] and phase II[12–15] drug metabolites, thus enabling characterization using techniques such as Nuclear Magnetic Resonance (NMR). These approaches often require the use of recombinantly expressed enzymes or tissue from animals that have been administered enzyme-inducing chemicals. Since there are currently no recombinantly expressed equine drug metabolizing enzymes available and because there are ethical issues surrounding the administration of enzyme-inducing chemicals to horses, these approaches are less viable in the horseracing industry. It would, however, be extremely desirable to use metabolite reference material generated *in vitro* to qualitatively confirm the presence of drugs in equine samples where no 'parent' drug can be detected and for which no chemically synthesized metabolite reference standard is available. Currently in this situation, International Laboratory Accreditation Cooperation-G7 guidelines on the *Accreditation Requirements and Operating Criteria for Horseracing Laboratories*[16] allow the use of *in vivo* urine/plasma samples resulting from a drug administration as qualitative reference material. This is not ideal, however, as it requires animal experimentation. In 2009, a new revision of the ILAC-G7 guidelines[16] stated for the first time (article 16.4) that *in vitro* incubations can now be used in place of *in vivo* post-administration samples. It is important to stress that under the ILAC-G7 guidelines, it is not necessary to produce large quantities of *in vitro* metabolite that can be isolated and subject to NMR. As with the existing *in vivo* post-administration paradigm, it is simply necessary to demonstrate that the analytical data are sufficient to fully justify the compound's identity as a metabolite by demonstrating the presence of a diagnostic analyte spectrum in post-administration samples but not in pre-dose samples or blanks.

Therefore, the aim of the current study was to perform qualitative *in vivo* and *in vitro* metabolism studies on a range of drugs of relevance to equine sports drug surveillance in order to ascertain the feasibility of using *in vitro* methods to refine and reduce the current *in vivo* paradigm. The primary objective was to identify *in vitro* at least the two major *in vivo* drug metabolites in order to demonstrate a good *in vivo-in vitro* qualitative correlation. A secondary objective was to perform structural characterization of the metabolites resulting from one of the drug incubations as proof of the concept of using the *in vitro* incubates as qualitative reference material in accordance with the new ILAC-G7 guidelines.[16] Eight drugs (Figure 1) representing a wide range of different structures and metabolic transformations commonly encountered in horseracing were chosen for study: acepromazine, azaperone, celecoxib, fentanyl, fluphenazine, mepivacaine, methylphenidate and tripelennamine. The metabolism of the androgenic-anabolic steroid stanozolol was also studied, but because the amount of data generated was substantial, the results of these analyses are reported elsewhere.[17]

Experimental

Chemicals and reagents

Deionized water was prepared using an SG Ultrachem TWF UV system (Barsbuttel, Germany). All organic solvents, acids and bases were analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Trizma base and HCl, NADPH, NAD, β-glucuronidase from *Helix pomatia* (type HP-2), pancreatin (8xUSP – product P7545), mepivacaine, fentanyl, fluphenazine, tripelennamine and methylphenidate were obtained from Sigma-Aldrich (Dorset, UK). Acepromazine was supplied by the US Pharmacopeia, (Rockville, MD, USA). Celecoxib was obtained from Searle (Chicago, IL, USA). Azaperone was provided by LGC Promochem (Teddington, UK). C18, 6 cc, 500 mg Sep-pak Vac solid phase extraction cartridges and a 100 mm $\times$ 2.1ID 3 µm Atlantis T3 column were supplied by Waters (Wexford, Ireland). Strata XC 3 ml 60mg solid phase extraction cartridges and 100 mm $\times$ 2.0 ID 2.5 µm HST Luna C18(2) columns were obtained from Phenomenex (Macclesfield, UK). Varian Nexus 3 mL 60 mg cartridges were obtained from Varian (Oxford, UK). Equine liver microsomes and S9 (both at 20 mg/mL protein and from a Quarter horse) were purchased from Xenotech Llc. (Lenexa, KS, USA), equine lung S9 (at 20 mg/mL and from a thoroughbred horse) was prepared by Asterand (Royston, UK) and equine plasma was obtained from a thoroughbred horse by a British Horseracing Authority Veterinarian (Newmarket, UK). The horses used were euthanized by ethically approved means for reasons not related to the current study.

In vitro experiments

Drug incubations were performed with equine liver microsomes, liver S9, lung S9 or plasma.

Plasma experimental volumes were 0.66 mL, contained drug only (25 μM) and were incubated in a water bath at 37 °C for 2 h. 330 μL aliquots were removed at 0 and 120 min and extracted immediately as follows: 2 mL of 2M pH 4.0 ammonium acetate and 5 mL tertiary-butyl-methyl-ether (TBME) were added and the samples rotated for 15 min. The TBME was then removed, blown down to dryness at 70 °C and the sample reconstituted in 10 μl propan-2-ol followed by 190 μl of water before being submitted to LC-MS analysis on the Thermo LTQ-Orbitrap.

Microsome/S9 experimental volumes were 0.33 mL and contained drug (25 μM), microsome or S9 (at 0.5 mg/mL protein), NADPH (0.63 mM for all drugs) and NAD (0.63 mM for fentanyl only, following the observation that this additional cofactor was required in order to produce a carboxy-metabolite of this drug) and pH 7.4 TRIS buffer (50 mM). Microsome/S9 samples were incubated in a water bath at 37 °C for 2 h and 50 μL aliquots were quenched at 0 and 120 min by the addition of 75 μL of ice-cold acetonitrile. Control experiments with no added cofactor were also performed. In the future, if performing experiments with compounds with previously undefined metabolic pathways, it would also be advisable to carry out control experiments in the absence of microsomes/S9 in order to obtain mechanistic information on the assay component/s that are responsible for producing any observed metabolites. For samples that were analyzed by LC/MS/MS, the quenched aliquots were then prepared in one of three ways (**1–3**):

1. Samples were diluted to 1125 μl by the addition of 1000 μl water (acepromazine, fentanyl, methylphenidate and tripelennamine).
2. Samples were centrifuged for 5 min at 11′000 rpm, all the supernatant transferred to a separate vial and blown down to dryness and reconstituted in 10 μl propan-2-ol followed by 190 μl of water (azaperone, fluphenazine and mepivacaine).
3. Samples were diluted to 2 mL with water and then added to 6 mL, 500 mg C18 solid phase extraction cartridges that had previously been primed with 5 mL methanol and 5 mL water. The cartridges were then washed with 5 mL water and 5 mL hexane before being dried under vacuum for 20 min and then eluted with 5 mL methanol. Extracts were then blown down to dryness, and reconstituted in 10 μl propan-2-ol followed by 190 μl of water (celecoxib).

Samples were then transferred to the appropriate vials and submitted for LC-MS analysis on either the Thermo LTQ-Orbitrap or the Sciex 5500 Q Trap.

In vivo experiments

Urine samples analyzed during the current study originated from *in vivo* administrations commissioned for previous studies unrelated to the current work. All *in vivo* administrations were performed using ethically approved protocols.

For acepromazine, an oral dose of 25 mg was administered to a thoroughbred male. A urine sample was taken at 21.5 h post-administration.

For azaperone, an oral dose of 1.2 g of azaperone was administered to a thoroughbred male. A urine sample was taken at 17 h post-administration.

For celecoxib, an oral dose of 1.7 g was administered to a thoroughbred female. A urine samples was taken at 3 h post-administration.

For fentanyl, an oral dose of 0.4 mg was administered to a thoroughbred male. Urine samples were taken at 20 and 24 h post-administration and were pooled prior to analysis.

For fluphenazine, an oral dose of 7 mg was administered to a thoroughbred male. A urine sample was taken at 4 h post-administration.

For tripelennamine and mepivacaine, the urine samples originated from 'past-positive' cases that were encountered during routine surveillance in our laboratories. Therefore, no further information regarding these animals is available.

No post-administration samples were available for methylphenidate, but it is known that it is metabolized or degraded to ritalinic acid in the equine.[18]

For the basic *in vivo* drug screen (covering acepromazine, fentanyl, fluphenazine, tripelennamine, mepivacaine, azaperone and methylphenidate), samples were prepared for analysis by mixing 2 mL of urine with 1 mL of 1 M pH 4.7 acetate buffer, 100 μL of a β-glucuronidase from *Helix pomatia* solution (40 000 units/mL in water) and 100 μL of a pancreatin solution (made by dissolving 1.25 g of Sigma product P7545 in 50 mL of 0.05 M pH 6.6 acetate buffer) and then hydrolysing overnight at 40 °C. Samples were then extracted the following day by conditioning a Phenomenex Strata XC 3 ml 60 mg solid phase extraction cartridge with 3 mL methanol and 3 mL water, loading the samples, washing with 3 mL 0.1M pH 9.0 acetate buffer, 3 mL water, 3 mL 0.1 M HCl, 3 mL methanol, 3 mL diethyl ether and then eluting with 2 × 1 mL of ethyl acetate : propan-2-ol : ammonia : 80 : 17 : 3. Samples were then evaporated to dryness and reconstituted in 10 μl propan-2-ol followed by 190 μl of water before being submitted to LC-MS analysis on the Thermo LTQ-Orbitrap.

For the more general drug screen (covering celecoxib), samples were prepared for analysis by mixing 2 mL of urine with 1 mL of 2 M pH 6.3 phosphate buffer, 100 μL of a β-glucuronidase from *Helix pomatia* solution (40′000 units/mL in water), 100 μL of a pancreatin solution (made by dissolving 1.25 g of Sigma product P7545 in 50 mL of 0.05 M pH 6.6 acetate buffer) and then hydrolyzing overnight at 40 °C. Samples were then extracted the following day by conditioning a Varian Nexus 3 mL 60 mg solid phase extraction cartridge with 1 mL methanol followed by 1 mL water, loading the samples, washing with 0.5 mL hexane, drying the cartridge under positive pressure for 30 s and then eluting with 2 × 1 ml 10% methanol in ethyl acetate. 1 mL water is then added, before samples are centrifuged for 5 min at 3000 rpm and the organic supernatant transferred to a new tube. Samples were then evaporated to dryness and reconstituted in 10 μl MeOH followed by 90 μl water before being submitted to LC-MS analysis on the Thermo LTQ-Orbitrap.

Thermo LTQ-Orbitrap high-resolution/accurate-mass LC-MS analyses

Samples from both the *in vivo* administrations and *in vitro* incubations were analyzed by two full-scan, high resolution accurate mass LC-MS screens. One screen covered basic drugs and the other, a more general screen, also covered acidic and neutral compounds.

For the basic drug screen (covering acepromazine, fentanyl, fluphenazine, tripelennamine, mepivacaine, azaperone and methylphenidate), 10 μL of sample was introduced onto a Thermo Accela autosampler/HPLC linked to a Thermo Discovery LTQ-Orbitrap. Chromatography was carried out using a Waters 100 mm

× 2.1 ID 3 µm Atlantis T3 column held at 40 °C. Flow rate was 400 µL/min and mobile phase A was 0.1% acetic acid with 300 µg/litre uracil (external calibrant) and mobile phase B was 0.1% acetic acid in acetonitrile with 300 µg/litre uracil (external calibrant). Mobile phase B was at 0% at 0 min, rising to 10% at 1.2 min, 35% at 2.0 min, 65% at 3.0 min, 98% at 3.5 min, held at 98% until 4.5 min before being reduced back to 0% at 4.51 min. Sample ionisation was carried out in the positive mode using the electrospray source at a capillary temperature of 200 °C, a sheath gas flow of 30 units, an auxiliary gas flow of 10 units and an ionspray voltage of 4.5 kV. Full scan centroid data over a range from 90 to 650 amu was then acquired by the LTQ Orbitrap using a resolution of 30 000 at FWHM. Data was acquired and processed using the Xcalibur version 2.0.7 software.

For the more general drug screen (covering celecoxib), 10 µL of sample was introduced onto a Thermo Accela autosampler/HPLC linked to a Thermo Discovery LTQ-Orbitrap. Chromatography was carried out using a Phenomenex 100 mm × 2.0 ID 2.5 µm HST Luna C18(2) column held at 35 °C. Flow rate was 400 µL/min until 4.0 min, at which time it was increased linearly to 500 µL/min at 4.20, before being held at this flow rate until 4.99 min, reduced back to 400 µL/min by 5.00 min and then held at this flow rate until the end of the run. Mobile phase A was 0.1% acetic acid with 300 µg/litre uracil (external calibrant), mobile phase B was 0.1% acetic acid in methanol with 300 µg/litre uracil (external calibrant) and mobile phase C was 99 : 1 methanol:2 M pH 4 acetate buffer with 300 µg/litre uracil (external calibrant). Mobile phase A was 90% at 0 min, dropping to 40% at 1.0 min and then 2% at 3.49 min, before being held at 2% until 4.99 min when it was increased to 90% over 0.01 min and held at 90% until 5.5 min. Mobile phase B was 10% at 0 mins, rising to 60% at 1.0 min, 98% at 3.49 min, before dropping to 23% at 3.50 min, held at 23% until 4.50 min when it was increased to 98% over 0.01 mins, held at 98% until 4.99 min, dropped to 10% over 0.01 min and then held at 10% until 5.5 min. Mobile phase C was 0% from 0 to 3.49 min, before being increased to 75% over 0.01 min at 3.49 min, held at 75% until 4.50 min, dropped back to 0% over 0.01 min and then held at

Table 1 A. Summary of the metabolites identified on the Orbitrap. The two major *in vivo* metabolites from the current study are highlighted in each case

Metabolite peak observed when using a mass tolerance of 5 ppm	Theoretical mass ($M + H^+$)	Retention time (mins)	Seen *in vivo*?	Seen in liver mics?	Seen in liver S9?	Seen in lung S9?
Acepromazine	327.1526	3.57	√	√	√	√
Hydroxy-ethyl-promazine	329.1682	3.49	√	√	√	√
Hydroxy-acepromazine	343.1475	3.37	√	√	√	×
Hydroxy-(hydroxy-ethyl)- promazine	345.1631	3.24	√ **(major)**	√	√	√
Hydroxy-(hydroxy-ethyl)- promazine	345.1631	3.30	√ **(major)**	×	√	×
Di-hydroxy-acepromazine	359.1424	3.19	√	√	×	×
Di-hydroxy-(hydroxy-ethyl)-promazine	361.1580	3.30	√	√	√	√
N-desmethyl-hydroxy-ethyl-promazine	315.1526	3.49	×	√	√	×
N-desmethyl-hydroxy-(hydroxy-ethyl)- promazine	331.1475	3.23	√	√	√	√
N-desmethyl-acepromazine	313.1369	3.53	×	√	√	×
Azaperone	328.1820	3.37	×	√	√	N/A
Hydroxy-azaperone	344.1769	3.29	√	√	√	N/A
Di-hydroxy-azaperone	360.1718	3.21	√	√	√	N/A
Di-hydroxy-azaperone	360.1718	3.29	√	√	√	N/A
Di-hydroxy-azaperone	360.1718	3.34	×	√	×	N/A
Tri-hydroxy-azaperone	376.1667	3.23	√	√	×	N/A
Tri-hydroxy-azaperone	376.1667	3.30	×	√	×	N/A
Tri-hydroxy-azaperone	376.1667	3.34	√	√	×	N/A
Azaperol	330.1976	3.28	√	√	√	N/A
Hydroxy-azaperol	346.1925	3.20	√ **(major)**	√	√	N/A
Hydroxy-azaperol	346.1925	3.33	√	√	√	N/A
Di-hydroxy-azaperol	362.1875	3.20	√	√	√	N/A
Di-hydroxy-azaperol	362.1875	3.26	√ **(major)**	√	×	N/A
Tri-hydroxy-azaperol	378.1824	3.08	√	√	√	N/A
Tri-hydroxy-azaperol	378.1824	3.20	√	√	√	N/A
Tri-hydroxy-azaperol	378.1824	3.29	√	√	×	N/A
Despyridyl-azaperone	251.1554	2.99	√	√	×	N/A
N-despyridyl-hydroxy-azaperone	267.1503	2.97	√	√	×	N/A
N-despyridyl-azaperol	253.1711	2.60	√	√	√	N/A
N-despyridyl-hydroxy-azaperol	269.1660	2.48	√	×	√	N/A
1-(2-pyridyl)-piperazine*	164.1182	3.37	√	√	√	N/A
Hydroxy-1-(2-pyridyl)-piperazine*	180.1131	3.29	√	√	√	N/A

* The 1-(2-pyridyl)-piperazine itself is believed to be an impurity since its concentration is 10–20-fold higher in the pre-dose than post-dose, but the hydroxy-1-(2-pyridyl)-piperazine concentration increases significantly in the post-dose samples and so is believed to be a metabolite. However, it is uncertain whether the hydroxy-1-(2-pyridyl)-piperazine is a metabolite of the 1-(2-pyridyl)-piperazine impurity or the azaperone itself.

0% until 5.50 min. Sample ionisation was carried out in the positive mode using the electrospray source at a capillary temperature of 200 °C, a sheath gas flow of 40 units, an auxiliary gas flow of 5 units and an ionspray voltage of 4.5 kV. Full scan centroid data over a range from 100 to 550 amu was then acquired by the LTQ Orbitrap using a resolution of 30 000 at FWHM. Data was acquired and processed using the Xcalibur version 2.0.7 software.

Applied Biosystems Sciex 5500 Q Trap LC-MS/MS analyses

The *in vitro* samples resulting from the fentanyl incubations were analyzed using product ion scanning LC-MS/MS techniques in order to structurally elucidate the major metabolites produced. In addition to the *in vitro* samples, *a* 10 ng injection of a fentanyl reference standard was also performed in order to match chromatographic retention time and mass spectra to that observed in the samples. The reason that the LTQ-Orbitrap was not used for this purpose was that the low-mass cut-off on the LTQ component of this instrument meant that some of the metabolically diagnostic low-mass ions could not be observed. This situation could theoretically be circumvented by the use of a similar LTQ-Orbitrap that makes use of a Higher-energy Collisional Dissociation (HCD) chamber.

10 µL of sample was introduced into an Applied Biosystems Sciex 5500 Q Trap using a Waters Acquity autosampler/HPLC. Chromatography was carried out using a Waters 100 mm × 2.1 ID 3 µm Atlantis T3 column held at 40 °C and using an elongated HPLC gradient relative to the Orbitrap analyses in order to provide some addition separation of the metabolites. Flow rate was 400 µL/min and mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. Mobile phase B was at 2% at 0 min, rising to 7% at 0.2 min, 10% at 1.2 min, 50% at 5.0 min, 100% at 5.2 min, held at 100% until 6.2 min before being reduced back to 2% at 6.25 min and then being held at 2% until 7.0 min.

Structural information regarding the metabolites was achieved using the 'enhanced product ion scan (EPI)' mode (uses collision-induced dissociation of precursor ions in the collision cell followed by product ion scanning using the linear ion trap function). Sample ionisation was carried out in the positive mode using the Turbo Ionspray source at a source temperature of 600 °C. Ion spray voltage was 5500 V, gas one had a flow of 50 units, gas two a flow of 50 units, the curtain gas had a flow of 10 units, the CAD gas setting was 'low', declustering potential was 120 V and entrance potential 10 V. Scan ranges were from 50 amu up to 5 amu above the chosen precursor ion. The Q1 resolution was 'unit', the Q3 entry barrier was 8V, the scan rate was 10 000 amu/s, the dynamic fill time setting on the linear ion trap was used and the step size was 0.12 amu. Collision energy was 30 eV, with a spread of 10 eV. Data was acquired in profile mode and processed using the Analyst version 1.5 software.

Results and Discussion

Comparison of *in vivo* and *in vitro* experiments

The results of the qualitative metabolite identification experiments on the Orbitrap are summarized in Table 1. As can be seen from this table, not all tissue fractions were used for the incubation of all drugs. This was because different tissues became available at different stages of the study. However, enough results were

Table 1 B. Summary of the metabolites identified on the Orbitrap. The two major *in vivo* metabolites from the current study are highlighted in each case

Metabolite peak observed when using a mass tolerance of 5 ppm	Theoretical mass ($M + H^+$)	Retention time (mins)	Seen *in vivo*?	Seen in liver mics?	Seen in liver S9?	Seen in lung S9?
Celecoxib	382.0832	3.89	×	N/A	√	N/A
Hydroxy-celecoxib	398.0781	3.20	×*	N/A	√	N/A
Carboxy-celecoxib	412.0573	3.50	√ **(major)**	N/A	√	N/A
Fentanyl	337.2274	3.50	×	√	√	√
Hydroxy-fentanyl	353.2224	3.31	×	√	√	×
Hydroxy-fentanyl	353.2224	3.36	√ **(major)**	√	√	×
Hydroxy-fentanyl	353.2224	3.41	×	√	√	×
Hydroxy-fentanyl	353.2224	3.61	×	√	√	×
Di-hydroxy-fentanyl	369.2173	3.22	×	√	×	×
Di-hydroxy-fentanyl	369.2173	3.30	×	√	×	×
Di-hydroxy-fentanyl	369.2173	3.45	×	√	×	×
N-des-(ethylbenzyl)-fentanyl	233.1648	3.18	×	√	√	×
N-des-(ethylbenzyl)-hydroxy-fentanyl	249.1598	2.75	×	√	×	×
N-des-(ethylbenzyl)- hydroxy-fentanyl	249.1598	2.83	×	√	×	×
N-des-(ethylbenzyl)- hydroxy-fentanyl	249.1598	3.00	×	√	×	×
N-des-(ethylbenzyl)- hydroxy-fentanyl	249.1598	3.32	×	√	×	×
N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA)	367.2016	3.37	√ **(major)**	×	√**	×
Fluphenazine	438.1821	3.70	√	√	√	N/A
Hydroxy-fluphenazine	454.1771	3.54	√ **(major)**	√	√	N/A
Di-hydroxy-fluphenazine	470.1720	3.33	√ **(major)**	√	√	N/A
Di-hydroxy-fluphenazine	470.1720	3.54	×	√	√	N/A
N-des-(2-hydroxyethyl)-fluphenazine	394.1559	3.70	×	√	√	N/A

* Hydroxy-celecoxib was detected *in vivo* by de Kock *et al.*[24] but only *in vitro* in the current study.
** Only detected when NAD is added to the incubation mixture.

available to observe trends between the different fractions. In general, the liver microsomes produced a greater number of hydroxy and desalkylated metabolites compared to the liver S9, whereas the liver S9 produced a greater number of reduced and carboxy metabolites. As expected, the lung S9 was only able to perform a limited number of metabolic transformations, consistent with the reportedly lower level of many drug metabolizing enzyme activities in the lung of most species.[19] Plasma samples were able to hydrolyze the ester bond of methylphenidate, whereas liver microsomes/S9 and lung S9 were not. These differing *in vitro* results highlight the need to use as many available tissue fractions as possible when studying the metabolism of a new drug.

If a broad level of success in correlating the *in vivo* and *in vitro* results is considered as the identification *in vitro* of the two most abundant *in vivo* metabolites, then for all drugs studied the success rate was 100%. In fact, for all the *in vivo* urine samples analyzed, all the metabolites that were observed in these samples were also identified in the *in vitro* samples. In several instances, it was also possible to detect minor metabolites *in vitro* that were not visible in the *in vivo samples.* However, the urine samples used in the current study were generally only taken from one time-point following administration of the drug. Since it is known that quantitative metabolite profiles often differ depending on the time following administration, a review of the published literature was also carried out in order to ensure the results were consistent with data from elsewhere.

For acepromazine, Dewey *et al.*[20] have reported that the drug is metabolized in the equine to 7-hydroxy-acetyl-promazine, 2-(1-hydroxy-ethyl)-7-promazine and 2-(1-hydroxy-ethyl)-promazine. In the urine sample analyzed within the current study, peaks consistent with the masses of these previously reported metabolites were identified using the Orbitrap. It was not possible, however, to give positional information to the different hydroxylations using just the accurate mass of the molecular ion data generated on the Orbitrap. As will be the case for the data relating to all the analytes from the current study, it would theoretically have been possible to postulate positional information based on the acquisition of product ion spectra, but this was not considered necessary for a simple *in vivo-in vitro* correlation. As is evident from Table 1A, several further metabolites were also observed *in vivo* and all these metabolites were detected in at least one of the *in vitro* tissue incubations.

For azaperone, Sams *et al.*[21] have reported that the drug is metabolized in the equine to 5′-hydroxy-azaperol and 5′-hydroxy-azaperone. Additionally, Chui *et al.*[22] reported that the drug is metabolized to N-despyridyl-azaperol, N-despyridyl-azaperone, hydroxy-azaperol, hydroxy-azaperone and azaperol. In the urine sample analyzed within the current study, peaks consistent with the masses of all of these previously reported metabolites were identified using the Orbitrap. As is evident from Table 1A, several further metabolites were also observed *in vivo* and all these metabolites were detected in at least one of the *in vitro* tissue incubations.

Table 1 C. Summary of the metabolites identified on the Orbitrap. The two major *in vivo* metabolites from the current study are highlighted in each case

Metabolite peak observed when using a mass tolerance of 5 ppm	Theoretical mass ($M + H^+$)	Retention time (mins)	Seen *in vivo*?	Seen in liver mics?	Seen in liver S9?	Seen in lung S9?
Mepivacaine	247.1805	3.18	√	√	√	N/A
Hydroxy-mepivacaine	263.1754	2.80	√	√	√	N/A
Hydroxy-mepivacaine	263.1754	2.89	√	√	√	N/A
Hydroxy-mepivacaine	263.1754	3.04	√ **(major)**	√	√	N/A
Hydroxy-mepivacaine	263.1754	3.09	√	√	√	N/A
Hydroxy-mepivacaine	263.1754	3.32	×	√	√	N/A
Hydroxy-mepivacaine	263.1754	3.37	×	√	√	N/A
Di-hydroxy-mepivacaine	279.1703	3.08	×	√	√	N/A
Di-hydroxy-mepivacaine	279.1703	3.16	√ **(major)**	√	√	N/A
N-desmethyl-mepivacaine	233.1648	3.14	×	√	√	N/A
N-desmethyl-keto-mepivacaine	247.1441	2.86	×	√	√	N/A
N-desmethyl-keto-mepivacaine	247.1441	3.12	×	√	√	N/A
N-desmethyl-keto-mepivacaine	247.1441	3.58	×	√	√	N/A
N-desmethyl-keto-mepivacaine	247.1441	3.68	×	√	√	N/A
Keto-mepivacaine	261.1598	3.80	√	√	√	N/A
Tripelennamine	256.1808	3.45	×	N/A	√	N/A
Hydroxy-tripelennamine*	272.1757	3.32	√ **(major)**	N/A	√	N/A
Hydroxy-tripelennamine	272.1757	3.36	×	N/A	√	N/A
Di-hydroxy-tripelennamine	288.1707	3.32	√ **(major)**	N/A	√	N/A
N-desmethyl-tripelennamine	242.1652	3.38	×	N/A	√	N/A
N-desmethyl-hydroxy-tripelennamine	258.1601	3.26	√	N/A	√	N/A
N-desmethyl-hydroxy-tripelennamine	258.1601	3.42	×	N/A	√	N/A
N-di-desmethyl-tripelennamine	228.1495	3.31	×	N/A	√	N/A

* The peak appearing in the hydroxy-tripelennamine window (*m/z* 272.1757) at 3.32 min has the same retention time as a peak that appears in the di-hydroxy-tripelennamine window. It is therefore possible that the peak in the hydroxy-tripelennamine window at 3.32 min is an analytical artefact from the in-source decomposition of an N-oxide of hydroxy-tripelennamine, but more work would be required to confirm this. For *in vivo* − *in vitro* comparison purposes however, the fact that the two peaks are seen both *in vitro* + *in vivo* is sufficient.

Table 1 D. Summary of the metabolites identified on the Orbitrap. No *in vivo* metabolism data was available for this compound, so no major *in vivo* metabolites can be listed

Metabolite peak observed when using a mass tolerance of 5 ppm	Theoretical mass ($M + H^+$)	Retention time (mins)	Seen in liver mics?	Seen in liver S9?	Seen in lung S9?	Seen in plasma?
Methylphenidate	234.1489	3.21	√	√	√	√
Ritalinic acid	220.1332	3.28	Impurity*	Impurity*	Impurity*	√*
Hydroxy-methylphenidate	250.1438	2.94	√	√	×	×
Hydroxy-methylphenidate	250.1438	3.02	√	√	×	×
Hydroxy-methylphenidate	250.1438	3.11	√	√	×	×
Hydroxy-ritalinic acid	236.1281	2.56	√	√	×	×
Hydroxy-ritalinic acid	236.1281	2.78	√	√	×	×
Hydroxy-ritalinic acid	236.1281	2.99	√	√	×	×
Hydroxy-ritalinic acid	236.1281	3.10	√	√	×	×
Hydroxy-ritalinic acid	236.1281	3.21	√	×	×	×
Hydroxy-ritalinic acid	236.1281	3.54	√	√	×	×
Di-hydroxy-methylphenidate	266.1387	3.05	√	×	×	×
Di-hydroxy-methylphenidate	266.1387	3.13	√	√	×	×
Di-hydroxy-methylphenidate	266.1387	3.74	√	×	×	×
Di-hydroxy-ritalinic acid	252.1230	2.94	√	×	×	×
Di-hydroxy-ritalinic acid	252.1230	3.07	√	√	×	×
Di-hydroxy-ritalinic acid	252.1230	3.37	√	×	×	×

* A large quantity of ritalinic acid was apparent as either an impurity or an analytical artefact in the pre-dose samples, the samples where no NADPH was added (negative control) and in an injection of pure reference standard. It was therefore not possible to assign ritalinic acid as a metabolite in the S9/microsomes experiments since there was no increase in the analytical response for this transition between pre- and post-dose samples. However, ritalinic acid was assigned as a metabolite in the plasma experiment because there was a 35-fold increase between pre- and post-dose, as expected from the high esterase activity present in equine plasma.

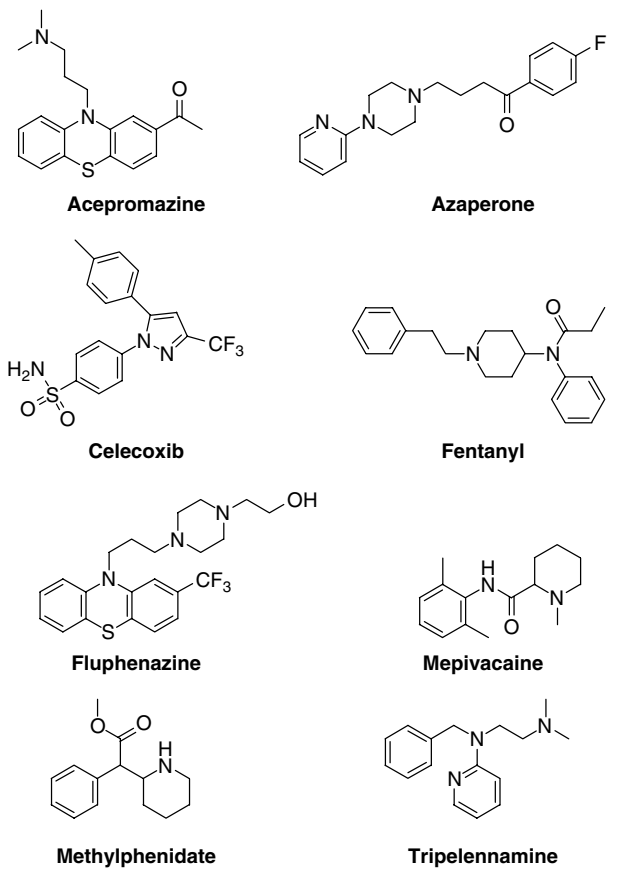

Figure 1. structures of the eight drugs used in this study.

For celecoxib, Dirikolu *et al.*[23] and de Kock *et al.*[24] have reported that the drug is metabolized in the equine to 4-hydroxy-celecoxib and 4-carboxy-celecoxib. In the urine sample analyzed within the current study, a peak consistent with the mass of 4-carboxy-celecoxib, but not for hydroxy-celecoxib, was identified using the Orbitrap. However, peaks consistent with the masses of both hydroxy-celecoxib and carboxy-celecoxib were identified *in vitro* (Table 1B).

For fentanyl, Frincke and Henderson[25] identified N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA) as the major metabolite in the equine. Russo *et al.*[26] have since compared the metabolism of fentanyl between man, horse and dog and found that PMA was the only metabolite formed in the equine and that PMA was unique to this species. In the urine sample analyzed within the current study, peaks consistent with the mass of PMA were identified using the Orbitrap. As shown in Table 1B, a hydroxy-fentanyl metabolite was also observed *in vivo* and both these metabolites were detected in at least one of the *in vitro* tissue incubations. The *in vitro* result was obtained by adding NAD as well as NADPH to the incubation mixture. PMA was not detected when only NADPH was added to the *in vitro* incubation, suggesting that enzymes preferring NAD as a cofactor, such as alcohol dehydrogenase and/or aldehyde oxidase, participate in the production of the carboxylic acid functionality. For future *in vitro* studies, it is therefore recommended that both NADPH and NAD be added to incubations in order to activate as many enzymes as possible.

For fluphenazine, no previously published equine studies were found, but as shown in Table 1B, all the metabolites that were observed in the *in vivo* urine sample analyzed on the Orbitrap in the current study were also detected *in vitro*.

For mepivacaine, Dumasia *et al.*[27] reported that the major metabolites of this drug in the equine were a number of hydroxy-

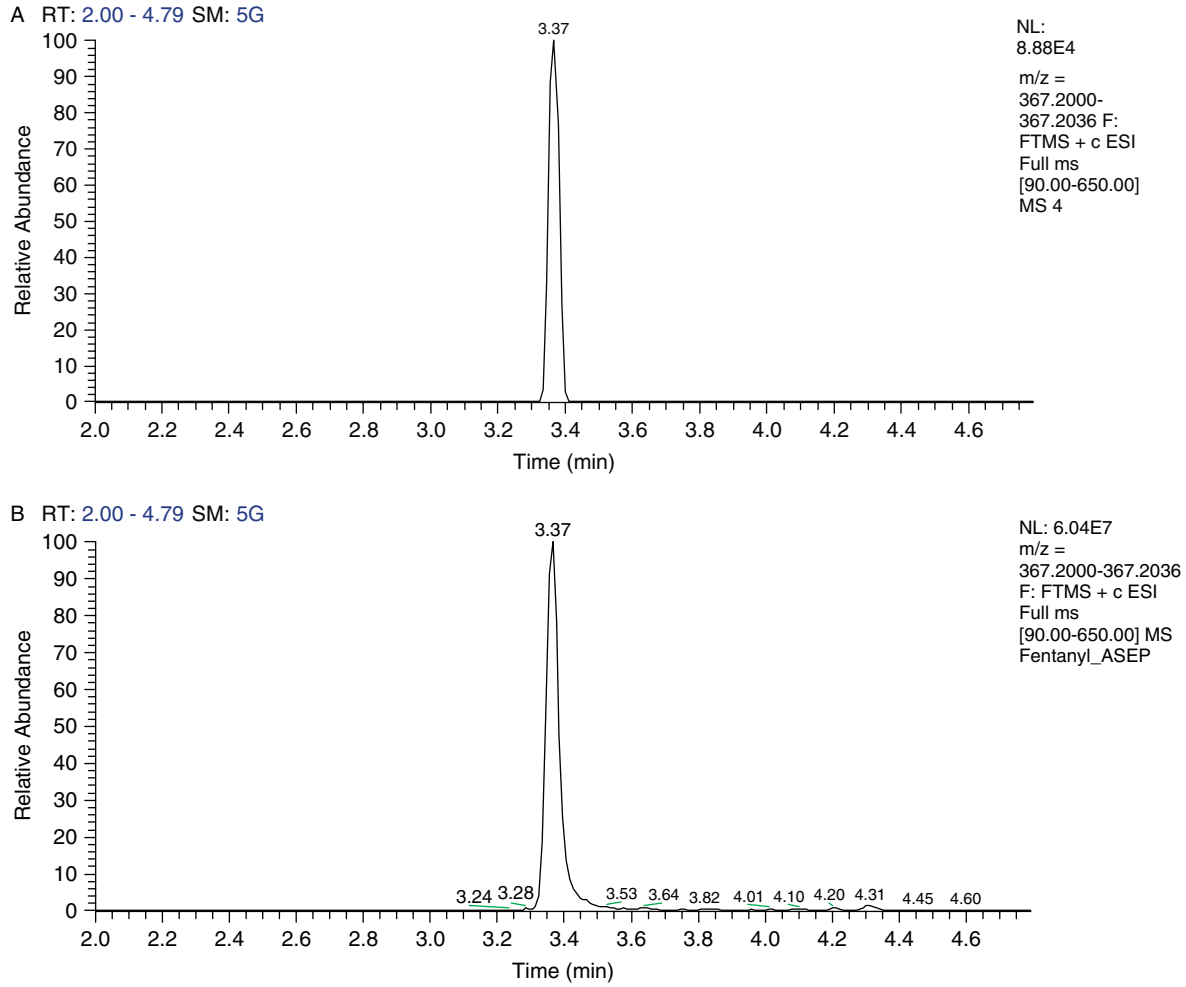

Figure 2. chromatograms for the major fentanyl metabolite, N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA), on the Orbitrap (*m/z* 367.2016 at a mass tolerance of 5 ppm) for; **A)** equine liver S9 with added NADPH and NAD cofactors and **B)** an equine *in vivo* post-administration sample.

mepivacaine and di-hydroxy-mepivacaine isomers. In the urine sample analyzed within the current study, peaks corresponding to the masses of both hydroxy-mepivacaine and di-hydroxyl-mepivacaine isomers were identified using the Orbitrap. As is evident from Table 1C, a keto-mepivacaine metabolite was also observed *in vivo* and all these metabolites were detected *in vitro*.

For Tripelennamine, Neils *et al*.[28] reported that the two major metabolites were hydroxy-tripelennamine and N-desmethyl-hydroxy-tripelennamine. Minor metabolites were despyridyl-tripelennamine, des-(ethylamino)-hydroxy-tripelennamine, di-desmethyl-hydroxy-tripelennamine and desmethyl-tripelennamine. In the urine sample analyzed within the current study, peaks corresponding to the masses of the two previously reported major metabolites hydroxy-tripelennamine and N-desmethyl-hydroxy-tripelennamine were identified using the Orbitrap. As shown in Table 1C, a di-hydroxy-tripelennamine metabolite was also observed *in vivo*, but none of the previously reported minor *in vivo* metabolites were observed. All the metabolites that were detected in the urine sample analyzed within the current study were also detected in at least one of the *in vitro* tissue incubations. Additionally, the *in vitro* incubates also produced several other tripelennamine metabolites, most notably N-desmethyl-tripelennamine, which was one of the previously reported minor metabolites.

For methylphenidate, although no post-administration samples were available, it is known that it is metabolized or degraded to ritalinic acid in the equine.[18] In accordance with this published study, ritalinic acid was identified *in vitro* in the current study, alongside a number of hydroxy-methylphenidate and hydroxy-ritalinic acid metabolites (Table 1D).

Structural characterization of the major fentanyl metabolite N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA)

In most cases, the exact structure of each of the metabolites identified was not studied because the major objective here was simply to correlate the results to ensure that *in vitro* methods were able to qualitatively match the *in vivo* profiles. In order to show that metabolite material generated *in vitro* could be used for structural elucidation according, however, a selection of the *in vitro* samples generated from the fentanyl incubations were subject to LC-MS/MS analysis on a Sciex 5500 Q Trap instrument. Fentanyl was chosen because its major *in vivo* metabolite was one of the smallest peaks identified *in vitro* on the Orbitrap and so if it were possible to generate good quality structural information on this minor metabolite, this would give confidence that the approach could be applied to most other drugs.

As shown in Figure 2, the major fentanyl metabolite, N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA), was detected on

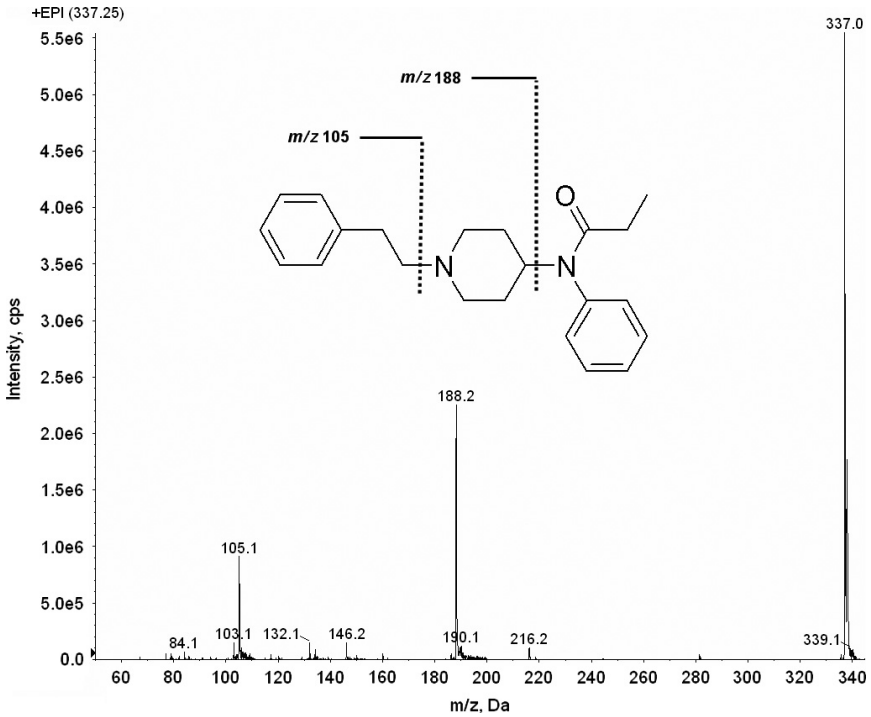

Figure 3. product ion spectrum (products of *m/z* 337.25) of fentanyl generated on the Q Trap 5500 (collision energy of 30 eV, with a spread of 10 eV).

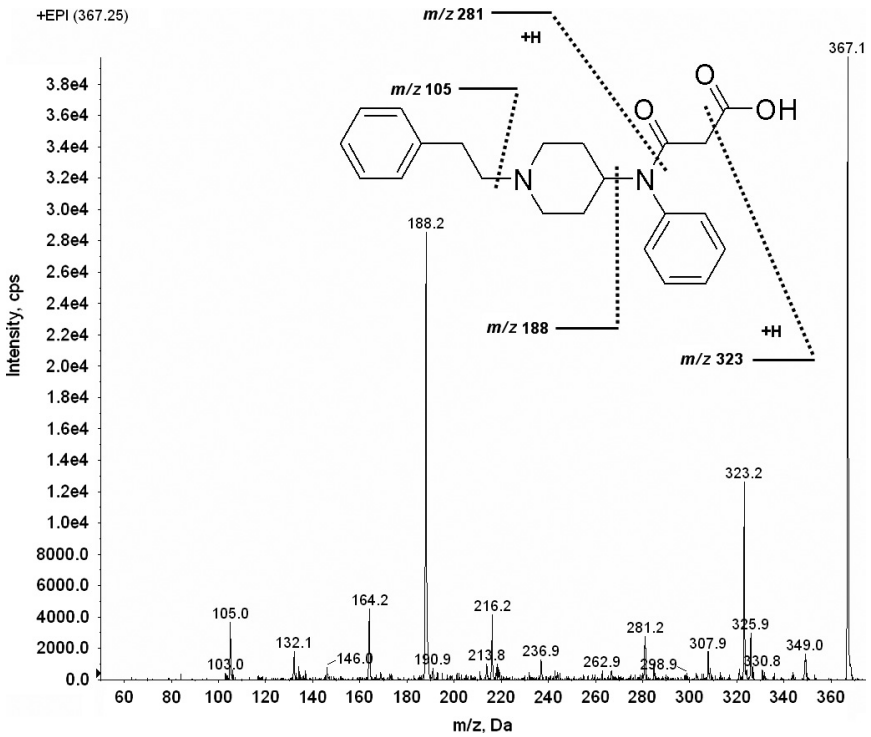

Figure 4. product ion spectrum (products of *m/z* 367.25) of the fentanyl metabolite, N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA), generated on the Q Trap 5500 (collision energy of 30 eV, with a spread of 10 eV) from the equine liver S9 incubation with added NADPH and NAD cofactors.

the Orbitrap with a relative instrumental response of three orders of magnitude lower than the peak detected *in vivo*. The product ion spectra for the parent drug fentanyl and for the proposed metabolite PMA generated on the 5500 Q Trap are given in Figures 3 and 4, respectively. Fragment ions at *m/z* 105 and 188 are common to both structures, confirming that the ethyl-benzene and the attached piperidine ring are not subject to metabolism. There is a peak at *m/z* 323 in the spectra of the proposed PMA metabolite (44 amu down from the precursor ion), consistent with the loss of CO_2 from a carboxylic acid moiety. There is also a peak at *m/z* 281 in the spectra of the proposed PMA metabolite (85 amu down from the precursor ion), corresponding to a loss of

CO_2 followed by a loss of CH_2CO, adding support to the proposal that the carboxylation occurs on the terminal carbon of the alkane side-chain. Russo *et al*.[26] have reported MS^3 experiments on the *m/z* 323 ion for this proposed metabolite, which also produce the *m/z* 281 ion and support the suggested successive nature of the fragmentation pathway from *m/z* 367 to 323 and then to 281.

In summary, at least the two major *in vivo* metabolites were found to be generated *in vitro* when analyzed by high resolution accurate mass on the Orbitrap. The major *in vivo* metabolite of fentanyl, N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA), was structurally characterized using MS/MS on the 5500 Q Trap, thus supporting the suggestion that such incubations could be used in place of *in vivo* post-administration samples as reference material of drug metabolites in accordance with new guidelines in the 2009 ILAC-G7 document.[16] This research also provides individual equine drug testing laboratories with some specific *in vitro* conditions from which to start when developing assays in their own facilities. Such *in vitro* techniques do not serve as a replacement for *in vivo* studies in all situations, but do offer the following advantages:

- The ability to reduce and refine the number of *in vivo* studies.
- As an aid to the identification of drug metabolites *in vivo* (producing a more concentrated, cleaner extract).
- For studying the fate of designer drugs where *in vivo* administrations are difficult to justify without previously defined toxicological profiles.
- For fast reaction to potential new threats.
- For studying the mechanisms of metabolism.
- To generate metabolite reference standards for use as an alternative to *in vivo* post-administration urine samples in accordance with the 2009 ILAC-G7 guidelines.[16]

It is therefore recommended that the use of *in vitro* techniques to study the metabolism of drugs and to produce reference material for equine drug surveillance purposes become routine.

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References

[1] R. Yuan, S. Madini, X. Wei, K. Reynolds, S. Huang, *Drug Metab. Dispos.* **2002**, *30*(12), 1311.
[2] G. N. W. Leung, E. N. M. Ho, D. K. K. Leung, F. P. W. Tang, K. C. H. Yiu, T. S. M. Wan, X. Xu, J. H. K. Yeung, H. N. C. Wong, *Proceedings of the 15th International Conference of Racing Analysts and Veterinarians* **2004**, p. 146.
[3] E. N. M. Ho, D. K. K. Leung, T. S. M. Wan, N. H. Yu, *Anal. Chim. Acta* **2005**, *540*(1), 111.
[4] E. N. M. Ho, W. H. Kwok, D. K. K. Leung, T. S. M. Wan, A. S. Y. Wong, *Anal. Chim. Acta* **2007**, *586*, 208.
[5] W. H. Kwok, G. N. W. Leung, T. S. M. Wan, C. H. F. Wong, J. K. Y. Wong, *Proceedings of the 16th International Conference of Racing Analysts and Veterinarians.* **2006**, p. 120.
[6] S. D. Stanley, K. Whitley, *Proceedings of the 16th International Conference of Racing Analysts and Veterinarians, Tokyo, Japan.* **2006**, p. 518.
[7] H. K. DiMaio Knych, S. D. Stanley, *Biochem. Pharmacol.* **2008**, *76*(7), 904.
[8] H. K. DiMaio Knych, C. DeStefano Shields, A. R. Buckpitt, S. D. Stanley, *Arch. Biochem. Biophy.* **2009**, *485*, 49.
[9] J. Lévesque, E. Templeton, L. Trimble, C. Berthelette, N. Chauret, *Anal. Chem.* **2005**, *77*, 3164.
[10] J. P. Lehman, L. Ferrin, C. Fenselau, G. S. Yost, *Drug Metab. Dispos.* **1981**, *9*, 15.
[11] R. B. Vail, M. J. Homann, I. Hanna, A. Zaks, *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 67.
[12] T. Kuuranne, O. Aitio, M. Vahermo, E. Elovaara, R. Kostiainen, *Bioconjugate Chem.* **2002**, *13*, 194.
[13] T. Kuuranne, M. Kurkela, M. Thevis, W. Schänzer, M. Finel, R. Kostiainen, *Drug Metabol. Dispos.* **2003**, *31*, 1117.
[14] S. E. Jäntti, A. Kiriazis, R. R. Reinilä, R. K. Kostiainen, R. A. Ketola, *Steroids* **2007**, *72*, 287.
[15] L. Hintikka, T. Kuuranne, O. Aitio, M. Thevis, W. Schänzer, R. Kostiainen, *Steroids* **2008**, *73*, 257.
[16] Accreditation Requirements and Operating Criteria for Horseracing Laboratories. *International Laboratory Accreditation Cooperation-G7 (ILAC-G7).* www.ilac.org/documents/ILAC_G7_06_2009.pdf accessed 19 October **2009**.
[17] J. P. Scarth, H. A. Spencer, S. C. Hudson, P. Teale, B. P. Gray, L. L. Hillyer, *Steroids* **2010**, *75*, 57.
[18] N. R. Srinivas, J. W. Hubbard, G. McKay, E. M. Hawes, K. K. Midha, *Chirality* **1991**, *3*, 99.
[19] G. G. Gibson, P. Skett, In *Introduction to Drug Metabolism* 3rd edn, Nelson Thornes: Cheltenham, UK **2001**, p. 185.
[20] E. A. Dewey, G. A. Maylin, J G. Ebel, J. D. Henion, *Drug Metabol. Dispos.* **1981**, *9*, 30.
[21] R. A. Sams, D. F. Gerken, R. L. Detra, S. D. Stanley, W. E. Wood, T. Tobin, J. M. Yang, H. H. Tai, A. Jegananthan, D. S. Watt *et al*. *J. Pharmaceut. Sci.* **1996**, *85*, 79.
[22] Y. C. Chui, B. Esaw, B. Laviolette, *J. Chromatog. B: Biomed. App.* **1994**, *652*, 23.
[23] L. Dirikoli, A. F. Lehner, J. Jacobs, W. E. Woods, W. Karpiessiuk, J. D. Harkins, W. G. Carter, J. Boyles, C. G. Hughes, J. M. Bosken, C. Holtz, C. Natrass, M. Fisher, T. Tobin, *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians, Cambridge,* **2000**, p. 162.
[24] S. S. De Kock, P. Jogi, R. Boshoff, *Proceedings of the 15th International Conference of Racing Analysts and Veterinarians, Dubai, United Arab Emirates,* **2004**, p. 365.
[25] J. M. Frincke, G. L. Henderson, *Drug Metabolism and Disposition,* **1980**, *8*, 425.
[26] C. I. Russo, S. J. Meagher, P. M. Wynne, *Proceedings of the 14th International Conference of Racing Analysts and Veterinarians, Florida, USA,* **2002**, p. 413.
[27] M. C. Dumasia, L. William, K. Silverthorne, E. Houghton, *Proceedings of the 12th International Conference of Racing Analysts and Veterinarians, Vancouver,* **1998**, p. 219.
[28] S. Neils, M. Jarrett, K. Hughes, D. Auer, *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians, Cambridge,* **2000**, p. 396.