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Drug metabolism in the horse: a review

James P. Scarth,[a]* Phil Teale[a] and Tiia Kuuranne[b]

A detailed understanding of equine drug metabolism is important for detection of drug abuse in horseracing and also in veterinary drug development and practice. To date, however, no comprehensive review of equine drug metabolism has been published.

The majority of literature regarding equine drug metabolite profiles is derived from sports drug detection research and is generally targeted at detecting marker metabolites of drug abuse. However, the bulk of the literature on equine drug metabolism enzymology is derived from veterinary studies aimed at determining the molecular basis of metabolism. In this article, the phase 1 and 2 metabolisms of seven of the most important classes of drugs monitored in horseracing are reviewed, including: anabolic-androgenic steroids (AAS), β_2-agonists, stimulants, sedatives/tranquilizers, local anesthetics, non-steroidal anti-inflammatory analgesics (NSAIDS)/cyclooxygenase-2 (COX-2) inhibitors, and opioid analgesics. A summary of the literature relating to the enzymology of drug metabolism in this species is also be presented.

The future of equine drug metabolism in the area of doping research will be influenced by several factors, including: a possible move towards the increased use of blood and other alternative testing matrices; the development of assays based on intact drug conjugates; the increasing threat of 'designer' and herbal- based products; advances in the use of *in vitro* technologies; the increased use of liquid-chromatography/high-resolution mass spectrometry; and the possibility of screening using 'omics' approaches. Also, the recent cloning of a range of equine cytochrome P450 (CYP) enzymes opens up the potential for carrying out more detailed mechanistic pharmacological and toxicological veterinary studies.

Keywords: equine; horse; drug metabolism; horseracing; cytochrome P450; steroids

Introduction

A survey of previously published drug metabolism literature shows that equine studies are generally much more scant than those on humans. Within the human field, the majority of published drug metabolism literature derives from studies relating to the pharmaceutical industry, with a smaller percentage coming from studies relating to the detection of drug abuse. However, within the equine field, proportionally more of the published literature derives from the detection of drug abuse (mainly in horseracing). This difference in the scope and volume of the human and equine literature may be partly due to the historically less demanding regulatory requirements of the veterinary drug industry for metabolism data prior to drug approval, and partly because of the fact that more drugs are prohibited in horseracing than human sports due to the different policies of the agencies/governing bodies involved.[1–5] Horses are also considered food-producing animals in some countries, which impacts on therapeutic drug usage patterns (and hence metabolism research) due to legislations regarding the residues of veterinary drugs entering the food chain.[6]

To date, no comprehensive review of equine drug metabolism has been published. Furthermore, much of the published equine drug metabolism literature is unavailable to the general scientific public because it is has largely been published in the proceedings of the International Conference of Racing Analysts and Veterinarians (ICRAV), which is not searchable on sites such as Scopus or Medline. An increased appreciation of the pathways of drug metabolism and the basic equine drug metabolizing enzymology could assist the veterinary industry in predicting the toxicology or pharmacology of new chemical entities and assist the horseracing industry to target the relevant metabolites in the detection of drugs of abuse. Therefore, the focus of the current review will be to provide a summary of the phase 1 and 2 metabolic pathways of seven of the most important classes of drugs monitored in horseracing, including: anabolic-androgenic steroids (AAS), β_2-agonists, stimulants, sedatives/tranquilizers, local anesthetics, non-steroidal anti-inflammatory analgesics (NSAIDS)/cyclooxygenase-2 (COX-2) inhibitors; and opioid analgesics. A summary of the literature relating to the enzymology of equine drug metabolism will also be presented. In order to provide a frame of reference for equine drug metabolism compared to other species, a selection of representative examples regarding human drug metabolism will also be reviewed.

Within equine sports drug testing, there are often different rules for the use and control of individual drugs depending on geographical location and the type of sport. For example, the International Federation of Horseracing Authorities (IFHA) aims to promote 'good regulation and best practices' amongst horseracing testing laboratories, and proposes an approach to the control of drug use and abuse in horseracing based upon a ban of the presence of drugs or metabolites at the time of racing.[1] This approach is formalized in Article 6 of the IFHA's *International Agreement on Breeding, Racing and Wagering*,[1] which differs markedly from the approach taken by the World Anti-Doping Agency (WADA) to human doping in that doping agents are banned by their potential affect upon a number of bodily systems

* *Correspondence to: James P. Scarth, HFL Sport Science, Newmarket Road, Fordham, Cambridgeshire, CB7 5WW, UK. E-mail: jscarth@hfl.co.uk*

a *HFL Sport Science (A Quotient Bioresearch Ltd Company), Newmarket Road, Fordham, Cambridgeshire, CB7 5WW, UK.*

b *Doping Control Laboratory, United Medix Laboratories Ltd, Höyläämötie 14, 00380 Helsinki, Finland*

rather than by specific name or structure. Local authorities have the ultimate decision on which parts of Article 6 they choose to implement as appropriate to their particular region and as a result there is a split between full[3] and partial signatories to Article 6.[2] Additionally, the rules for Olympic equestrian sports such as dressage and eventing are distinct from those of the horseracing authorities.[4] While a full discussion of the treatment of each individual drug by different sports regulatory authorities is beyond the scope of this review, an insight to the regulatory background of each drug class is given by describing their classification by the Association of Racing Commissioners International (ARCI) as an example of a horseracing authority[2] and the Federation Equestre Internationale (FEI) for Olympic disciplines.[4]

Under FEI rules, prohibited substances are classed as either 'banned' (outright) or 'controlled' (their use is restricted), depending on whether they have legitimate therapeutic use in certain contexts.[4] The ARCI classification is somewhat more complex, with prohibits substances being classified in either of five different groups, as determined by a combination of pharmacology, patterns of drug use, and appropriateness of drug use in the racing horse.[2]

- Category 1: Stimulant and depressant drugs that have the highest potential to affect performance and that have no generally accepted medical use in the racing horse. Many of these agents are Drug Enforcement Agency (DEA) schedule II substances.
- Category 2: Drugs that have a high potential to affect performance, but less of a potential than drugs in Class 1. These drugs are 1) not generally accepted as therapeutic agents in racing horses, or 2) they are therapeutic agents that have a high potential for abuse.
- Category 3: Drugs that may or may not have generally accepted medical use in the racing horse, but the pharmacology of which suggests less potential to affect performance than drugs in Class 2.
- Category 4: Therapeutic medications that would be expected to have less potential to affect performance than those in Class 3.
- Category 5: Therapeutic medications for which concentration limits have been established by the racing jurisdictions as well as certain miscellaneous agents such as dimethylsulfoxide (DMSO) and other medications as determined by the regulatory bodies.

The equidae family

The term 'equine' itself encompasses all species of animal within the equidae family. The major extant species within the equidae family fall into one genera (*Equus*). Most members of the equidae family are able to interbreed and produce viable offspring, although these are almost always sterile.[7] The horse (*Equus caballus*) is now virtually extinct naturally in the wild but survives in large numbers in human captivity and in some feral populations that have developed on several continents. Due to the selective breeding of horses by humans that has been occurring for thousands of years, distinct breeds have become apparent. For example Shire horses have been selected for their strength and ability to do work, while Thoroughbreds have been bred for their speed.[8]

The Thoroughbred is one of the oldest pedigrees with records spanning three centuries and is of particular importance in horseracing. A feature of this breed is the narrow genetic variation with ten founder females accounting for 72% of maternal lineages and one founder stallion being responsible for 95% of paternal lineages.[9] Genetic variability, measured as average heterozygosity, is significantly lower in Thoroughbred horses ($H = 0.353 +/- 0.065$) than in Argentine Creole horses ($H = 0.585 +/- 0.131$).[10] This narrow genetic stock of Thoroughbreds may therefore be expected to lead to reduced polymorphism in many characters when compared to other breeds.

The differences that exist in overall morphology between horse breeds may also be relevant to aspects of drug metabolism. As an example, it has been reported that significant differences in the pH of Standardbred and Thoroughbred horse urine exist and that this could be expected to lead to the differential excretion of some drugs.[11] Thoroughbred urine showed a bimodal distribution with a major peak at pH 5.5 and a minor peak at pH 8.0 while Standardbreds had a single peak at pH 8.0.

Donkeys are closely related to the horse (a domestic breed of the species *Equus asinus)* and a notable difference between the two species is the increased ability of donkeys to survive large losses in body water.[12] Only a very small amount of qualitative literature is available on the donkey and comparisons are mainly based on pharmacokinetics of the parent drug. Although the clearance of some drugs such as caffeine show similar excretion profiles between the two species, donkeys generally have a much greater capacity for drug metabolism. The clearance of phenylbutazone for example has been shown to be between 5 and 15 times higher in donkeys than in horses.[12]

Several factors may lead to differences in drug metabolism between horses and humans. The diet of the two species is different as humans are typically omnivores and horses herbivores.[8] While a hindgut fermentation chamber is absent in humans, members of the equidae family have a very well-developed caecum rich in a flora of micro-organisms that potentially contribute to the metabolism of a drug.[13] One particular feature of the caecal flora is that they are know to produce relatively large quantities of monoamines, which are absorbed into the bloodstream and have the potential to influence a number of physiological variables.[13,14] Another factor that differs substantially from horse to man is the capacity of the spleen to sequester red blood cells in times of inactivity or conversely to expel red blood cells into the circulation during periods of stress or exercise. The horse has a huge spleen that can alter the red blood cell content of blood by up to 50%.[15] The human spleen, however, is relatively much smaller, with a significantly reduced capacity for altering blood volume. The potential for massive changes in blood volume in a horse depending on the exercise and/or excitation status of the animal has obvious implications for variations in the pharmacokinetics of drugs.

Methods Used for Studying Drug Metabolism in the Horse

Studies on drug metabolism in equine species have been confined to *Equus caballus* (horses) and *Equus asinus* (asses and domestic donkeys). Within these studies, there exists a large body of *in vivo* and a very much smaller body of *in vitro* drug metabolism literature. Unless specified otherwise, all studies reviewed from this point onwards were carried out using *Equus caballus* species. A specific breed will only be referred to when there exists a point of comparison with another breed or sub-species.

A large proportion of the published data on drug metabolism in horses relates to the detection of drug abuse. Although an understanding of enzymology would help in analyzing data, it is not the primary aim of these studies and much of the data leaves questions about the underlying biology unanswered.

Analytical techniques for studying equine drug metabolism

In general, unless an authentic commercial reference standard is available or is easily synthesized, the structures of equine metabolites in surveillance research studies are generally inferred from their analytical behaviour or through comparison with known metabolic information from other species. The analytical techniques used for the actual metabolite identification have evolved significantly over the last 50 years. Initially, thin layer chromatography-ultraviolet detection (TLC-UV), often using radio-labelled drug as tracers or to provide quantitative data, were very popular techniques.[16] While TLC-based detection has now largely been superseded, a minority of laboratories still use some TLC for screening or confirmation of specific substances. High-performance liquid-chromatography linked to ultraviolet detection (HPLC-UV) was very popular until the 1980s/1990s,[17] after which gas chromatography and then liquid-chromatography linked to mass spectrometry (GC- and LC-MS respectively) became the techniques of choice.[18,19] While immunoassay techniques can be very useful for drug screening, they are generally of less use when it comes to performing metabolism studies as they provide little information on the structure of the metabolites. Nuclear magnetic resonance (NMR) analysis has occasionally been used to definitively identify metabolites,[20] but this typically requires extensive purification of large sample volumes and thus takes a significant amount of time and money and is not always feasible.

Although there has been a major shift from GC-MS to LC-MS in the past decade, GC-MS has remained an important tool because certain compounds, especially saturated steroid metabolites, suffer from poor ionization properties under, for example, electrospray ionization conditions of LC-MS.[18,19] The ability of many modern instruments to carry out MS^n experiments makes them particularly useful for identifying metabolites. Especially useful are triple quadrupole instruments since they allow a combination of full scan MS, selected reaction monitoring (SRM), product ion scanning, and more generic neutral loss or precursor ion scans to be performed; each with their own benefits depending on the type of study involved. For both GC- and LC-MS, the availability of deuterated or other labelled analogues of the drug can significantly aid the metabolite identification process, both in terms of the 'shift' technique[21] and the observation of peak doublets if a mixture of labelled/unlabelled versions of the drug is administered.[22] While selective derivatization procedures prior to GC-MS analysis have been used as aid in the structural elucidation process for several decades, the application of derivatization to LC-MS structural elucidation has been more recent. For example, the selective derivatization of steroidal ketone groups using a methoxyamine moiety has recently be used to infer the position of metabolic reduction in the 'designer' steroid estra-4,9-diene-3,17-dione.[23] Also, the recent emergence of higher resolution LC equipment including reduced dead volumes and the ability to run with sub-2 μM particle sizes now means that metabolites with similar molecular masses and retention times can now be more easily resolved.[24]

Most recently, robust high-resolution-accurate-mass LC-MS (LC-HRMS) systems have become commercially available and have started gaining popularity for sports drug surveillance screening and research.[25–27] Because the data acquired are full-scan analyses of intact $[M+H]^+$ or $[M-H]^-$ species at very high resolution (therefore not requiring targeted MRM analyses), the results can be analyzed using statistical packages designed to pick out subtle differences between pre- and post-administration samples. Some systems also allow in-source or collision-activated dissociation to be performed; with the result that the accurate mass assignments of the fragment ions can significantly aid the structural elucidation process.[23] Further advantages of using LC-HRMS include the ability to retrospectively analyze data once new information comes to light and the extended analyte coverage through the use of full-scan MS.

In light of the fact that equine drug metabolism studies rarely utilize radio-labelled dosing (discussed further later), it is important to design sample preparation, extraction, and analysis techniques that are not optimized too specifically on the 'parent' drug since metabolites often differ significantly in their behaviour. For these reasons, the best designed equine studies typically involve the use of sequential or parallel sample preparation, extraction, and analysis steps in order to encompass, for example, the extremes of polarity, ionic nature, volatility, phase 2 conjugation, metabolite lability to temperature and pH.[28] A particular feature of equine urine, which impacts on the choice of sample preparation or extraction technique, is its wide range of pH, which is affected by factors such as diet and exercise.[29] Also, equine plasma contains relatively high esterase activity,[27] which means that sample handling conditions can be important in determining the percentage hydrolysis of an amide- or ester-based compound *ex vivo* if enzyme activity is significant.

A particularly important consideration regarding sample preparation relates to the hydrolysis of urine samples prior to extraction to cleave the phase 2 conjugates. While this is beneficial in the extraction and analysis of the drugs, it means that the mode of conjugation is often undetermined. This is not always the case, however, and selective hydrolytic procedures or LC-MS techniques have sometimes been able to determine the nature of a conjugate. The conjugation pathways for endogenous and exogenous steroids in the horse, for example, are particularly well characterized.[30] The particular method used for hydrolyzing phase 2 conjugates prior to analysis is of critical importance in measuring the metabolite profile. For example, recombinantly produced β-glucuronidase from *E. coli* is relatively selective for hydrolysing glucuronides, whereas β-glucuronidase from *Helix pomatia* also contains aryl sulfatase and other enzymes leading to hydrolysis of some aromatic sulfates and various phase 1 reactions.[30] Simultaneous hydrolysis of glucuronide and sulfate conjugates is possible using strongly acidified methanol (methanolysis), although the harsh conditions may lead to losses or byproducts.[31] At present, there are very few drug screening assays based on the analysis of intact phase 2 conjugates.[32,33] This may be partly due to a lack of reference standards but also because they are not generally suitable for analysis by GC-MS. However, combining LC-MS analysis with *in vitro* production of conjugated metabolites (see later discussion) may now allow development of phase 2 conjugate screening assay on a larger scale. This would be advantageous since it could in theory significantly reduce the amount of sample preparation required and, in the case of urinary steroid metabolites, mean that they could be detected efficiently using electrospray ionization more efficiently than is the case for the free steroid.

In vivo equine metabolism studies

The most comprehensive *in vivo* metabolism studies involve the administration of a radio-labelled analogue of the drug to trace its fate with maximum certainty. This offers several benefits including: the ability to study the mass balance of the drug in different excreted products, the ability to focus the analysis on sample fractions containing increased levels of radioactivity; and to ensure the suitability and high recovery of sample preparation and extraction techniques as an analytical aid.[34] The drawbacks of radio-labelled studies are the cost of preparing the material, the ethical considerations regarding the administration of radioactivity, the requirements for specialized analytical equipment and the precautionary measures in handling the samples. In drug surveillance laboratories, the number of radiolabelled *in vivo* equine metabolism studies is limited and reducing in frequency, whereas they appear to have been carried out during veterinary drug development as a convenient way to study the mass balance and pharmacokinetics.

With regard to *in vivo* drug administration studies in drug surveillance research, the range of studied matrices generally mirrors the most suitable ones for routine testing. Equine urine has been the most extensively studied matrix to date; partly because of its applicability to post-race testing protocols, but also because it typically contains a much higher proportion of metabolites than plasma. Other matrices that have been studied include faeces,[35,36] hair[37] and saliva.[38] Although not typically containing as large a proportion of metabolites as urine or faeces, blood analysis is gaining importance due to its ease and speed of collection during training or pre-race, a general requirement for less sample purification compared to urine and a greater relevance of the observed parent drug concentrations to evaluation of their pharmacological effect.[39] There are also regional differences, such that testing protocols in the USA place greater emphasis on blood analysis compared to some other parts of the world.[40]

In vitro equine metabolism studies

While *in vivo* metabolism studies are the mainstay of equine metabolism research, the use of *in vitro* techniques utilising *ex vivo* liver or lung preparation is gaining popularity.[27] *In vivo* experiments have the advantage that the whole plethora of possible transformation can be considered and allows the most representative picture of the situation for real-life samples. However, *in vivo* experiments require animal experimentation 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; compounds 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. By comparison, *in vitro* methods do not require animal experimentation, although there can be ethical issues related to the supply of tissues; can be carried out quickly; produce a 'cleaner' extract for analysis;[27] can be used to study 'designer' drugs;[23] and can be more easily tailored to study mechanistic aspects.[21] Some of the disadvantages of using *in vitro* methods include the lack of an intact biological system and the inability to generate quantitative *in vivo-in vitro* correlation. It is therefore important to recognize these limitations as well as the advantages.

In vitro studies are used in equine surveillance laboratories to compliment the *in vivo* studies,[23,27,41–43] but proportionally more *in vitro* studies have been conducted in the veterinary arena, where they are useful, for example, in determining possible routes of metabolism. These studies will be discussed in detail in the 'Enzymology' section of this review. Of the published equine drug surveillance studies, *in vitro* techniques have been used to study the biosynthetic pathways of C18 androgens in testicular tissue[44] and the B-ring unsaturated estrogens in placental tissue.[45] More recently, equine liver microsomes have been used to study the phase 1 metabolism of the anabolic-androgenic steroids clostebol acetate and mesterolone,[47] methenolone acetate,[41] turinabol,[42] a range of designer steroids[46] and the non-steroidal anti-inflammatory drug phenylbutazone.[48] Most recently, several equine cytochrome P450 (CYP) enzyme isoforms, named CYP2D50, CYP2C92, CYP3A89, CYP3A96, and CYP3A97 and have for the first time been sequenced and their activity compared to human CYP2D, CYP2C and CYP3A isoforms.[49–51]

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 1 and 2 metabolism of the designer steroid tetrahydrogestrinone (THG).[52] Several authors have also reported the scaling up of *in vitro* conditions to allow the production and purification of mg quantities of human phase 1[53,54] and phase 2[55–58] drug metabolites, thus enabling characterization using techniques such as 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. Other factors that have limited the use of *in vitro* technologies in equine drug surveillance include the availability of equine tissues and a limited knowledge of the application of *in vitro* techniques. However, equine tissues are now becoming more readily available from specialist suppliers and there is increasing demand from many angles to reduce the use of animals in experimentation. The increased usage of LC-HRMS also provides a strong incentive to utilize *in vitro* methods for studying drug metabolism. The technique not only provides a powerful tool for the rapid interpretation of drug metabolism but is also increasingly used as a screening technique within drug control laboratories. Where only knowledge of the qualitative profile of metabolites is required, it is relatively straightforward to insert the relevant masses and retention times into a detection database, *in vitro* techniques are ideally suited to this application.[27]

In order to confirm the presence of a metabolite in the case of a positive drug finding, it is necessary to compare the mass spectral and chromatographic performance of the analyte against a standard material. Currently in this situation, International Laboratory Accreditation Cooperation (ILAC)-G7 guidelines on the *Accreditation Requirements and Operating Criteria for Horseracing Laboratories*[59] allow the use of *in vivo* urine/plasma samples resulting from a drug administration as qualitative reference material. However, this is not ideal as it requires animal experimentation. In 2009, a new revision of the ILAC-G7 guidelines[59] 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. It is therefore envisaged that a major benefit of *in vitro* methods will be to provide reference materials where no 'parent' drug can be detected and for which no chemically synthesized metabolite reference standard is available.

Equine Metabolism of Model Drug Classes

Since it would not be possible to do justice to all the *in vivo* and *in vitro* literature in this review, select examples of the phase 1 and 2 metabolism of several classes of drugs important to horseracing will be presented, including; anabolic-androgenic steroids (AAS), β_2-agonists, stimulants, sedatives/tranquilizers, local anesthetics, non-steroidal anti-inflammatory analgesics (NSAIDS)/cyclooxygenase-2 (COX-2) inhibitors, and opioid analgesics. However, it is important to highlight that metabolism studies have been performed on many other drugs not covered in the current review, including, but not limited to, diuretics, beta-blockers, canabinoids, corticosteroids, anti-ulcerative drugs, anti-haemorrhagic drugs, angiotensin converting enzyme (ACE) inhibitors, anti-infective drugs, and, increasingly, natural components/contaminants of feed that may affect performance. The metabolism of biological macromolecules such as growth hormone, insulin-like-growth-factor 1, erythropoietin, human chorionic gonadotrophin, etc., will not be covered since they are generally detected as 'parent' drug (or downstream biomarker/s) and are generally metabolized by proteolytic enzymes that are distinct from the enzymes involved in the metabolism of the 'small' molecules discussed later.

Anabolic-androgenic steroids

Because of their importance as ergogenic drugs of abuse within the horseracing industry and due to their involvement in equine reproduction, steroid metabolism has received more focus in the horse than any other class of compounds. Anabolic-androgenic steroids are banned under FEI rules (and also by many horseracing authorities),[4] but they are not given the highest level of classification by the ARCI and are placed in category 3.[2] This is partly because some anabolic-androgenic steroid preparations are permitted for use outside of competition.

*In vivo and in vitro s*tudies of both endogenous and exogenous steroid metabolism in horses have been conducted and there is now a strong focus within the veterinary field to understand the molecular biology of their action as well as measuring their concentrations in biological fluids. The presence and metabolism of endogenous anabolic-androgenic steroids (AAS) in meat-producing animals, including horses, has recently been reviewed[60] and a review article addressing the metabolism of both endogenous and exogenous AAS in horses has recently been produced.[19] Also, a review of the analytical methodologies used to detect steroid abuse has also recently been published.[18] Because these aforementioned articles already provide a thorough coverage of the field, the current section of this review will serve to summarize the overall trends and to provide some further information on the enzymology of horse steroid metabolism.

All the major mammalian steroid biosynthetic pathways have been demonstrated to be present in horses: equine follicular cDNAs from CYP11A1, 3β-hydroxysteroid dehydrogenase/Δ^5-/Δ^4-isomerase[61] and 17β-hydroxysteroid dehydrogenase have been cloned;[62] equine steroid acute regulatory protein (STAR) and CYP17 mRNA have been detected in theca cells;[63] CYP17 has been immunolocalized in the mare corpus luteum;[64] equine CYP19 cDNA has been cloned[65] and also immunolocalized in testicular tissue.[66] The existence of many of the remaining steroidal CYPs and oxidoreductases is based on the detection of their metabolites in biological fluids.[67]

In order to provide a frame of reference for highlighting some of the distinct features of equine steroids metabolism, the following section will compare equine steroid metabolism with that in the human. A major difference in steroid biosynthesis between human and equine steroid metabolism involves CYP17, which catalyzes both the 17α-hydroxylation and 17,20-cleavage of pregnenolone and progesterone in two distinct steps.[68] The 17α-hydroxylation step is efficient for both pregnenolone and progesterone in all species but the 17,20-lyase enzyme is often selective for one particular substrate. In the horse, both 17α-hydroxyprogesterone and 17α-hydroxypregnenolone can be substrates, but in man activity is selective for 17α-hydroxypregnenolone. This leads to humans using predominantly the Δ^5 pathway and horses both the Δ^4 and Δ^5 pathways for the biosynthesis of the androgens and oestrogens.[68] Man and horse are both able to produce the oestrogens oestrone and oestradiol (from AAS precursors), but the concentrations produced by horse testis are much higher than levels in man.[69] The pregnant horse also uniquely produces the B-ring unsaturated steroids equilin, equilenin and related metabolites.[69] Using testosterone as an example of an endogenous AAS, 5α/5β-reduction and 17-oxidation are important catabolic pathways in both species, but the horse tends to secondarily reduce 17-keto groups to from a mixture of 17α- and 17β-hydroxy isomers. Also, whereas 3α reduction of the 3-keto function is important in man, horses tend to reduce this oxygen to the 3β isomer. Hydroxylation at carbon 16 has also been shown to be a much more prominent phase 1 metabolic pathway in horses, while 6-hydroxylation is generally of less quantitative significance compared to man.[30] For synthetic steroid analogues in which many of the classical oxidative and reductive routes of metabolism are hindered, multiple carbon skeleton hydroxylations can become major excretory pathways in many species and the horse is no exception.

Phase 2 conjugations of steroids involve glucuronidation and sulfation in both man and horse, but there is a trend for sulfation to predominate in horses and glucuronidation in man. In horses, there is a preference for steroids with a 17β-hydroxy group to form sulfate conjugates and for steroids with a 17α-hydroxy group to form glucuronide conjugates.[19]

Figure 1 summarizes the major routes of anabolic-androgenic steroid catabolism in horses, while Table 1 summarizes the major phase 1 and 2 metabolites of the steroids that have to date been studied in this species.

β_2-agonists

β_2-agonists are used as bronchodilators in order to treat pulmonary disorders, but in addition to enhancing respiratory function, they also have a repartitioning effect at higher doses and promote muscle synthesis over fat.[70] Because of their potential to affect performance, they are controlled by most sports authorities and are classified as either class 2 or 3 agents by the ARCI.[2] FEI classification for the β_2-agonists is variable depending on the individual drug and whether it has a licensed use in horses[4] with the use

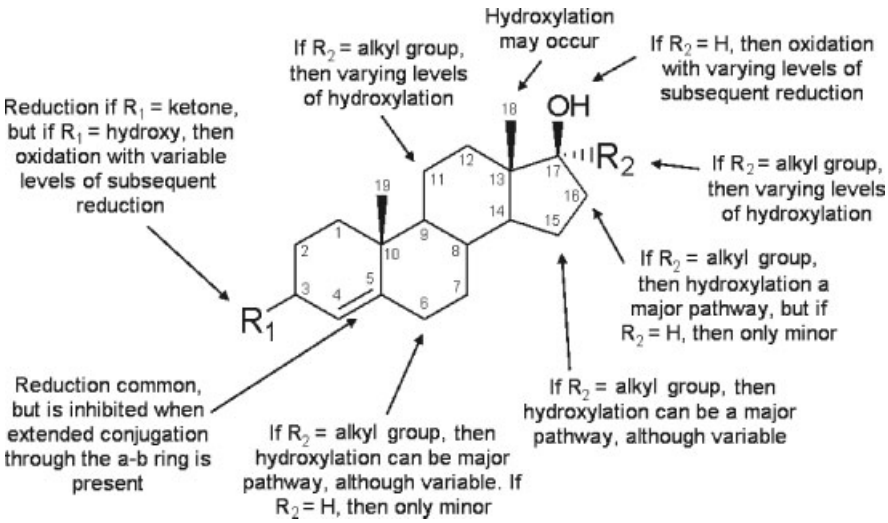

Figure 1. summary of some of the common pathways of AAS metabolism in the horse. See table 1 for a more extensive summary for individual steroids (including those with various substitutions not shown above).

Table 1A. Summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in the horse

Steroid	References	Phase 1 metabolites	Phase 2 metabolites	Comments
Boldenone	[252,253]	A range of metabolites result from modifications including oxidation and subsequent reduction at C17, reduction at Δ1 and Δ4 and hydroxylation at C6 and C16.	Boldenone predominantly as sulphate. Minor metabolites predominantly as glucuronides.	Boldenone and boldenone sulphate endogenous in intact males at low levels.
Clostebol	[254]	Major = 4-chloroandrost-4-ene-3α,17β-diol and 4-chloroandrostane-3α,17β-diol. Minor = various metabolites resulting from reduction at C3 and Δ4, oxidation at C17 and hydroxylation at C6.	The majority of metabolites were detected in the sulphate fraction.	
Danazol	[255]	Major = ethisterone, 6-hydroxyethisterone and 2-(hydroxymethyl)ethisterone. Minor metabolites include a number of other oxidised metabolites following isoxazole ring cleavage.	Mixture of free, glucuronide and sulphate	Cleavage of the isoxazole ring a major feature of danazol metabolism; something that is not observed for the pyrazole ring in stanozolol
Desoxyvinyltestosterone (DVT) (designer steroid analogues)	[46]	A mixture of metabolites resulting from the addition of a ketone or hydroxy at C2 and/or C3, reduction or shifting of the double bond within the A-ring and reduction and hydroxylation of the vinyl group	Predominantly in the glucuronide fraction.	A range of other analogues with various substitutions at C17 were also studied *in vitro*, but not *in vivo*
Estra-4,9-diene-3,17-dione (a designer steroid)	[23]	Major = reduction at C17. A range of minor metabolites result from modifications including reduction at C3 and hydroxylation at various sites, including C15/16.	Unknown (*in vitro* results only)	
Ethylestrenol	[256,257]	Mixture of metabolites including norethanodrolone and a range of 3-hydroxy-Δ4-reduced metabolites	Ethylestrenol in the free fraction, with the other metabolites predominantly in the glucuronide fraction, but with some sulphation	

Table 1B. Summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in horses

Steroid	References	Phase 1 metabolites	Phase 2 metabolites	Comments
Fluoxymesterone	[258,259]	Major = 9α-fluoro-11β-hydroxy-17,17-dimethyl-18-norandrosta-4-13-dien-3-one.	Predominantly in the free fraction for all metabolites, with a smaller proportion in the glucuronide fraction	Loss of the 17-hydroxy group with subsequent migration of the C18 methyl group to the C17-position is an interesting feature of fluoxymesterone metabolism.
		Minor = 17-epifluoxymesterone, 16- and 20-hydroxyfluoxymesterone		
Furazabol	[260]	Major = 16α-hydroxyfurazabol.	Data not available.	
Mestanolone	[261]	Forms the A-ring reduced metabolites of methyltestosterone.	Forms the A-ring reduced metabolites of methyltestosterone.	Mestanolone a metabolite of methyltestosterone and oxymetholone.
Mesterolone	[43]	Major = 1α-methyl, 3,16-dihydroxy-5α-androstan-17-one and 1α-methyl, 3,18-dihydroxy-5α-androstan-17-one.	Metabolites split between both the glucuronide and sulphate fractions, but the tentatively identified 17α-hydroxy isomers predominantly as glucuronides and 17β-hydroxy isomers predominantly as sulphates.	
		Minor = a range of metabolites resulting from reduction at C3, epimerisation at C17 and hydroxylation at C16.		
Methandienone	[262,263,264,265]	Major = 16-hydroxymethandieneone, Δ4-reduced + 6 and 16-hydroxylated as well as Δ4-reduced and 6,16-dihydroxylated methandienone.	16-hydroxymethandieneone predominantly as sulphate, epimethandienone predominantly as free and the remainder of metabolites split between the glucuronide and sulphate fractions.	Some sulphate conjugates unstable in urine, leading to 'apparent' unconjugated metabolites if storage conditions not adequate
		Minor = a number of metabolites, including epimethandienone, and a range of A-ring fully reduced metabolites (being the longest detected metabolites).		
Methandriol	[265,266]	As the A-ring reduced metabolites of methyltestosterone.	A mixture of glucuronide and sulphate for parent methandriol. As methyltestosterone for the A-ring reduced metabolites.	

of some β_2-agonists banned and the use of others controlled. Unlike some other drugs, many β_2-agonists can be delivered in inhaled formulations in order to act directly on the lungs and therefore reduce systemic side-effects.[71] Inhaled dosing may have consequences for the quantitative ratio of metabolites compared to other routes of administration. One of the most important implications as far as the racing analysis in concerned is that drug concentrations in biomatrices are often much lower after inhalations than systemic routes of administration, and hence more difficult to detect. With the widespread application of high sensitivity instrumentation, parent drug can be detected, for example, in plasma in many cases. However, at the time that many of the metabolism studies concerning β_2-agonists were conducted, it was not possible to detect parent drug in plasma and detection in urine was often the most practical option.

A β-hydroxyphenethylamine structure (or a derivative thereof) is a characteristic of the β_2-agonists and these drugs also contain various meta- or para-substitutions of the aromatic ring (Figure 2). A number of β_2-agonists have been studied in the equine and the major pathways of excretion apparently depend on the presence of free hydroxy groups attached to the phenyl ring that are available for direct conjugation. For compounds such as clenbuterol and mabuterol, which do not bear a hydroxy function on their phenyl rings, unconjugated parent drug appears to be the predominant urinary product, whereas the majority of the remaining β_2-agonists studied in the horse (all bearing hydroxy groups on their phenyl ring), phase 2 conjugates are the major urinary metabolites (Figure 2). Theoretically, the β-hydroxyphenethylamine hydroxy or amine functions could be conjugated to form O or N-glucuronides respectively. Indeed, O-glucuronides have been reported as minor metabolties of clenbuterol in several species.[72] Clenbuterol N-glucuronide has been reported as a minor metabolite in both dog and calf urine,[73,74] but a recent study has called these findings into question as the authors of the latter study were unable to form N-glucuronides using either chemical synthesis or *in vitro* techniques.[72] To date, β-hydroxyphenethylamine O or N-glucuronides of β_2-agonists in the equine have not been reported. However, the finding that clenbuterol and mabuterol appear to be excreted predominantly unconjugated in this species suggests that these are not major pathways.[71,75]

Table 1C. Summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in horses

Steroid	References	Phase 1 metabolites	Phase 2 metabolites	Comments
Methenolone	[41]	Mixture of epimethenolone, 17-keto-16-hydroxymethenolone, 16-hydroxymethenolone and 2,6-dihydroxy-methenolone (this metabolite was only observed *in vitro*).	Metabolites split between both the glucuronide and sulphate fractions.	
Methyltestosterone	[267,268,269]	Hydroxylation at C6, C15, C16 and C20, epimerization at C17 and reduction in the A-ring, leading to a range of metabolites, including mestanolone.	Predominantly as sulphates.	
Nandrolone	[270,271,272,273]	Major = 5α-estrane-3β,17α-diol.	5α-estrane-3β,17α-diol predominantly as glucuronide, while nandrolone predominantly as sulphate. Other metabolites split between both fractions.	Some C18 androgens endogenous in intact males and pregnant females. Now thought to be predominantly analytical artefacts caused by degradation of 19-oic acids.
		Minor = a number of metabolites, including epinandrolone, norepiandrosterone, 5α-estrane-3β,17β-diol and various C16-hydroxylated metabolites.		
Norethandrolone	[274]	Mixture of metabolites resulting from reduction of the A-ring and hydroxylation at C16, C20 + C21 (also forming a carboxylic acid at C21)	Carboxylic acids in free fraction. The remainder of metabolites in either the sulphate, glucuronide or a mixture of both fractions.	Norethandrolone also a metabolite of ethylestrenol
Normethandrolone (normethandrone)	[275]	Mixture of metabolites including, epinormethandrolone, A-ring reduced metabolites, 6- and 16-hydroxynormethandrolone and A-ring reduced 16-hydroxynor-methandrolone	C6- and C16-hydroxylated metabolites predominantly in the glucuronide fraction, with the remainder split between the glucuronide and sulphate fraction	71% of the overall radioactive dose excreted in glucuronide fraction
Oxymetholone	[266,276]	Mixture of mestanolone, C3-reduced mestanolone isomers, 2-hydroxymethyl-17α-methyl-5α-androstan-3,17β-diol and 2,17α-di(hydroxymethyl)-5α-androstan-3,17β-diol.	Metabolites split between both the glucuronide and sulphate fractions.	

Clenbuterol. Following oral administration, clenbuterol has been reported to be excreted into equine urine predominantly as unconjugated parent drug (no increase in concentration following enzyme hydrolysis).[71] A relative lack of phase 2 metabolism is not unexpected, since unlike many other β_2-agonists, clenbuterol does not bear a phenolic group to which conjugates could directly bind. Unchanged parent drug has also been detected in unhydrolyzed urine samples following inhaled administration of clenbuterol (no comparison with hydrolyzed urine samples available).[76] In a more recent study following intravenous and oral administrations to horses, urinary concentrations of clenbuterol were found to be 100-fold higher than those in plasma for both routes of administration.[77]

In a veterinary drug development study carried out by Boehringer Ingelheim,[78] the authors reported that although clenbuterol was the major metabolite found in equine urine following oral administration of the drug, a small quantity of 1-(4-amino-3,5-dichlorophenyl)-1,2-ethanediol was identified as a phase 1 metabolite. In another report from Boehringer Ingelheim, approximately 70–77% of an oral radio-labelled clenbuterol dose was recovered in the urine, while the majority of the remaining radioactivity was accounted for in the faeces.[79] Of the urinary radioactivity, parent clenbuterol accounted for 31–49%, 1-(4-amino-3,5-dichlorophenyl)-1,2-ethanediol for 0–11% and another metabolite, 1-(4-amino-3,5-dichlorophenyl)-2-(hydroxymethyl-*tert*-butylamino)-ethanol, accounted for 10–16%.

Isoproterenol. The metabolic fate of Isoproterenol (isoprenaline) has been studied after oral administration to the equine.[80] Parent drug could not be detected in unhydrolyzed plasma or urine, but was observed at trace levels in samples enzyme hydrolyzed with extracts of *Helix pomatia*. Relatively larger instrumental responses in hydrolyzed urine samples were obtained for an isoproterenol metabolite, with a confirmed structure of 3-methylisoproterenol. The formation of 3-methylisoproterenol is not unexpected in horses, since isoproterenol contains a catechol structure and other drugs bearing this structure (such as isoxsuprine) are known to be methylated in horses, most probably by orthologs of catechol-O-methyltransferase enzymes.[71,81] Also, since the *Helix pomatia* enzymes used for hydrolyzing samples in this study do not cleave methyl esters, the detection of both parent drug and metabolite in only the hydrolyzed samples suggests that both are conjugated with either glucuronic acid and/or sulfate.

Mabuterol. The metabolic fate of mabuterol has been studied after oral administration to the equine.[75] Parent drug was identified in unhydrolyzed urine, but not in plasma. No phase 1 metabolites were observed in either plasma or urine and

Table 1D. Summary of the phase 1 and 2 metabolism of the androgenic-anabolic steroids studied in horses

Steroid	References	Phase 1 metabolites	Phase 2 metabolites	Comments
6-Oxo (androst-4-ene-3,6,17-trione)	[277]	An extensive array of metabolites resulting from reduction at C3, Δ4, C6, and C17.	Some detected in both free and glucuronide fractions, while others in glucuronide only. Extent of sulphation not assessed due to the acid lability of the compounds.	
Stanozolol	[21,278,279]	Major = 15-, 16α- and 16β-hydroxystanozolol.	Stanozolol and 15-hydroxystanozolol predominantly as glucuronide, while 16α- and 16β-hydroxystanozolol predominantly as sulphates.	16α- and 16β-hydroxylation proposed to be mediated by an enzyme related to human CYP2C8s
		Minor = 16-ketostanozolol and 3′, 4α, 4β and 6α-hydroxystanozolol.		
Testosterone	[280,281]	Major = 5α-androstrane-3β,17α-diol.	5α-androstrane-3β,17α-diol predominantly as glucuronide, while testosterone predominantly as sulphate. Other metabolites split between both fractions.	Testosterone endogenous in intact males, geldings and females.
		Minor = a number of metabolites, including epitestosterone, epiandrosterone, 5α-androstrane-3β,17β-diol and various C16-hydroxylated metabolites.		
Testosterone precursors (DHEA or androst-4-ene-3,17-dione)	[282]	Increased excretion of testosterone metabolites for both DHEA and androst-4-ene-3,17-dione administration. DHEA also increased levels of androst-5-ene-3,17-diols.	Authors report concentrations as 'total' only, but do state that urinary DHEA sulphate 'attained high levels after DHEA administration.'	
Trenbolone	[30,283]	Major = epitrenbolone.	Mixture of glucuronide and sulphate.	A-ring metabolism inhibited by the extended A-B-C-ring conjugation.
		Minor = various C16-hydroxylated metabolites.		
Turinabol	[42]	Major = epiturinabol, 20-hydroxyturinabol, 6β,20-dihydroxyturinabol and a range of A-ring reduced metabolites.	Excreted as a mixture of sulphated and free steroids	
		Minor (*in vitro* only) = 6βhydroxy and 6β, 16-dihydroxyturinabol.		

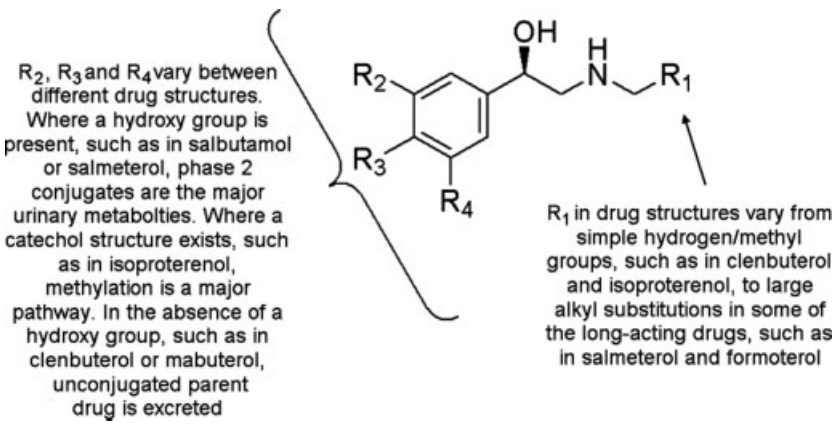

Figure 2. summary of some of the structures and related routes of metabolism of $\beta_2$agonists in the horse. The structure presented in generic for most, but not all, $\beta_2$agonists. See text for details of individual drugs.

comparison of enzyme hydrolyzed with unhydrolyzed urine samples suggested that mabuterol was present in the 'free' form. As with clenbuterol, this lack of phase 2 metabolism is not unexpected, since mabuterol does not bear a phenolic hydroxy to which the conjugating group could directly attach onto.

Ractopamine. The urinary excretion of ractopamine and its metabolites has been studied after oral administration to horses.[82] Although the reported results were not quantitative, phase 2 pathways appeared extensive, with glucuronide, methyl, mixed methyl-glucuronide, sulfate and mixed methyl sulfate metabolites all directly identified in urine. No phase 1 metabolites were reported. These results are consistent with those in sheep and cattle, where the vast majority of ractopamine was excreted in the conjugated form and enzyme hydrolysis with extracts from *Patella vulgata* (possessing both glucuronidase and aryl sulfatase activity) was necessary to detect the drug's administration effectively.[83] The authors also discussed the possibility that two further metabolites might relate to amino acid or creatine conjugates of ractopamine, but the exact identity of these compounds remained uncertain. Also, the observation of doublet metabolite peaks of similar intensity for each compound in the GC-MS chromatograms of TMS derivatized hydrolyzed urine sample extracts suggested that no stereoselective metabolism had occurred and that the original ratio of diastereomers observed in the pharmaceutical preparation had remained intact.

Salbutamol. The metabolic fate of salbutamol (albuterol) has been reported following both oral and inhaled administration to horses.[71] Parent drug could not be detected in unhydrolyzed plasma or urine following either administration, but it was identified in samples enzyme hydrolyzed with extracts of *Helix pomatia* in both cases. In samples only from the oral administration, a carboxylic acid metabolite (resulting from oxidation of the hydroxymethyl group attached to the phenyl ring) was identified in hydrolyzed urine samples. Although this metabolite was not formally quantified, it was detected for only one-third of the time compared to the parent drug, suggesting (although not proving) that it was less abundant. The detection of this metabolite only from the oral administration (at a 33.3% lower dose than the inhaled administration) is suggestive of 1st pass metabolism in the gut, but further experiments would be required to clarify this. Overall therefore, phase 2 conjugation appears to be the major pathway of salbutamol excretion in the horse, although the exact nature of the conjugate/s for both the parent drug and the carboxy metabolite remains undetermined.

Salmeterol. Following inhaled administration, salmeterol has been reported to be excreted into equine urine predominantly conjugated (no free drug was detected).[84] The exact nature of the conjugate/s remains undetermined.

Terbutaline. The metabolic fate of terbutaline has been reported following both oral and inhaled administration to the equine.[71] Parent drug could not be detected in unhydrolyzed plasma following either administration, but it was identified in both hydrolyzed and unhydrolyzed urine samples in both cases. Comparison of the instrumental responses for terbutaline from enzyme hydrolyzed and unhydrolyzed urine samples showed that for both routes of delivery, conjugates accounted for around 90% of the excreted dose. The exact nature of the conjugate/s remains undetermined.

Stimulants

The drugs categorized as class 1 agents by the ARCI are considered as having no medical use in racing horses but high risk of abuse due to their pharmacologic potential for altering the performance, and approximately 30% of these compounds are stimulants.[2] Stimulants also form the largest individual group of the list of prohibited substances of the FEI.[4] Although any drug enhancing any physiological function can be considered as 'stimulant', in performance sport the enhancing properties of stimulants are based on the ergogenic effect of these compounds, i.e. the administration of stimulants and their effect on central nervous system increases the psychomotoric activity and eliminates the sense of fatigue, or enhances, for example, cardiovascular or muscular function by affecting the sympathetic nervous system.[85]

From a chemical point of view, the group of stimulants is heterogeneous, although the majority of compounds are structurally related to amphetamine, i.e. nitrogen-containing small molecules which mimic the structures of endogenous neurotransmitters such as dopamine or noradrenaline. For centrally acting stimulants there are several suggested modes of action,[86] from which the most typical are (1) inhibition of transmitter reuptake; (2) enhanced release of these transmitters; or (3) inhibition of the degradative enzyme (e.g. monoamine oxidase, MAO) to enhance and prolong the effect of the neurotransmitter in the synaptic area. Agonistic properties which mimic the action of neurotransmitter at adrenergic receptor have not been systematically investigated but proposed at least for amphetamine,[86] benzylpiperazine[87] and methylenedioxymethamphetamine.[88] In most cases, nevertheless, the stimulating effect is mediated via several overlapping pathways.

With respect to morphine-like opiates, the therapeutic use of which is narcotic analgesia in human, horses respond differently as the effect mediated by μ-subtype of opiate receptors is stimulation. This section focuses mainly on the metabolism of amphetamine-like stimulants and methylxanthines, whereas the group of narcotic analgesics is discussed in detail in the section on opioid analgesics.

Phenylalkylamine structured stimulants

Amphetamine, methamphetamine, ephedrine, and methylephedrine. Several reports on the metabolism of amphetamine-like compounds have been published in human or various animal species and despite of the small size of the molecules, there are several functionalities available for metabolic reactions. These modifications may involve both the aromatic ring and the propyl side-chain and include, for example, (1) aromatic hydroxylation (typically in the 4′ position); (2) aliphatic hydroxylation; (3) N-dealkylation; and (4) oxidative deamination.

The available excretion studies in horse cover, for example, intravenous and intramuscular administration routes, and also studies with ^{14}C labelled compounds. Following an intravenous administration of ^{14}C amphetamine about 5% of the dose was found unchanged in urine, and 7% as 4′-hydroxyamphetamine. The other prevailing metabolic routes included N-dealkylation and concurrent aliphatic hydroxylation resulting in the formation of 1-phenylpropan-2-one (29%). Other observed metabolites of amphetamine were hippuric acid (19%), 1-phenylpropan-2-ol (12%) and glucuronide-conjugated benzoic acid (12%).

Approximately 84% of the administered dose was excreted within 28 h post-administration.[89]

Several routes have been reported in the metabolism of methamphetamine in man, namely aromatic or/and aliphatic hydroxylation in combination or without N-demethylation.[90] However, in an intramuscular excretion study of methamphetamine (200 mg) only the parent compound was detected in horse urine up to 24 h, but the concentration of the suggested N-demethylated metabolite (amphetamine) was too low for the GC-MS detection. In the same study this reaction was, however, observed for ephedrine (i.m. 600 mg) resulting in formation of norephedrine, and for methylephedrine (i.m. 600 mg) resulting in the formation of both ephedrine and norephedrine, but in most cases the concentration of the most abundant urinary compound was the parent drug.[91] In another excretion study, carried out with ephedrine, 1,2-dihydroxy-1-phenylpropane (following by oxidative deamination) was observed in ponies.[92]

Benzylpiperazine. The mode of action of benzylpiperazine is based on the reuptake inhibition of noradrenaline, dopamine and especially that of serotonin.[87] The metabolism of N-benzylpiperazine in horse is not described, but the studies in rats and humans have shown that this compound is excreted mainly as unchanged parent compound in urine. The main metabolic routes target the aromatic ring (mono- or dihydroxylation), benzyl carbon (N-dealkylation) and piperazine hetero-ring (double N-dealkylation). Hydroxylated metabolites were recovered also from the conjugated fraction, but as a mixture of β-glucuronidase and aryl-sulfatase enzymes was applied, the formation of either glucuronide- or sulfated conjugates could not be confirmed.[93]

Fencamfamine is a dopamine uptake inhibitor[94] and an excellent example of metabolic differences between species. According to an excretion study in horses, no parent compound was observed in urine following oral administration of fencamfamine (100 mg). The main human metabolite, N-desethylfencamfamine (2-amino-3-phenylnorbornane), represented only 1% of the total dose and the main equine metabolites were parahydroxylated metabolites of fencamfamine. It was also observed that the pH of urine had an effect on the excretion of the parent compound and N-desethylated metabolite.[95,96]

Mephentermine. Clinical use of mephentermine relies on its potency to prevent post-spinal hypotension.[97] There is a lack of *in vivo* studies on the metabolism of mephentermine in horse, but *in vitro* assays with rat liver enzymes have revealed the pathways of N-demethylation and *p*-hydroxylation as potential ones, producing metabolites with these phase-1 modifications.[98] In human the main metabolite is phentermine, resulting from N-demethylation.[99]

Methylenedioxymethamphetamine and methylenedioxyamphetamine. From the beginning of 1980s 3,4-methylenedioxymethamphetamine (MDMA, Ecstacy) has been available on black market as a recreational drug.[100] There are difference in metabolic fate of MDMA between animal species, but in horse MDMA has been reported to be metabolized after oral administration by N-demethylation to 3,4-methylenedioxyamphetamine (MDA), followed by oxidative demethylenation of both MDMA and MDA, 3-*O*-methylation of the corresponding cathecols and finally N-oxidation of the amine groups to form the hydroxylamines. For the phenolic and N-hydroxy metabolites also phase 2 metabolic reactions have been reported.[101,102] The involvement of cytochrome P450 isoenzymes in phase 1 metabolism of MDMA has been studied using human isoenzymes and according to these studies the highest contribution in demethylenation was obtained for CYP2D6 and in N-demethylation for CYP1A2 and CYP2B6, and interestingly, enantioselective metabolism was suggested for CYP2C19 and CYP2D6.[103]

Methylphenidate. Therapeutic use of methylphenidate is aimed at treating attention-deficit hyperactivity disorder (ADHD) in humans owing to the release and reuptake of dopamine in the striatum.[104] In horses, a short detection time (less than 6 h) has been observed for methylphenidate in blood following either IV or IM administration, whereas the detection of parent compound was possible between 12–24 h in urine.[105] Although data on the *in vivo* metabolism of methylphenidate in horses is lacking, *in vitro* studies using equine plasma have shown that the drug is rapidly dealkyated to ritalinic acid.[27] In the same study, a number of hydroxymethylphenidate and hydroxyritalinic acid metabolites were also identified when liver S9 or microsomes were used.

Modafinil possesses wake-promoting properties and its clinical use in human is aimed at the treatment of daytime sleepiness and in human less than 10% of the dose is excreted in urine as unchanged. The metabolic pathway is mainly amide hydrolysis, resulting into formation of modafinil acid and the contribution of cytochrome P450 oxidative reactions is not significant.[106] The identical behaviour was also observed in horse, where modafinil acid was the main component and detected for four days post-administration whereas parent compound only for 24 h after oral administration.[107]

Phendimetrazine and phenmetrazine. Usually the metabolic reactions terminate the pharmacologic activity of drugs, but an example of an active metabolite is described for phendimetrazine, an anorectic agent the therapeutic use of which is as an appetite suppressant. By N-demethylation phendimetrazine is metabolized to phenmetrazine and these compounds are potent substrates for noradrenaline and dopamine transporters.[108] The suggested metabolic reactions have been published only as the results from the excretion studies in man where a further modification by N-hydroxylation has also been suggested for both phendimetrazine and phenmetrazine.[109]

Prolintane is a centrally acting stimulant and the therapeutic use is to stimulate appetite. The metabolism of prolintane has been reported in greyhounds [110] and horses[111] and differences in metabolic routes between these two species has been highlighted. In horses, a 150 mg single oral dose of prolintane was completely metabolized. The main urinary metabolite was 4-hydroxyprolintane, which was modified further to 3,4-dihydroxyprolintane and 3-methoxy-4-hydroxyprolintane (minor metabolite). The high aromatic hydroxylase activity of horse was shown also with prolintane, as one additional hydroxylated metabolite was observed, although the precise location of this alternative hydroxylation was not revealed. Formation of lactam metabolite has been reported as major metabolite in greyhound and man,[112] but was exceptionally not excreted by horse. β-glucuronidase enzyme of H. Pomatia was applied in the sample preparation and the prolintane metabolites were

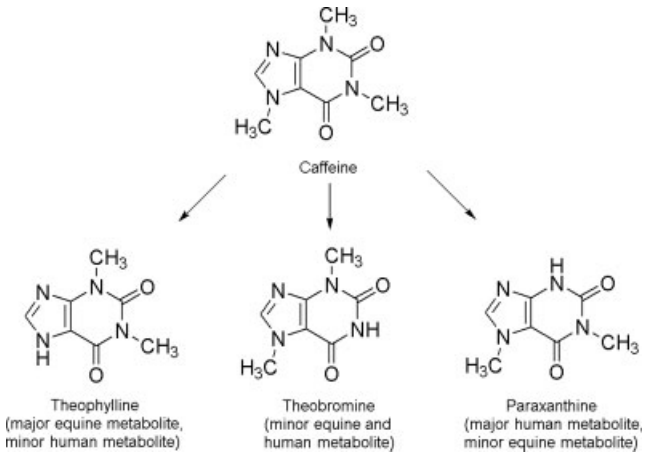

Figure 3. summary of caffeine metabolism in the equine and human.

almost completely further conjugated as their glucuronides or sulfates.

Selegiline. In human medicine selegiline is used in the treatment of Parkinson's disease as monotherapy or in combination with levodopa owing to its properties as MAO-B inhibitor as well as reuptake inhibitor of dopamine and noradrenaline.[113] In an excretion study (40–50 mg, oral dose) the parent selegiline was observed only in low concentration, whereas main urinary metabolite was identified as N-desmethylselegiline. N-dealkylation was also observed for the complete propynyl group, resulting into low concentrations of methamphetamine and also of amphetamine via further N-demethylation.[114]

Methylxanthines

Caffeine. A variety of pharmacological effects has been suggested to stimulating effect of caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione), but the most potential one is the adenosine receptor antagonism.[115] The reported urinary half-life for caffeine in horses varies by author, but is typically at around 12 h.[116] The major metabolic pathway of caffeine in horses is demethylation and the major urinary dimethylxanthine (i.e. mono-desmethyl) metabolite is theophylline with smaller amounts of theobromine, paraxanthine and parent caffeine excreted, all in the unconjugated form. The uric acid derivatives of some of the dimethylxanthines have also been detected in horses.[116] Figure 3 summarizes the major catabolic pathways for caffeine in horses and compares the metabolite profile to that in humans.

In most cases, the stimulants and performance-enhancing compounds are of exogenous origin and the analytical approach is qualitative, i.e. targeted on the reliable identification of the prohibited substances. The case with the methylxanthines, however, is an exception as some of them are natural plant constituents. Theobromine is a particular problem as it can be present at relatively high concentrations in cocoa husks that may be constituents of feed products consumed by horses.[117] The excretion of theobromine after feeding has been carefully evaluated and based on these studies a quantitative urinary threshold of 2 μg/ml has been established.[117]

Micellaneous stimulants

Nikethamide. In certain cases the abused drugs may be metabolized to physiological compounds, which makes the detection window of the potential abuse narrow and complicates the interpretation of analytical data. This is the case with nikethamide (N,N-diethylnicotinamide), which as an analeptic agent has a stimulating effect on respiratory function[118] and which is metabolized very rapidly to nicotinamide. In an excretion study in horse, and intermediate, N-ethylnicotinamide, was observed as a urinary marker for nikethamide, although the detection time after IM administration was still only within hours.[119]

Strychnine is a plant alkaloid, a competitive antagonist of glycin receptor, and a convulsant with respect to its action and the therapeutic use in human focuses on the treatment of sleep apnea. The studies on the *in vivo* equine metabolism of strychnine are not reported, but based on *in vitro* models applying animal liver preparations (S9 and microsomal enzyme fractions) the metabolism of strychnine is suggested to proceed mainly via CYP450-mediated routes.[120] A wide spectrum of metabolites has been identified and significant differences have been observed in metabolic profiles between various animal species. In these assays the main metabolites were identified as strychnine-N-oxide (main metabolite in rabbit and dog), 2-hydroxystrychnine (in rabbits and guinea pigs), and 16-hydroxystrychnine (in rats and mice) together with minor fractions of 21,22-dihydroxy-22-hydrostrychnine, strychnine 21,22-epoxide, and 18-oxostrychnine.[120,121]

Sedatives and tranquilizers

Aside from the AAS and NSAIDs, sedatives and tranquilizers are the most widely metabolically studied classes of drugs appropriate to equine drug testing. This is partly due to their wide range of structures and modes of action, but also because of their importance as therapeutic agents and the need to control their abuse in competitive horseracing. Sedatives/tranquilizers clearly have the ability to affect performance, but may also be legitimately used in a veterinary context outside of competition. Therefore, some drug classes are banned by the FEI (i.e. the barbiturates and reserpine), some drugs are controlled (such as acepromazine, detomidine, and ketamine) and others have members that fall into both banned and controlled categories depending on the individual drug (i.e. the benzodiazepines).[4] Similarly, the ARCI classifications are a mixture of categories, with the majority falling into either category 2 (i.e. the barbiturates, benzodiazepines, ketamine and reserpine) or category 3 (i.e. acepromazine and detomidine).[2]

Due to the enormity of the literature, the following section serves as a summary of the metabolism of a representative selection of these drugs from some of the different chemical classes and modes of action on which these compounds are based.

α_2-agonists

Detomidine. The metabolism of the α_2-agonist sedative detomidine has been reported by several authors.[34,122–124] In one early study, the major urinary metabolite detected following a radio-labelled dose of the drug was 3-carboxydetomidine, which accounted for 27% of the total dose.[34] No parent drug was detected, and a minor metabolite was found to be the glucuronide conjugate of 3-hydroxydetomidine, accounting for 6%

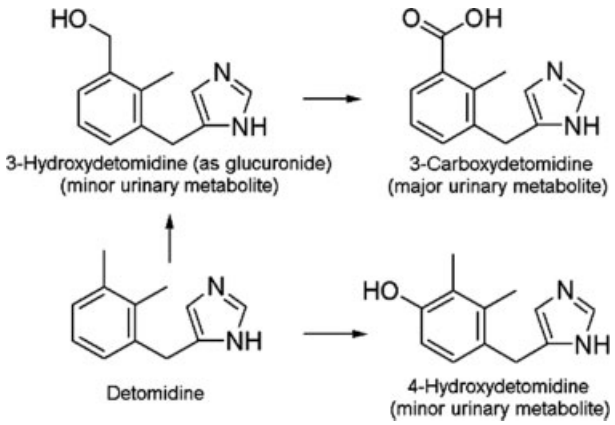

Figure 4. summary of the metabolism of detomidine in the horse.

of the total dose. In a later study, which also involved administering radio-labelled detomidine, approximately 50% of the total radioactivity was excreted into urine over the first 12 h. Of the urinary metabolites, 3-carboxydetomidine accounted for over two-thirds of the radioactivity, 3-hydroxydetomidine glucuronide accounted for 10 to 20%; and free 3-hyroxydetomidine was present at only trace levels.[122] In a third study, 4-hydroxydetomidine was also identified as a minor metabolite in enzyme-hydrolyzed urine samples.[123] Most recently, the equine plasma pharmacokinetics of detomidine and 3-carboxydetomidine have been reported.[124] Compared to 3-carboxydetomidine peak plasma concentrations of parent drug following IM and IV administration of detomidine were approximately ten- and a hundred-fold higher, respectively, but the excretion profile of the metabolite was shallower than that of parent drug. The metabolic fate of detomidine in the horse is summarized in Figure 4.

Xylazine. Following its administration, xylazine is reported to be extensively metabolized in the horse.[125] Urine samples from two separate horses were hydrolyzed with enzyme preparations of *Helix pomatia*, extracted, and analyzed, which allowed detection of parent xylazine for only a short period of time after administration. However, much greater instrumental responses were achieved for seven different xylazine metabolites, which allowed detection for an extended period of time. 4-hydroxyxylazine produced the greatest response in both animals, with six further metabolites identified as 3′-hydroxyxylazine, 4′-hydroxyxylazine, *N*-(2,6-diemethylphenyl)thiourea, 4-ketoxylazine, 2,6-dimethylaniline and 5,6-dehydro-4-ketoxylazine. Mutlib *et al*.[126] also identified several of the equine urinary metabolites reported in the aforementioned study,[125] but also determined through comparison of β-glucuronidase hydrolyzed and unhydrolyzed sample extracts that 3′- and 4′-hydroxyxylazine were glucuronide conjugated and that *N*-(2,6-diemethylphenyl)thiourea was unconjugated. In another study following administration of xylazine, the parent compound could be detected in plasma, but no metabolites were detected.[127] In urine, however, an additional major metabolite was tentatively identified in both the hydrolyzed and unhydrolyzed samples as 4′-hydroxy-4-ketoxylazine. The metabolic fate of xylazine in the horse is summarized in Figure 5.

Antihistamines

Chlorpheniramine. There are two major sub-types of the histamine receptor; drugs that antagonize predominantly the H_1 receptor are primarily used as anti-allergy drugs while those antagonizing predominantly the H_2 receptor are used as anti-ulceratives. In addition to their effects on the body's response to allergic reactions, many of the H_1 receptor antagonists, such as chlorpheniramine and tripelennamine, also produce varying degrees of sedation and/or drowsiness. Following its administration to horses, the metabolic profile of chlorpheniramine has been studied in urine samples that were hydrolyzed with *Helix pomatia* prior to extraction and analysis.[128] Parent chlorpheniramine was detected in urine, but it produced instrumental responses much lower than that of four different metabolites. In the early time-points following administration of the drug, N-desmethylchlorpheniramine produced the greatest response, while in the later stages di-desmethylchlorpheniramine, hydroxychlorpheniramine and N-desmethylhydroxychlorpheniramine were more significant.

Tripelennamine. The urinary metabolic profile following administration of the H_1 receptor antagonist tripelennamine to horses has been studied in urine samples that were hydrolyzed with extracts of *Helix pomatia* prior to extraction and analysis.[128] Tripelennamine was detected at very low concentrations initially following its administration, but analytical response of its metabolites were far in excess of the parent drug. The major metabolite detected at all time-points was a hydroxytripelennamine, with the site of oxidation proposed to occur on the pyridine ring. Three other minor metabolites also resulting from pyridine ring hydroxylation were N-desmethylhydroxytripelennamine, N-didesmethylhydroxytripelennamine and N-des(dimethylaminoethyl)hydroxytripelennamine. Two further minor metabolites were identified as N-despyridyltripelennamine and N-desmethyltripelennamine. In a separate equine study, three of the pyridine ring hydroxylation metabolites identified in the former study, namely hydroxytripelennamine, N-desmethylhydroxytripelennamine and N-des(dimethylaminoethyl)hydroxytripelennamine, were identified as glucuronide conjugates, while N-despyridyltripelennamine, N-desmethyltripelennamine and tripelennamine-N-oxide were excreted in the free fraction.[251] In a more recent study, a di-oxygenated metabolite of tripelennamine, proposed to be a pyridine-hydroxy and ethylamine-N-oxide, was identified in both urinary extracts and following *in vitro* incubation with liver S9.[27]

Barbiturates

Barbiturates produce their sedative effect through agonism of the GABA receptors and are also know to be potent hepatic enzyme

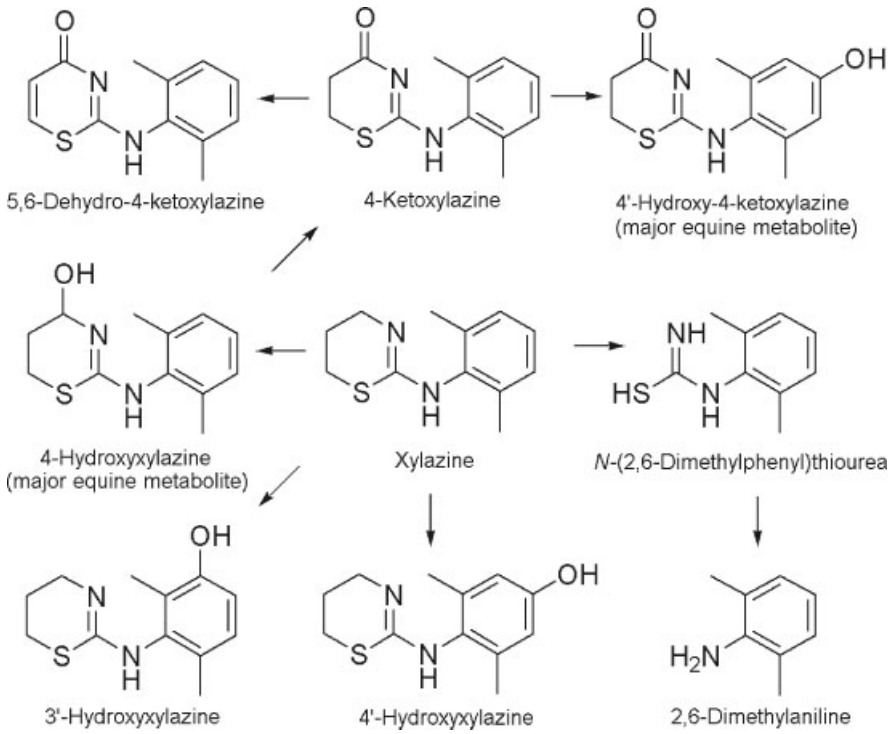

Figure 5. summary of the metabolism of xylazine in the horse.

inducers.[129] Relatively little published data exists on the equine metabolism of the barbiturates, but the available literature for those drugs that have been studied suggests that metabolism is extensive. In an early study of hepatic drug metabolizing capacity in the horse, the rate of hexobarbital oxidation, as measured by its disappearance in an *in vitro* liver model, was significantly more rapid in females compared to males (1.41 and 0.93 µmol/g liver respectively), but no metabolite characterization was performed.[130] Following the administration of phenobarbital to the horse, parent drug has been detected in the free fraction of urine, but the major urinary metabolite was a glucuronide conjugate of para-hydroxyphenobarbital.[131] In comparison, pentobarbital was reported to be so extensively metabolized that none of the parent drug could be detected in urine. Instead, two metabolites resulting from successive oxidation of the butyl side-chain were isolated from the urinary free fraction; namely 5-ethyl-5-(3-hydroxy-1-methylbutyl)-barbituric acid and 5-ethyl-5-(1-methyl-3-carboxypropyl)-barbituric acid.[131] Consistent with these findings, it has been reported that while pentobarbital is cleared at a rate of 4%/hour in humans, the corresponding figure in horses is 50%/hour.[132] An isomer of hydroxysecobarbital has been reported to be the major metabolite of secobarbital in the horse, but little further information was reported.[133]

Benzodiazepines

Diazepam. Diazepam is a member of the benzodiazepine classes of drugs, which produce their tranquilizing and sedating effects by agonizing the GABA receptor. Following its administration, parent diazepam and the demethylated metabolite nordiazepam have been detected in equine plasma samples hydrolyzed with extracts of *Helix pomatia*.[134] Although the concentrations of parent diazepam in plasma were initially higher than nordiazepam, the excretion profile for the metabolite was much slower and hence it could be detected for a longer period of time. No other metabolites were detected in plasma. In urine, concentrations of parent diazepam in hydrolyzed and unhydrolyzed samples were much lower than its metabolites. Three major urinary metabolites of diazepam were identified as nordiazepam (mainly as a conjugate), temazepam (solely as a conjugate), and oxazepam (solely as a conjugate). Concentrations of nordiazepam, oxazepam, and temazepam were initially of a similar order of magnitude, but the excretion profile for oxazepam was much slower and hence it could detected for a longer period of time. It is worth noting that there are several other benzodiazepine drugs with structures similar to diazepam and which lead to similar metabolite profiles compared to diazepam. For example, temazepam and oxazepam are used also as therapeutic drugs, while prazepam is metabolized to nordiazepam. Sample pretreatment with hot acid conditions causes the diazepine ring to open, forming the corresponding aminobenzophenones.[135] This needs to be borne in mind when designing sample preparation protocols, although it can be an advantage in certain situations since it can intensify the analytical signal through the conversion of several different metabolites into the same aminobenzophenone.[135] The metabolic fate of diazepam in the equine is summarized in Figure 6.

Lorazepam. While information regarding the phase 1 metabolism of lorazepam in the equine is lacking, the drug is known to undergo significant glucuronide conjugation.[136] The concentrations of lorazepam glucuronide in the plasma, urine and bile and the degree of the enterohepatic circulation of the conjugate have been studied in ponies.[136] In plasma, following a short initial period after IV administration where the unconjugated parent drug was more concentrated than the glucuronide metabolite, the conjugate was approximately twice as abundant as the parent drug. Excreted quantities of the parent drug were negligible, but significant amounts of the

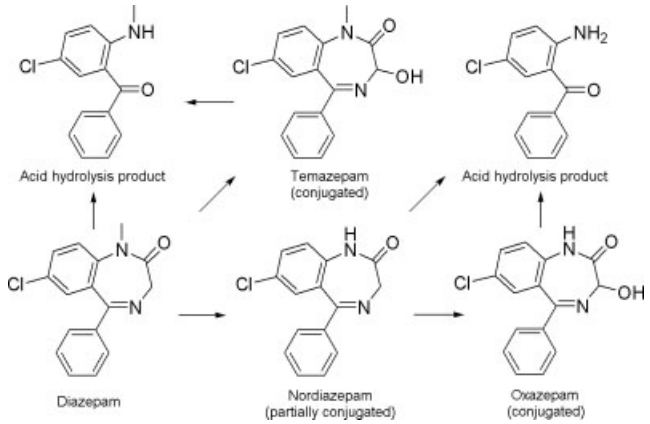

Figure 6. summary of the metabolism of diazepam in the horse.

glucuronide conjugate were excreted into both urine and bile. When the enterohepatic circulation was allowed to remain intact, an average of 41% of the administered dose was recovered in urine as the glucuronide, but as soon as the enterohepatic circulation was interrupted and bile collected, only 36% of the dose was recovered in urine and an average of 24.5% of the dose was recovered in bile as the glucuronide. Following reintroduction of the collected bile back into the digestive tract of the animal, 20% of the lorazepam glucuronide contained in this 'dose' was then recovered in urine. Additionally, the plasma half-life of lorazepam was significantly shorter when the enterohepatic circulation was interrupted (2.3 versus 3.4 h), again consistent with a significant degree of enterohepatic circulation of the drug.

Phenothiazines

Acepromazine. The phenothiazines are characterized by the presence of two benzene rings linked by nitrogen and sulfur. The different phenothiazine-based drugs contain various structural modifications and are used as both antipsychotics (such as chlorpromazine) and antihistamines (such as promethazine). Like other drugs in this class, acepromazine possesses sedative properties. In an early study of its metabolism in the horse (not quantitative), urinary free fraction metabolites were reported to be 2-(1-hydroxyethyl)promazine and 2-(1-hydroxyethyl)promazine sulfoxide while urinary conjugated fraction metabolites (conjugate status unknown) were 7-hydroxyacetylpromazine and 2-(1-hydroxyethyl)-7-hydroxypromazine.[137] In a more recent study, the major urinary metabolites detected from the total (combined free and conjugated) urinary fraction were reported to be two separate isomers of oxygenated 2-(1-hydroxyethyl)promazine.[27] While positional information regarding the sites of oxygenation in these two metabolites (including whether they were located on a nitrogen, sulfur, or carbon) were not determined, the masses of these two metabolites were consistent with them relating to the 2-(1-hydroxyethyl)promazine sulfoxide and 2-(1-hydroxyethyl)-7-hydroxypromazine structures reported previously.[137] A number of less abundant metabolites were also identified in this later study, including parent acepromazine, 2-(1-hydroxyethyl)promazine, oxygenated-acepromazine, dioxygenated-acepromazine, dioxygenated-2-(1-hydroxyethyl)promazine, N-desmethyl-2-(1-hydroxyethyl)promazine, N-desmethyl-oxygenated-2-(1-hydroxy-ethyl) promazine and N-desmethylacepromazine.[27]

Propionylpromazine. In propionylpromazine the acetyl group of acepromazine is replaced by a propionyl group. Its metabolism has been studied by several authors; all of which have reported major differences in the excreted metabolite profile.[138,139] In an early study utilizing enzyme hydrolyzed of urine samples with extracts of *Helix pomatia*, parent propionylpromazine could be detected following propionylpromazine administration.[138] However, the metabolite producing the greatest response was 2-(1-hydroxypropyl)promazine sulfoxide, with 2-(1-hydroxypropyl)promazine further identified as a minor metabolite. In a subsequent study, propionylpromazine could be detected in both unhydrolyzed plasma/urine and urine hydrolyzed with extracts of *Helix pomatia* following administration of the drug.[139] Again, however, instrumental responses for propionylpromazine in the urine samples were lower than those of its metabolites, identified as 2-(1-propenyl)promazine (in unhydrolyzed urine samples), 2-(1-hydroxypropyl)promazine and 7-hydroxypropionylpromazine (both in hydrolyzed urine samples only). In contrast to the earlier study,[138] 2-(1-hydroxypropyl)promazine sulfoxide was not detected. Previously unpublished data from our laboratory suggests that there is significant variation in the observed urinary metabolite profile between samples that have been found to be positive for propionylpromazine during doping control screens. Further, carefully designed, experiments are required in order to ascertain whether the reported differences between these studies are due to sample preparation, instrumental conditions, dosing regimens, time of sample collection or inter-animal variation.

Tricyclic antidepressants

Amitriptyline. Like other tricyclic antidepressants, amitriptyline acts on multiple neurochemical targets and exerts a tranquilizing effect as part of its pharmacological action.[140] Following its administration to the horse, trace concentrations of parent amitriptyline and the metabolite nortriptyline have been detected in the urinary free fraction.[140] In the conjugated (acid hydrolyzed) urinary fraction, a large number of peaks were identified as potential metabolites (with instrumental responses much greater than in the free fraction indicating extensive phase 1 and 2 metabolism). However, because of the relatively uncharacteristic fragmentation of these compounds (leading to large *m/z* 44 and 58 peaks and relatively few large mass ions under the EI-GCMS conditions used) it was not possible to assign structures to all the metabolites. Of the observed metabolites in the conjugated urinary fraction, the presence of a derivative of nortriptyline and two hydroxylated amitriptyline metabolites (one phenolic and one aliphatic) were postulated based on their mass spectra. No amitriptyline-related compounds were detected in plasma and the authors suggested that this might have been due to extensive protein binding or distribution to erythrocytes. A subsequent qualitative metabolism study in the horse, reported the presence of an additional metabolite, whose mass spectrum was consistent with the structure of a dihydroxynortriptyline isomer.[141] Soft ionization techniques coupled to MS/MS could assist in the full characterization of the metabolic pathway of amitriptyline.

Doxepin. The excretion of parent doxepin has been reported following its administration to the equine (representing a 5 : 1 mixture of its *trans* and *cis* configurations).[142] While both the serum and urinary *cis* and *trans* isomer ratios were observed to remain unchanged throughout the excretion profile, serum concentrations of the parent drug were found to be significantly

greater than urinary concentrations throughout. In a subsequent study by the same authors, the presence of several doxepin metabolite isomers in serum and urine was reported.[143] In the serum-free fraction, the only metabolite detected was desmethyldoxepin, with a *cis : trans* ratio approximating that of the parent drug. Although the study was not quantitative, several urinary metabolites were also detected, producing instrumental responses greater than that of the parent drug. In the urinary-free fraction, both *cis* and *trans* isomers of desmethyldoxepin were also detected. However, the *cis : trans* ratio was observed to deviate four-fold in the favour of the *trans* form compared to serum. These results contrast to those in the human where the *cis* form of desmethyldoxepin predominates in both plasma and urine.[144] In the enzyme hydrolyzed urinary fraction, four abundant peaks corresponding to hydroxydoxepin and four less-abundant peaks corresponding to hydroxydesmethyldoxepin were detected, but in both cases the exact positions of hydroxylation were not confirmed. The *cis* and *trans* identities of these hydroxydoxepin and hydroxydesmethyl isomers were not established, so it is unclear whether the four peaks observed for each metabolite relate to both *cis* and *trans* isomers or to different positions of hydroxylation from a single *cis* or *trans* isomer. In this respect, it is interesting to observe that in a human study, only *trans*-2-hydroxydoxepin and *trans*-2-hydroxydesmethyldoxepin were detected in urine, with the *cis* forms not observed.[144]

Others

Azaperone. Azaperone is a pyridinylpiperazine and butyrophenone neuroleptic drug with dopamine, histamine, and acetylcholine antagonistic effects and is used in veterinary medicine as a sedative and tranquilizer. Its metabolism has been studied in the horse by several authors.[27,145,146] In an early study that employed enzyme hydrolysis with β-glucuronidase/sulfatase from limpets (*Patella vulgata*), 5′-hydroxyazaperol and 5′-hydroxyazaperone were identified in urinary extracts following azaperone administration.[146] In a separate study, fractionation was used to identify conjugated and unconjugated metabolites in urine following acid hydrolysis as well as the direct detection of some of the intact conjugates.[145] In this study, trace responses were obtained in unhydrolyzed urine samples for the parent drug as well as two N-dealkylated metabolites identified as N-despyridinylazaperol and N-despyridinylazaperone. However, greater instrumental responses were obtained from the hydrolyzed urinary fraction where azaperol, hydroxyazaperol, two hydroxyazaperones, N-despyridinylazaperol and N-despyridinylazaperone were all identified. The direct detection of the glucuronide conjugates of azaperol, hydroxyazaperol and hydroxyazaperone was also achieved. In a more recent study, azaperone itself was not observed in extracts from a total urinary fraction, but 19 distinct metabolites were identified.[27] Although detailed characterization of the individual metabolites was not performed, the following structures were suggested: hydroxyazaperone, two dihydroxyazaperones, two trihydroxyazaperones, azaperol, two hydroxyazaperols, two dihydroxyazaperols, three trihydroxyazaperols, N-despyridinylazaperone, N-despyridinylazaperol, N-despyridinylhydroxyazaperone, N-despyridinylhydroxyazaperol, 1-(2-pyridinyl)-piperazine and hydroxy-1-(2-pyridinyl)-piperazine. Of these 19 metabolites, the greatest instrumental responses were obtained for one of the hydroxyazaperols and one of the dihydroxyazaperols.

Chloral hydrate. The metabolic profile of the GABA-agonist chloral hydrate[147] in equine plasma, blood cells, urine (hydrolyzed and unhydrolyzed) and saliva has been reported.[148] Similar to other species such as man and dog, chloral hydrate was observed to metabolize to trichloroethanol, trichloroethanol glucuronide (urochloralic acid) and trichloroacetic acid. Chloral hydrate itself was detected at only very low concentrations in plasma and saliva. Of the plasma metabolite, trichloroethanol was initially the most abundant and concentrations in this matrix were roughly equivalent to those in saliva throughout the excretion profile. Plasma concentrations of trichloroacetic acid were initially slightly lower than trichloroethanol, but the excretion profile was very shallow and from 8 h onwards this became the most abundant plasma metabolite. In contrast to trichloroethanol, salivary concentrations of trichloroacetic acid were much lower compared to plasma (at least ten-fold lower), possibly due to a combination of high protein binding and very low pK_a.[38] Concentrations of chloral hydrate and trichloroethanol were found to be roughly equivalent between plasma and blood cells, but trichloroacetic acid concentrations in cells could not be reliably measured so no concentrations were reported. In urine, over 99% of the metabolites detected were present as urochloralic acid, with trace concentrations of free trichloroethanol and trichloroacetic acid also present. Chloral hydrate itself was not detected in urine.

Ketamine. The metabolism of the N-methyl *d*-aspartate (NMDA) antagonist sedative ketamine has been studied extensively in the equine. In the first study to report the metabolism of ketamine in the equine, ketamine, norketamine and 5,6-dehydronorketamine were identified from urine samples hydrolyzed with β-glucuronidase from *Patella vulgate*.[149] In this study, ketamine was only detected up to 2 h post-dose, whereas the more intense peaks for norketamine 5,6-dehydronorketamine allowed detection for up to 8 and 12 h, respectively. In a later study, equine plasma concentrations of ketamine and its metabolites were quantified following either IV bolus or continuous rate infusion of ketamine.[150] In addition to ketamine, norketamine and 5,6-dehydronorketamine, a hydroxyketamine isomer was also determined. Since it has been postulated that 5,6-dehydronorkeatmine may be a degradation product of hydroxynorketamine (produced during either sample preparation or analysis), the true *in vivo* ratios of these two products has not been definitively determined. The ratio is therefore also likely to vary depending on the analytical procedure involved. Following continuous rate infusion of ketamine, parent drug was initially the most abundant compound measured in plasma. However, at later timepoints concentrations of the different compounds were in the order of 5,6-dehydronorketamine > hydroxynorketamine > norketamine > ketamine. Following the IV bolus injection of ketamine, parent drug concentrations were again initially higher than its metabolites. After 20 min, the order of concentrations had changed to norketamine > ketamine > 5,6-dehydronorketamine > hydroxyketamine. At 120-minutes the order of concentrations had further changed to 5,6-dehydronorketamine > norketamine > ketamine > hydroxynorketamine.

Many of the recent ketamine equine studies have focused on determining the chiral status of ketamine and its metabolites because the S-isomer has a greater affinity for NMDA receptors. Following IV bolus administration of racemate R/S-ketamine, plasma concentrations of the R and S parent drug isomers have been shown to be equal, while S-norketamine is found in larger amounts than R-norketamine.[151] In a subsequent study

using target-controlled infusion of ketamine, pharmacokinetic modelling of the resulting data was used to suggest that the rate of R and S-norketamine formation was similar, but that the rate of subsequent metabolism of R-ketamine to further metabolites was greater, thus resulting in higher plasma concentrations of S-norketamine.[152] This proposal was further supported by a subsequent *in vitro* metabolism study, which reported a similar rate of metabolism of both R and S-ketamine, but different rates of subsequent metabolism of R and S-norketamine enantiomers.[153] Furthermore, these findings were confirmed in an *in vivo* study, where it was reported that in both urine and plasma, the S : R ratio of norketamine enantiomers was significantly greater than 1 : 1, while the S : R ratio of 5,6-dehydronorketamine was significantly less than 1:1.[154] Most recently, in a study using a combination of *in vivo* and *in vitro* equine experiments, a large number of different hydroxyketamine and hydroxynorketamine isomers and a small amount of deaminonorketamine were identified.[155] However, reference standards for all the different products were not available, so their relative proportions and isomeric natures could not be confirmed.

Reserpine. Reserpine produces its tranquilizing and sedative effects by depleting the concentrations of monoamine neurotransmitters in the synaptic cleft. The disposition of reserpine following radio-labelled administration of the drug to horses has been reported.[17] In this study, parent reserpine was detected in plasma, but not urine. Of the radio-labelled dose, 77% was excreted into the faeces, while only 2% was recovered in urine within the first 13 days. The authors suggested that the radioactivity in urine may have taken the form of metabolites. The major urinary metabolite of reserpine in the equine has since been identified as desmethylreserpine (syringomethyl reserpate) glucuronide; formed by oxidative demethylation of the 4-methoxy position of the 3,4,5-trimethoxybenzoyl moiety of the drug followed by glucuronidation.[156,157]

Local anaesthetics

The local anaesthetics produce local anaesthesia through blockade of voltage-gated sodium channels.[158] However, they also produce varying effects on the heart and a central nervous system (CNS) stimulatory effect.[159] While the majority of topical anaesthetics are listed as class 3 agents according to the ARCI, because of their potential for use as nerve blocking agents, most of the injectable local anesthetics, including bupivacaine, butanilicaine, lidocaine, mepivacaine, prilocaine and ropivacaine are listed as class 2 agents.[2] In view of its CNS stimulating properties, cocaine is listed as a class 1 agent, while procaine (which is also used in complex with penicillin) is listed as a class 3 agent.[2] Similarly, the FEI classifications depend on the individual drug and are a mixture of banned (i.e. butanilicaine, cocaine and ropivacaine) and controlled (i.e. bupivacaine, lidocaine, mepivacaine, prilocaine and procaine) substances.[4]

Many of the local anaesthetics contain a hydrophilic amino group linked to a substituted benzyl residue via an ester or amide linkage. Hydrolysis of the ester linkage is a common pathway of phase 1 metabolism, but amide linkages are generally more resistant to hydrolysis. Hydroxylation of the benzyl moiety is also a common feature and N-dealkylations from hydrophilic amino substituents have also been reported. 'Free' parent drug can often be detected in plasma or urine, but metabolites are often conjugated as glucuronides. Figure 7 summarizes the major routes of catabolism in horses for the local anaesthetics discussed below.

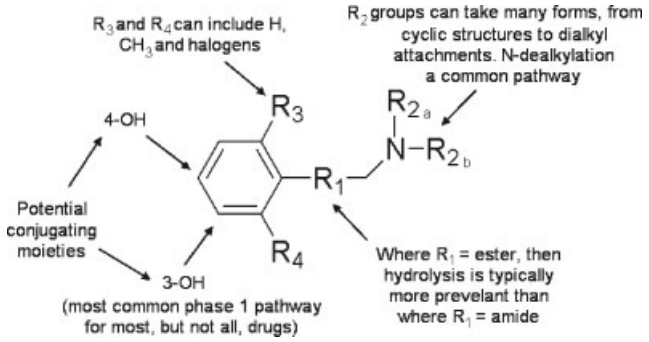

Figure 7. summary of some of the common pathways of local anaesthetic metabolism in the horse. The structure presented in generic for some, but not all, local anaesthetics. See text for details of individual drugs.

Bupivacaine. Following the analysis of β-glucuronidase hydrolyzed urinary extracts, 3′-hydroxylation has been reported as the major pathway of biotransformation for the amide-based local anaesthetic bupivacaine in horses.[158] The presence of further metabolites was not detected but comparison with reference standards suggested that 3′-hydroxybupivacaine was the only hydroxy-isomer formed in the horse.

Butanilicaine. No reports on the *in vivo* metabolism of butanilicaine were found, but the drug's metabolism has been reported following the *in vitro* incubation with either horse plasma, or liver/lung homogenates.[160] At 37 °C the plasma half-life of this amide-based local anaesthetic was 80 min, which compares with the much shorter half-life (9 min) of the ester-based local anaesthetic procaine.[161] The liver and lung homogenate half-lives were much shorter, with values of 3.2 and 4.6 min respectively. The metabolites were not identified, but the breakdown in plasma is suggestive of amide hydrolysis. The reduced half-life in liver and lung could indicate an increased esterase activity relative to plasma or a reaction not found in plasma, such as the type of aromatic-hydroxylation observed with some of the other local anaesthetics.

Cocaine is both a local anaesthetic and a CNS stimulant.[162] The drug has two ester linkages and hydrolysis of each of these two functional groups leads to the major urinary metabolites benzoylecgonine and ecgonine-methyl-ester, with benzoylecgonine being of greater quantitative significance.[163] The quantitative excretion of cocaine and its metabolites in the free fraction of urine and plasma has been reported.[164] Although cocaine itself was initially the compound of highest abundance in plasma following IV administration, its concentration dropped to lower than that of benzoylecgonine within 30 min. Other minor metabolites detected in plasma were ecgonine-methyl-ester > para-hyroxybenzyolecgonine > norcocaine. The major metabolites in urine were shown to be benzoylecgonine > ecgonine-methly-ester > cocaine > norcocaine. A range of other metabolites, namely para- and meta-hydroxybenzoylecgonine and hydroxycocaine analogues were also identified. The stability of cocaine in urine at different pHs, temperatures, and storage times has also been studied.[165] Degradation of cocaine to benzoylecgonine and ecgonine-methyl-ester showed that degradation was increased in

alkaline samples whereas neutral or acidified urine samples were relatively stable. The authors therefore proposed that if sample storage conditions were carefully controlled, then a high ratio of benzoylecgonine to cocaine could be used as a distinguishing factor between environmental contamination and cocaine administration to horses. However, the authors also acknowledged that that the presence of hydroxybenzoylecgonine or hydroxycocaine metabolites, especially in the hydrolyzed urine fraction, could be used as supporting evidence of cocaine administration.

Lidocaine. The metabolism of lidocaine has been studied and unconjugated lidocaine, monoethylglycinexylidide, 2,6-xylidine, and glycinexylidide have been detected in horse urine following its administration.[166] Enzyme hydrolysis of urine samples with extracts of *Helix pomatia* yielded further metabolites tentatively identified as 3′-hydroxylidocaine, 3′-hydroxymonoethylglycinexylidide and 4-hydroxy-2,6-xylidine. Quantitatively, 3′-hydroxylicocaine in the enzyme-hydrolyzed fraction appeared to be the most important metabolite in horses, but N-deethylation and hydrolysis of the amide link were also significant. It has subsequently been reported that the plasma clearance of lidocaine is over twice as fast in horses compared to man, but the authors could not ascribe the different rates to any particular cause.[167]

Mepivacaine. Mepivacaine metabolism in horses is extensive and a number of authors have reported that the equine urinary metabolites are a range of different hydroxymepivacaine, ketomepivacaine, and dihydroxymepivacaine isomers, with hydrolysis of the amide group not observed.[27,168,169] The major metabolite, representing over 75% of the excreted dose and excreted as a glucuronide, is 3′-hydroxymepivacaine.[168,169]

Prilocaine. The plasma and urinary metabolite profiles resulting from administration of the amide-based local anaesthetic drug prilocaine in horses have been studied (previously unpublished data from our laboratory). In the 'free' fraction of plasma, only parent prilocaine was detected. Enzyme hydrolysis of plasma was not carried out, so the extent of any phase 2 conjugates in this matrix could not be assessed. In the urinary 'free' fraction, trace levels of prilocaine and a hydroxy-metabolite, tentatively identified as 4-hydroxyprilocaine, were reported. When hydrolyzed with extracts from *Helix pomatia*, significantly greater instrumental responses for prilocaine and its metabolites were observed and 4-hydroxyprilocaine was tentatively identified as the major metabolite. Three further hydroxyprilocaine metabolites, a single dihydroxyprilocaine and two hydroxy-ortho-toluidine metabolites (resulting from cleavage of the ester group) were also identified.

Procaine. The ester-based local anaesthetic procaine is rapidly hydrolyzed in plasma (half-life of 9 min) to form the primary urinary metabolites para-aminobenzoic acid and diethylaminoethanol.[161] The presence of these metabolites in plasma or urine is not fully diagnostic of procaine administration as they can also arise from other legitimate sources. Therefore, detection of procaine use is predominantly based on the detection of free parent drug, which is excreted predominantly unconjugated.[170] Procaine can also be administered as a benzylpenicillin complex for dual use as a local anaesthetic/anti-infective.[171] The procaine is liberated from its penicillin complex and is then subject to similar metabolism as the latter drug, but with a significantly longer plasma half-life.[171] A related compound, procainamide, is the amide-analogue of procaine and is more often used as an anti-arrhythmic.[159] Procainamide is much more resistant to hydrolysis than its ester analogue and its predominant metabolite is N-acetylprocainamide.[159]

Ropivacaine. The major metabolite of this amide-based local anaesthetic that has been identified in urine by LC-MS/MS as its intact glucuronide conjugate is hydroxyropivacaine.[172] Hydrolysis of the urine samples with β-glucuronidase allowed detection of free hydroxyropivacaine. A subsequent study by the same laboratory demonstrated that the hydroxylation of this metabolite was in the 3′-position.[173]

NSAIDS/COX-2 inhibitors

Aside from the anabolic-androgenic steroids and sedatives/tranquilizers, the non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely metabolically studied class of drugs in horses. This is partly due to their wide range of structures, but also because of their importance as therapeutic agents and the need to control their use in competitive horseracing. Different regulatory authorities take varying standpoints on the control of NSAID use, depending on which drug has been applied; whether it is during or outside of competition; and whether use of the drug has been declared. The ARCI places a large number of NSAIDs into class 4, but some (i.e. etodolac and several of the COX-2 inhibitors) are upgraded to class 2 or 3 if they are not licensed for use in horses or if they produce other effects on equine performance.[2] FEI rules follow a similar pattern and many of the licensed equine NSAIDS fall into the controlled category, but with a number of other drugs banned.[4]

Due to the enormity of the literature, a full coverage of the equine metabolism of NSAIDs would require a dedicated review article. Therefore, the following section serves as a summary of the metabolism of a representative selection of NSAIDs from some of the different chemical classes on which these drugs are based. Following the review of two examples from each chemical class (selecting drugs with either high importance or detailed studies), an extended review of a newer breed of NSAIDs, namely the COX-2 inhibitors, is presented.

NSAIDS with COX-1 and COX-2 inhibiting properties

The majority of 'traditional' NSAIDs are able to inhibit cyclooxygenase enzymes types 1 and 2 (COX-1 and -2 respectively) to varying extents. While inhibition of COX-2 is considered to be the major mechanism for the anti-inflammatory effects of the NSAIDs, inhibition of COX-1 can lead to gastrointestinal side-effects as this enzyme is important in maintaining the integrity of the gut mucosa. The majority of NSAIDs are acidic moieties and many can be broadly classified into the following four reviewed classes.

Propionic acid based NSAIDs (profens)

With the exception of naproxen, the profens are generally marketed as racemate preparations.[174] In horses, a number of the profens are known to depart in concentration from the racemate 50 : 50 ratio in plasma over time. For example, shortly after IV dosing with carprofen and ketoprofen, plasma R(−):S(+) ratios for both drugs were close to 50 : 50, whereas after 1 h these ratios had departed in opposite directions, reaching values of 74 : 26 and 28 : 72 for carprofen and ketoprofen, respectively.[174] In the

same study, the administration of an enantiomerically pure S(+) form of carprofen did not show any conversion into the R(−) isomer, suggesting that a differential rate of excretion between the R(−) and S(+) isomers could be causative. In a separate study it has been shown that the phase 2 glucuronidation and biliary excretion of carprofen favoured the S(+) isomer, supporting the theory of an increased clearance of the S(+) isomer causing a departure from the 50 : 50 racemate over time.[175] More recently, it has been shown that ibuprofen enantiomers display a similar effect in horses.[176] The authors reported that the ratio of R(−):S(+) ibuprofen isomers in plasma decreased over time and that pharmacokinetic calculations confirmed an increased rate of clearance of the R(−) isomer. The results from the aforementioned studies raise the possibility that the isomer ratio could be used to differentiate environmental contamination of samples from true administrations of the drug. More work on the steroselective metabolism of a wider range of drugs, especially the profens, might therefore be warranted.

Naproxen. The metabolic profile of naproxen and its major metabolites have been studied quantitatively in equine plasma and urine.[177] Parent drug was the most abundant compound in unhydrolyzed plasma following naproxen administration. In comparison, relative plasma concentrations of its major metabolite, 6-O-desmethylnaproxen, were approximately 5% of those of naproxen. In urine, however, concentrations of total 6-O-desmethylnaproxen were slightly higher than those of parent drug. Parent naproxen was excreted into urine as mainly as it glucuronide conjugate (approximately 75%) whereas the relative proportion of conjugated 6-O-desmethylnaproxen was much lower (approximately 30%). An additional metabolite, 6-methoxy-2-naphthalene acetic acid, was also detected in urine, but its concentrations were below the quantitation limit.

Vedaprofen. The metabolic profile of vedaprofen and its metabolites has been studied in plasma, urine and faeces following the administration of radio-labelled vedaprofen.[178] The authors reported that around 73% of the dose was excreted in urine, with a smaller proportion in faeces. Using extensive HPLC fractionation and other experiments to identify phase 1 and phase 2 excretion products, a total of 14 metabolites were identified, most of which were detected in both plasma and urine. In plasma, unconjugated parent drug was by far the most abundant compound, whereas in urine, less than 1% of the dose was excreted as unchanged vedaprofen. Of the urinary metabolites, structures were proposed for eight of the 14 metabolites initially identified from the radioactivity chromatogram. The most abundant urinary metabolite was a hydroxyvedaprofen, with the site of oxidation located on the cyclohexane group. Two further hydroxyvedaprofen and two hydroxyvedaprofen glucuronides (one ether and one ester glucuronide; all with the site of hydroxylation proposed to be on the cyclohexane ring) were also identified at slightly lower concentrations compared to the major hydroxyvedaprofen metabolite. Lastly, three dihydroxyvedaprofen isomers were identified; again at slightly lower concentrations compared to the major hydroxyvedaprofen metabolite. The sites of oxidation of these dihydroxy metabolites were proposed to be on the cyclohexane and propionyl groups in both cases. A subsequent study employing a similar vedaprofen dosing regimen (not radioactive) also identified some of the same phase 1 metabolites as the earlier study, but also identified a glucuronide-conjugate of one of the dihydroxyvedaprofen isomers (positions unknown).[179] A major difference between this and the previous study was that parent vedaprofen extracted from urine was observed to produce greater instrumental responses than its metabolites (not formally quantified). Although physiological variation cannot be excluded, it is considered more likely that the reported differences are due to the alternative extraction and analytical approaches used by the two authors, especially as both studies used similar doses and Thoroughbred horses. In a further study analyzing the metabolite profile of vedaprofen in unhydrolyzed horse faeces, a hydroxyvedaprofen isomer produced instrumental responses greater than twenty-fold higher than those of the parent drug.[36]

Acetic-acid-based NSAIDs

Etodolac. Following etodolac administration, free and conjugated parent drug have been detected as the sole compounds present in equine serum.[180] Approximately 30–50% of the etodolac detected in serum was unconjugated and the other 50–70% was proposed originate from an glucuronide ester. In urine, the proportion of free etodolac was less than 5% relative to its proposed glucuronide ester conjugate. Three further metabolites, namely 6-hydroxyetodolac, 7-hydroxyetodolac and 8-(1′-hydroxyethyl)-etodolac, were also identified in both unconjugated and conjugated forms in urine (conjugate status not proposed). While the concentrations of these hydroxyetodolac metabolites were not formally quantified, inspection of an HPLC-UV chromatogram at 227 nm from a post-administration hydrolyzed urine sample suggests that etodolac was the most abundant compound present in this matrix.

Fenclofenac. The metabolic fate of fenclofenac following a radioactive dose to the horse has been reported by Marsh *et al*.[181] In plasma, parent fenclofenac accounted for 90–95% of the radioactivity, with the remainder being identified as a fenclofenac acyl glucuronide. A total of 83–85% of the radioactive dose was excreted into urine, but only 11–13% of this urinary output was attributed to unchanged fenclofenac. A further 69–70% of the urinary dose was identified as the fenclofenac acyl glucuronide, with the remainder of the dose attributed to a mixture of 5-hydroxyfenclofenac and a proposed dihydroxyfenclofenac (structure unconfirmed). Interestingly, the authors noted that β-glucuronidase hydrolysis only liberated a proportion of free drug from the fenclofenac acyl glucuronide and that base hydrolysis was required in order to produce 100% hydrolysis. More detailed HPLC studies differentiated several fenclofenac acyl glucuronide peaks and the authors attributed the peaks to rearrangement products of the acyl glucuronide, all of which could be hydrolyzed through base hydrolysis, but some of which were resistant to enzyme hydrolysis. The authors proposed that the alkaline pH conditions of some equine urine samples could promote this glucuronide rearrangement and therefore lead to differential recoveries of free fenclofenac. Other authors have also shown that alkaline pH conditions can promote positional rearrangement of acyl-glucuronides into β-glucuronidase resistant forms.[182,183] However, the extent to which this phenomenon was fully responsible for the results obtained by Marsh *et al*.[181] remains undetermined since acidic or alkaline conditions may lead to acyl-glucuronide hydrolysis in addition to positional re-arrangement.

Fenamic-acid-based NSAIDs

Meclofenamic acid. The equine metabolism of meclofenamic acid has been studied in several different matrices. In a study monitoring the metabolic profile of meclofenamic acid in unhydrolyzed equine plasma, parent drug produced instrumental responses approximately three- to four-fold higher than two hydroxy metabolites.[184] In studies comparing the excretion of meclofenamic acid between base hydrolyzed equine urine and unhydrolyzed faeces, the drug has been found to be extensively metabolized.[35,36] In the urine samples, parent meclofenamic acid produced instrumental responses similar to those of its two principle metabolites; 3′-hydroxymeclofenamic acid and 4′-hydroxymeclofenamic acid. In faeces, however, while responses for all three compounds were similar at 4 h post-dose, after this time 3′hydroxymeclofenamic increased proportionally and produced responses much greater than either parent drug or 4′-hydroxymeclofenamic acid.

Tolfenamic acid. Following the administration of tolfenamic acid in the equine, both parent drug and a hydroxy metabolite, proposed to be phenolic in nature, have been detected in both plasma and urine.[185] The study did not report the relative abundances of parent drug and metabolite or the extent of any phase 2 conjugation in either matrix, but inspection of summed EI-GC-MS ion chromatograms of methylated extracts from a 10-hour post-dose plasma sample suggest that parent drug was much more abundant than its hydroxy metabolite, at least in this matrix.

Enolic-acid(oxicam)-based NSAIDs

Lornoxicam. Following its administration to the equine, unconjugated lornoxicam has been shown to be the major urinary metabolite up to 1 hr post-dose.[186] From 2 onwards, however, 5′-hydroxylornoxicam produced greater instrumental responses, with enzyme hydrolysis experiments suggesting that approximately 50% of 5′-hydroxylornoxicam was glucuronide conjugated. Trace responses for a further metabolite, 5-chloro-3-methylsulfamoylthiophen-2-carboxylic acid, were also observed. The authors reported that the qualitative profile of metabolites in urine was similar to man, but that proportionately more parent drug could be detected in the equine.

Meloxicam. Following administration of meloxicam, parent drug has been reported as the most abundance urinary excreted product.[187] However, two further major metabolites were identified as 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam; with 5′-carboxymeloxicam generally producing instrumental responses slightly higher than 5′-hydroxymethylmeloxicam. No phase 2 metabolites were observed, either directly or through inference following enzyme hydrolysis. The authors reported that the profile of metabolites in urine was qualitatively similar to man, but that relative amount of parent drug was higher in equine urine. In a separate equine study from the same year,[188] similar equine phase 1 metabolic pathways were observed. However, in contrast to the former study, the use of a dual enzyme/base hydrolysis procedure (therefore hydrolyzing both glucuronic acid and sulfate conjugates) produced an increase in the instrumental response for both 5′-hydroxymethylmeloxicam and 5′-carboxymeloxicam. While the authors did not report the quantitative significance of these increases, they commented that all compounds were excreted 'mainly unconjugated.' In a more recent study,[189] the presence of a second minor isomer of hydroxymeloxicam and also a minor dihydroxymeloxcam isomer in equine urine were reported. The hydroxylation site of the former metabolite was proposed to occur on the benzothiazine moiety but the exact location could not be concluded. The hydroxylation sites of the latter dihydroxmetabolite were proposed to be on the thiazolyl and the benzothiazine moieties. Also, an intact hydroxymeloxicam glucuronide isomer was detected in horse urine for this first time.

Other NSAIDs (with structures not falling into the above categories)

Acetylsalicylic acid (aspirin). In the equine, acetylsalicylic acid is rapidly hydrolyzed to salicylic acid, which is the predominant urinary metabolite.[191] The authors of this study found that urinary concentrations of both acetylsalicylic acid and a hydroxy salicylic acid metabolite, gentisic acid, were less than 5% of those of salicylic acid, which is in contrast to the human where the glycine conjugate of salicylic acid, salicyluric acid, represents around 64% of the excreted products. In a separate study administering radio-labelled salicylic acid to both horses and ponies, around 98% of the dose was excreted in urine and similar quantitative metabolite profiles were obtained in both equine breeds.[190] In horses, 94.3% of the excreted dose was unchanged salicylic acid, 0.4% as salicyluric acid, 0.1% as salicyl-acyl-glucuronide, 2.2% as salicyl-ether-glucuronide and 3.0% as gentisic acid. In contrast, the same study reported that around 95% of a dose of benzoic acid was excreted as the glycine conjugate hippuric acid, highlighting the fact that small differences in analyte structure (presence of absence of a hydroxy group) can lead to significant differences in enzyme activity towards different molecules. Salicylic acid is also known to be a metabolite of other administered salicylates such as methyl salicylate. Most importantly, salicylic acid is also naturally present in many plants and therefore leads to detectable concentrations of salicylic acid in the plasma and urine of animals not deliberately dosed with acetyl or other salicylate-based drugs.[192] For these reasons, population studies of 'natural' salicylic acid concentrations have been conducted in the equine and international thresholds for salicylic acid of 750 µg/mL in urine and 6.5 µg/mL in plasma established in order to control the use of salicylate-based drugs in horseracing.[192,193] The metabolic pathways and threshold approaches for controlling acetylsalicylic acid use are summarized in Figure 8.

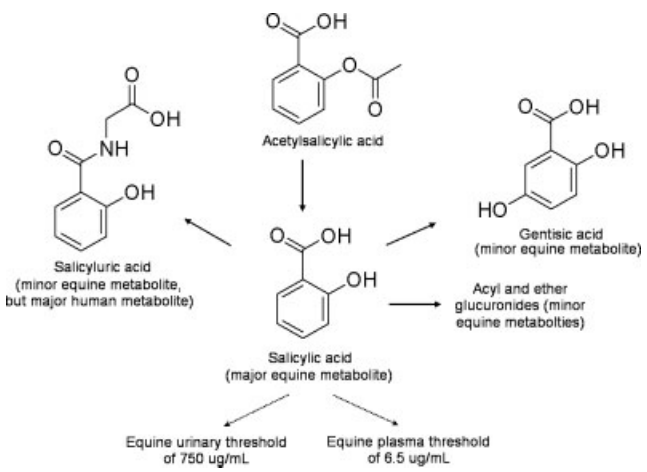

Figure 8. summary of the metabolism of acetylsalicylic acid in the horse and the IFHA thresholds mechanisms in place for controlling its use in horseracing.

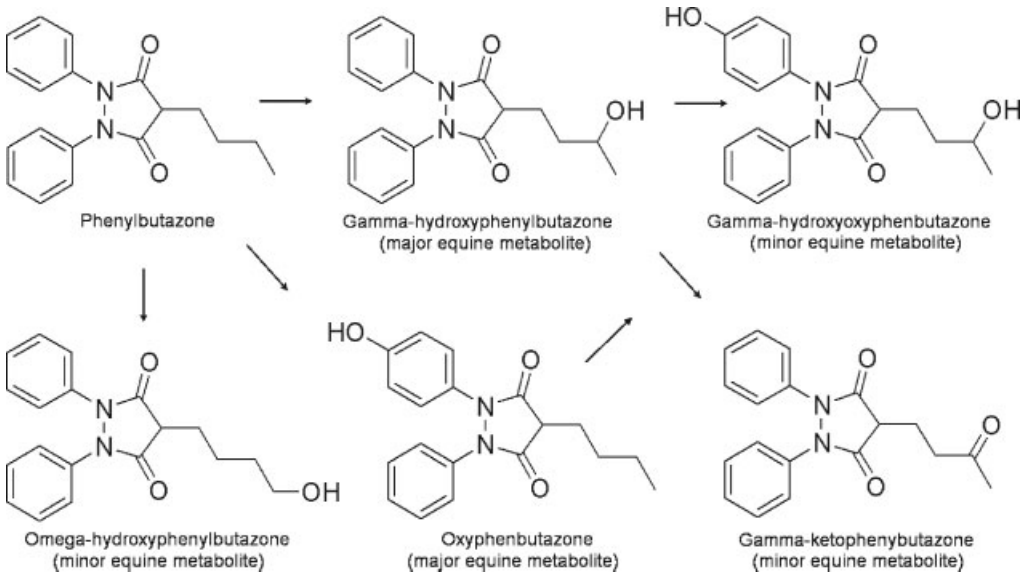

Figure 9. summary of the metabolism of phenylbutazone in the horse.

Phenylbutazone. Phenylbutazone has long and well-documented history of use in horseracing and has therefore been the subject of extensive metabolism and pharmacokinetic studies. Following administration of radio-labelled phenylbutazone, around 55% of the dose has been reported to be excreted into urine, with the majority of the remaining radioactivity recovered in faeces.[194] In this study, approximately 80% of the radioactivity in plasma could be attributed to parent phenylbutazone, with the remaining percentage being split similarly between the unconjugated phase 1 metabolites oxyphenbutazone and γ-hydroxyphenylbutazone. In urine, phenylbutazone metabolites were reported to be unconjugated, with parent phenylbutazone representing only a minor overall percentage at around 7% of the urinary products. In terms of total excreted radioactivity, the major urinary metabolites were oxyphenbutazone and γ-hydroxyphenylbutazone, accounting for around 36 and 15% of urinary radioactivity respectively. More detailed analysis revealed that while urinary concentrations of γ-hydroxyphenylbutazone were highest up to 10 h post-dose, after this time-point concentrations of oxyphenbutazone were quantitatively more significant. The majority of the remainder of the urinary radioactivity was accounted for in a number of minor metabolites identified as γ-hydroxyoxyphenbutazone, γ-ketophenylbutazone and ω-hydroxyphenylbutazone. Phenylbutazone and its metabolites contain an acidic hydrogen and it has been reported that urinary concentrations of phenylbutazone, oxyphenbutazone and γ-hydroxyoxyphenbutazone correlate positively with urinary pH.[195] The clearance of phenylbutazone has been reported to be between 5 and 15 times higher in donkeys than in horses due to an increased rate of formation of oxyphenbutazone.[12,196] The metabolic fate of phenylbutazone in the horse is summarized in Figure 9.

COX-2 inhibitors

COX-2 inhibitors are a relatively new breed of anti-inflammatory agent and are distinguished from the 'traditional' NSAIDs by virtue of their selectivity for inhibiting the COX-2 enzyme, therefore reducing their gastrointestinal side-effects. The laboratory of the National Horseracing Authority of Southern Africa has been particularly prevalent in publishing the metabolism of COX-2 inhibitors in horses.

Celecoxib. Following the administration of celecoxib, unconjugated parent drug has been detected in equine plasma.[197] In equine urine, however, concentrations of the parent drug were low and the major metabolite detected was 4-carboxycelecoxib. The authors of this study also reported trace responses from urine samples for a presumed intermediate in 4-carboxycelecoxib formation, 4-hydroxymethylcelecoxib. Both of these metabolites were found to be excreted predominantly unconjugated. Subsequent studies by other authors have confirmed these findings,[198] with both of these phase 1 metabolites also having been produced *in vitro* following incubation with horse liver S9.[27] The metabolic fate of celecoxib in the horse is summarized in Figure 10.

Etoricoxib. The metabolic profile of etoricoxib has been studied in both equine plasma and urine.[199] Although the study was not quantitative, relative LC-MS/MS instrumental responses were reported for both etoricoxib and its metabolites. Parent etoricoxib was detected in plasma at instrumental responses slightly higher than its major metabolite, 6′-hydroxymethyletoricoxib. In unhydrolyzed urine, however, etoricoxib was detected at only very low instrumental responses relative to its metabolites, which were detected with relative response order of 6′-hydroxymethyletoricoxib $>$ 6′-hydroxymethyletoricoxib glucuronide $\gg$ 6′-hydroxymethyletoricoxib-1′-N-oxide $\gg$ etoricoxib. The authors noted that a major human metabolite, carboxyetoricoxib, was not detected in horses and that the detection time for etoricoxib metabolites in plasma was longer than that in urine.

Firocoxib. The equine metabolism of firocoxib has recently been studied.[200] Parent firocoxib could be detected in plasma along with lower instrumental responses for descyclopropylmethylfirocoxib and a hydroxyfirocoxib (position of hydroxylation proposed to be on the cyclopropyl ring). In urine, relative responses for detected metabolites were descyclopropylmethylfirocoxib $\gg$ hydroxyfirocoxib $\gg$ firocoxib. Preliminary evidence for the presence

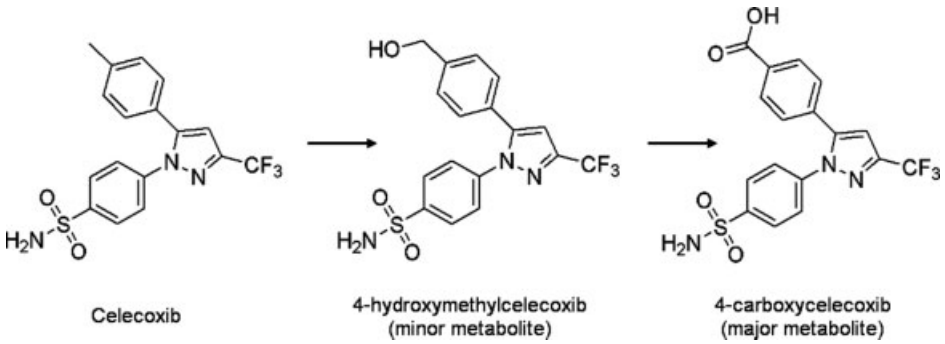

Figure 10. summary of the phase 1 metabolism of celecoxib in the horse. See text for details of other COX-2 inhibitors.

of urinary glucuronide conjugates of descyclopropylmethylfirocoxib and hydroxyfirocoxib was also obtained, although enzyme hydrolysis did not increase the instrumental response for either of these metabolites. Therefore, the authors concluded that hydrolysis was not necessary for detection of firocoxib administration. In a subsequent study, the metabolism of firocoxib was studied following the administration of a radioactively labelled dose of the drug.[201] From the radioactive dose 68% was excreted in urine and 15% in the faeces. This study concurred with the results of de Kock *et al*.[200] in terms of the phase 1 urinary metabolites, but using the more definitive technique of monitoring radioactive traces, the authors found that glucuronide metabolites were of increased quantitative importance. In urine, 56% of the dose was excreted as descyclopropylmethylfirocoxib glucuronide, 17% as hydroxyfirocoxib, 19% as descyclopropylmethylfirocoxib and $<2\%$ as parent firocoxib. Descyclopropylmethylfirocoxib glucuronide was also the major metabolite detected in faeces.

Lumiracoxib. Whilst it has been reported that lumiracoxib is extensively metabolized in humans, such that only around 3% of the dose is excreted unchanged in urine, equine studies have shown that parent lumiracoxib is the major metabolite detected in both plasma and urine.[202] In this equine study, a range of lumiracoxib metabolites were, however, also identified. The most prominent of these metabolites was lumiracoxib glucuronide, producing LC-MS/MS instrumental responses in both plasma and urine of approximately half that of the parent drug. Four minor metabolites detected in both urine and plasma were two hydroxylumiracoxib isomers, proposed to be 4′-hydroxylumiracoxib and 5-hydroxymethyllumiracoxib, as well as their corresponding glucuronides. Further minor metabolites tentatively identified in urine were 5-carboxylumiracoxib, 4′-hydroxy-5-carboxylumiracoxib, 5-carboxylumiracoxib lactam, 4′-hydroxy-5-carboxylumiracoxib lactam and a hydroxylumiracoxib lactam glucuronide. Neutral loss LC-MS/MS experiments failed to identify any sulfate metabolites of lumiracoxib.

Rofecoxib. Following the administration of rofecoxib and the subsequent analysis of β-glucuronidase/base hydrolyzed equine urine samples, parent rofecoxib has been detected as the major urinary product.[198] Low concentrations of a phase 1 metabolite, 5-hydroxyrofecoxib, were also detected, which is in contrast to human where metabolism to 5-hydroxyrofecoxib (as well as other metabolites) is quantitatively more important.[203]

Valdecoxib and parecoxib. The urinary elimination of valdecoxib and its pro-drug analogue parecoxib have been studied in horses.[204] Following valdecoxib administration, parent valdecoxib was by far the most abundant urinary product, with a much lower instrumental response for a postulated hydroxyvaldecoxib metabolite observed. Following administration of the valdecoxib pro-drug parecoxib, the most abundant urinary metabolite detected at 1 and 3 h following administration was parent parecoxib. However, urinary concentrations of valdecoxib increased steadily such that by 6 h onwards, it had surpassed parecoxib as the most abundant metabolite. The hydroxyvaldecoxib metabolite observed following valdecoxib administration was also detected following parecoxib administration; again at much lower concentrations than the other compounds. The authors noted that a previously reported human valdecoxib metabolite, carboxyvaldecoxib, could not be detected in equine urine following administration of either valdecoxib or parecoxib, but acknowledged the possibility that this could have been influenced by the targeted nature of the sample extraction procedure used. The authors also reported that neither enzyme hydrolysis nor targeted LC-MS/MS experiments were able to identify the presence of sulfate or glucuronide metabolites of either drug.

Opioid analgesics

The opioids produce their pharmacological effects by acting on different opioid receptor subtypes and their chemical structures can be broadly classified as either endogenous (i.e. endorphins and enkephalins), opium alkaloids (e.g. codeine and morphine), semi-synthetic (e.g. oxycodone and diacetylmorphine), or synthetic (e.g. butorphanol, dextromoramide, etorphine, fentanyl, propoxyphene, remifentanil and tramadol). Depending on the dose and drug in question, the opioid analgesics may produce analgesia, sedation, CNS stimulation and psychological dependence and are therefore typically classed by the US Drug Enforcement agency as Schedule 2 substances. Because of these effects, the opioid analgesics are also considered to have a high potential for abuse in equine sports. This is recognized by the inclusion of the majority of the opioid analgesics at the highest level of classification within the rules of both the ARCI (predominantly category 1 substances)[2] and the FEI (predominantly banned substances).[4]

Opium and semi-synthetic opioids

Morphine, codeine, and diacetylmorphine. Although published details of the phase 1 metabolism of morphine following its administration to horses are lacking, its pharmacokinetics in hydrolyzed urine and unhydrolyzed plasma have been studied.[205] In terms of phase 2 metabolism, previously unpublished data from

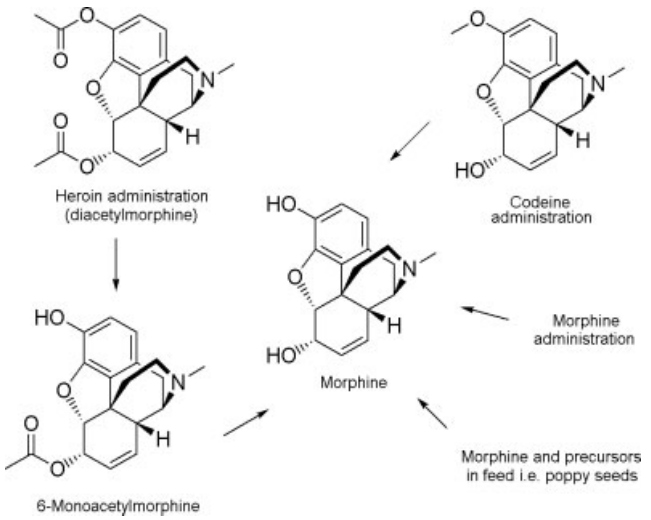

Figure 11. summary of some of the proven sources of morphine in the equine.

our laboratories suggests that similarly with human metabolism, morphine is extensively conjugated prior to excretion also in equine and the major product being morphine-3-glucuronide. The predominant excretion of the 3-glucuronide isomer is significant since it is inactive in many species, whereas the 6-glucuronide is known to be a pharmacologically active compound.[206]

In addition to the presence of morphine in equine samples being the result of morphine administration, it is also known to be a metabolite from several other sources. For example, the administration of codeine leads to detectable urinary concentrations of both conjugated codeine and morphine (conjugation status not determined), while administration of diacetylmorphine (heroin) leads to 6-monoacetylmorphine and morphine as metabolites.[207] Furthermore, morphine and codeine are known to occur naturally in certain plants, which leads to the possibility that the presence of these opioids in equine samples could derive from dietary intake.[208] A discussion on the analytical approaches that may be used to distinguish a morphine administration from dietary intake are beyond the scope of this review and have already been given adequate coverage elsewhere.[207–209] The different possible causes for the presence of morphine in equine samples are summarized in Figure 11.

Oxycodone. Following administration of oxycodone to the horse, low concentrations of unconjugated parent drug have been detected in urine for several hours.[210] However, the major excreted metabolite is the demethylated and subsequently phase 2 conjugated product oxymorphone (conjugate type unknown). Concentrations of this metabolite in urine were reported to be approximately 50 times those of the parent drug and were detectable for a much longer period of time.

Synthetic opioids

Butorphanol. In an early study of the metabolism of butorphanol in horses, neither of the previously reported human metabolites, norbutorphanol and hydroxybutorphanol, could be detected in hydrolyzed urine or unhydrolyzed plasma samples.[240] However, parent drug was readily detected in both matrices. In a recent study using more sensitive instrumentation, both norbutorphanol and hydroxybutorphanol were detected at trace levels in plasma for a short period of time.[211] Definitive studies on the phase 1 and 2 metabolite profile of butorphanol in equine urine using modern instrumentation are lacking in the literature.

Dextromoramide. Dextromoramide appears to be extensively metabolized in the equine and detection of its abuse in urine is reported to require analysis of its two major metabolites hydroxydextromoramide (aromatic) and 2,2-diphenyl-3-methyl-4-morpholinobutyramide (formed through loss of the pyrrolidine ring).[212] Published data regarding the presence of dextromoramide/metabolites in plasma or phase 2 metabolites in urine are currently lacking.

Etorphine. The urinary excretion of the potent opioid etorphine has been studied in horses following administration of a radioactively labelled analogue of the drug.[213] Approximately 85% of the administered radioactivity was recovered in urine within the first 168 h. Extraction of unhydrolyzed urine recovered only 5% of the radioactivity, whereas additional hydrolysis of the samples with β-glucuronidase from *Patella vulgata* recovered 95%. Only parent etorphine was detected in the hydrolyzed/unhydrolyzed urine samples; suggesting that direct phase 2 conjugation (exact conjugate unidentified) is the major pathway of excretion in this species.

Fentanyl. N-(1-phenethyl-4-piperidyl)-malonanilinic acid (PMA) has been identified as the major urinary metabolite of fentanyl in the equine and appears to be present predominantly in the unconjugated form.[214] The authors also highlighted that this metabolite was thermally labile and when analyzed at temperatures above 150 °C by GC-MS breaks down to form despropionylfentanyl in the source. The metabolism of fentanyl has also been compared between man, horse, and dog, where it was found that PMA was the only metabolite formed in horses and that PMA was unique to this species.[215] Most recently, the metabolism of fentanyl has been studied both *in vivo* and *in vitro* in horses.[27] The authors found that PMA was the major *in vivo* metabolite, but they also identified a minor hydroxyfentanyl metabolite *in vivo* and a number of additional hydroxy-, dihydroxy-, desalkyl and desalkylhydroxy-metabolites *in vitro*. The metabolic fate of fentanyl in horses is summarized in Figure 12.

Propoxyphene. Following administration of propoxyphene to the horse, parent drug has been detected for several hours in urine.[216] However, much greater instrumental responses were obtained for several metabolites, which allowed detection for extended periods of time relative to the parent drug. Identified metabolites included norpropoxyphene and two aromatic ring hydroxypropoxyphene isomers. Responses for the two hyroxypropoxyphene isomers were significantly increased following enzyme hydrolysis, suggesting that these metabolites were excreted predominantly as phase 2 conjugates.

Reminfentanil. When administered to the horse, parent remifentanil has been detected in the urinary-free fraction.[217] The major urinary metabolite in this species has been reported as the ester hydrolysis product 4-methoxycarbonyl-4[(1-oxopropyl)phenylamino]-1-piperidinepropionic acid, which produced instrumental responses approximately ten-fold higher

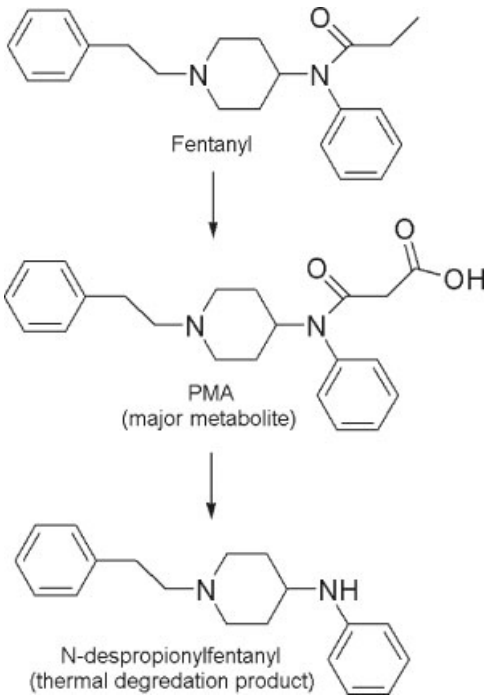

Figure 12. summary of the metabolism of fentanyl in the horse. PMA = N-(1-phenethyl-4-piperidyl)-malonanilinic acid.

than the parent drug. No further phase 1 metabolites or any phase 2 conjugates were detected.

Tramadol. The urinary excretion of tramadol has been directly compared between a human volunteer and an equine subject.[218] In the human, the percentage of the dose excreted as parent tramadol was much greater than in the horse, where the proportion of metabolites was higher. A total of 12 metabolites were identified in the equine, resulting from combinations of O- and N-demethylation and aliphatic/aromatic ring hydroxylation. Comparison of equine urine samples with and without β-glucuronidase (*E. coli*) hydrolysis suggested that the hydroxylated and O-demethylated metabolites were 'extensively conjugated' with glucuronic acid. Overall, the percentage of hydroxylated metabolites was higher in the equine and the percentage of N-demethylated metabolites was higher in the human, while O-demethylation appeared to be similarly prevalent for both species. A number of proposed degradation/analytical artefact products of some of the metabolites were also observed in both species, but the precise origins of these moieties could not be confirmed. The circulating pharmacokinetics of tramadol and its metabolites in the equine have also been reported recently.[219–221]

Equine Drug Metabolism Enzymology

A number of *in vitro* and *in vivo* studies aimed at probing the basic enzymology of drug metabolism in horses have been conducted over the past 40 years, but to date there is still a relative dearth of sequenced enzyme data compared to many other species. However, the recent publishing of the equine genome promises to make rapid advances in this area. The following section serves to review the studies that have been carried out to date before a summary of the inferences that can currently be made regarding the existence and activity of different enzymes in horses is presented.

In vivo metabolic probe substrate studies in horses

Certain compounds are considered to act as 'probe substrates' for particular drug metabolizing enzymes in some species,[222] i.e. they are characteristically metabolized by one type of enzyme. In the absence of sequenced enzyme data, extrapolation of the ability of particular substrate to probe the metabolic capability of an enzyme in another species, such as the equine, is a first step in guiding possible metabolic capacity.

The pharmacokinetics of antipyrine can give a good measure of total metabolic clearance capacity in the liver.[222] Antipyrine clearance is around 10 times greater in horses (5.83 mL/min/kg) than in man (0.536 mL/min/kg).[167] Since antipyrine is oxidatively metabolized by several CYP forms in most species,[223] it can be speculated that horses generally have greater oxidative phase 1 metabolic capacity than man although dominance from a particular enzyme isoform/s cannot be ruled out.

Although the excretion of warfarin and antipyrine from horses is reported to be much faster than from humans, the plasma clearance of paracetamol (acetaminophen), which is predominantly cleared by phase 2 glucuronide and/or sulfate conjugations in most species,[167] is similar in both horse and man (4.84 and 4.68 mL/min/mg respectively). This suggests that horses and man may have similar overall capacity for phase 2 conjugations, but since the exact nature of the metabolites in the horse is not determined, no firm conclusions can be drawn.

In a PhD thesis at the University of London in 1983[224] entitled *Characterisation of some metabolic conjugation pathways in the horse*, it was demonstrated the phase 2 conjugations of some carboxylic acid drugs in the horse. Glycine conjugation was shown to be quantitatively important in the metabolism of phenylacetic, benzoic, and 2-napthylacetic acid. Glucuronic acid was the major conjugate of fenclofenac while glucuronide conjugates of benzoic acid and 2-napthylacetic acid were also detected. Taurine conjugation was a minor pathway in the metabolism of 2-napthylacetic acid but was the major conjugate observed for isoxepac. The addition of a two-carbon fragment to benzoic acid (thought to be associated with the endogenous pathways of fatty acid elongation) was also observed with 3-hydroxyphenylpropionic acid and acetophenone (a possible decarboxylated product of 3-keto-3-phenylpropionic acid) being detected.

In vitro metabolic probe substrate studies in horses

In an early study,[130] the *in vitro* metabolism of six substrates in whole tissue homogenates (phase 2 metabolism) and S9 fractions (phase 1 metabolism) from 34 horse livers was reported. The reactions monitored were (1) glucuronide conjugation of p-nitrophenol; (2) oxidation of zoxazolamine; (3) oxidation of hexobarbital; (4); N-dealkylation of aminopyrine; (5) hydrolysis of procaine; and (6) reduction of p-nitrobenzoic acid. On comparison with other data in the literature, Yeary concluded that the horse has much greater p-nitroreductase activity than the dog, rabbit, rat, guinea pig or mouse and that, with the exception of p-nitroreductase activity, the values obtained for the other compounds were generally lower than those reported for commonly used laboratory animals. The rate of oxidation of hexobarbital was significantly ($P < 0.05$) greater in female than in male horses.

In 1991, Park and Plapp[225] studied the enzymology of horse liver alcohol dehydrogenase by cloning the cDNA for two distinct isoenzymes (E and S) and expressing the genes in *E. coli* to produce proteins for sequencing, x-ray crystallography and activity analysis. Isoenzyme E was active on ethanol but not steroids, and isoenzyme S was active on ethanol and steroids. Human gamma alcohol dehydrogenase is also active in metabolizing steroids, but the amino acid residues in the substrate pocket are altered from those in the horse and this leads to different substrate specificities and catalytic activities in the two species.

In 1993, Komuri[226] reported the purification and partial characterization of a CYP (named P450h-1) from male and female horse liver microsomes. The electrophoretically homogenous preparation contained 14.8 nmol/mg protein and the recovery was 0.38% of the microsomal CYP content. The apparent molecular weight was 52 kDa and a reconstituted system could catalyze benzphetamine N-demethylation, 7-ethoxycoumarin O-deethylation and testosterone 16α-hydroxylation. The N-terminal amino acid sequence was highly conserved when compared with rat CYP2C11 and an anti-P450h-1 antibody revealed that this antibody most strongly recognized rat CYP2C13. The anti-P450h-1 antibody inhibited over 90% of testosterone 16α-hydroxylation in horse liver microsomes but did not affect 6β-hydroxylation. These results indicate that P450h-1 belongs to the CYP2C subfamily and contributes to testosterone 16α-hydroxylation.

In 1996, Byard[227] used electrophoresis and a lauric acid affinity column to purify a protein from horse liver microsomes. The separated components were not fully homogenous but the conversion of p-nitrophenol to 4-nitrocatechol and visualization by western blot using a rat anti-CYP2E1 antibody suggests that at least one of the components was related to a CYP2E. Subsequent work by the same author[228] determined the existence of an epoxide hydrolase in horses.

In 1997, Chauret[229] compared the *in vitro* metabolic activity of CYPs in horses human, dog, and cat liver microsomes. Markers for CYP mediated reactions were measured: phenacetin O-deethylase (CYP1A1/A2), coumarin 7-hydroxylase (CYP2A6), tolbutamide hydroxylase (CYP2C8/9), S-mephenytoin 4′-hydroxylase (CYP2C19), dextromethorphan O-demethylase (CYP2D6), Chlorzoxazone 6-hydroxylase (CYP2E1) and testosterone 6β-hydroxylase (CYP3A4). CYP1A1/2 marker activity was not significantly different between horse and man although the human standard deviations were large (possibly due to varied degrees of expose to chemical inducers). Furafylline effectively inhibited CYP1A1/2 activity in both species, but to a greater extent in man. CYP2A6 marker activity was significantly lower in horse than man (four-fold) and an anti-CYP2A6 monoclonal antibody effectively inhibited marker activity in both species. CYP2C9 marker activity was significantly higher in horse than man (two-fold), and while sulfaphenazole effectively inhibited marker activity in both species, this was to a lower extent in the horse. CYP2C19 marker activity was not significantly different between horse and man and tranylcypromine effectively inhibited marker activity in both species. The most significant difference between horse and man was that CYP2D6 marker activity was over twenty-fold higher in the horse. CYP2D6 marker activity inhibition was not reported for the horse. CYP2E1 marker activity was significantly higher in the horse than man (two-fold) and while diethyldithiocarbamic acid effectively inhibited marker activity, the effect was lower in the horse. CYP3A4 marker activity was significantly lower in the horse than man (seven-fold) and while troleandomycin effectively inhibited CYP3A4 marker activity in man, it only partially inhibited activity in the equine. The lower degree of CYP marker activity inhibition in some cases in the horse could be the result of additional enzymes being involved with the metabolic transformation of marker substrate in this species. It could alternatively (or additionally) be due to an altered CYP amino acid sequence in the horse that leads to a reduced affinity of enzyme for the inhibitor.

In 1997, Court[230] studied the biotransformation of chlorzoxazone by hepatic microsomes from humans and ten other mammalian species including horses. In line with results obtained by Chauret,[229] Court reported that chlorzoxazone 6-hydroxylation catalytic activity was higher in horses than humans. An Eadie-Hofstee plot of enzyme activity in both species suggested that only one enzyme was involved in the biotransformation. The K_m in the horse (n=1) was 35 μM and those for two human subjects were 64 and 77 μM. The V_{max} in the horse was 2.1 nmol min^{-1} mg^{-1} and for the two humans was 1.8 and 1.4 nmol min^{-1} mg^{-1}. When using diethyldithiocarbamate to inhibit 6-hydroxychlorzoxazone formation, the $IC_{50\ max}$ and I_{max} (%) were not significantly different between the two species.

In 2000, Lakritz[231] reported a comparison of hepatic and pulmonary enzyme activities in horses. Pulmonary and hepatic tissues from 22 horses aged from 4 months to 32 years were used to prepare cytosolic fractions for glutathione-S-transferase (GST) and epoxide hydrolase activity and microsomal fractions for CYP activity. Total CYP content was determined by monitoring the CO bound difference spectrum of dithionate-reduced microsomes. GST activity was measured by monitoring the formation of chlordinitrobenzene (CDNB) glutathione conjugate while soluble epoxide hydrolase activity was measured by detecting the formation of the hydrated product of trans stilbene oxide (TSO). Markers for CYP activity were ethoxyresorufin O-deethylase (EROD) for CYP1A, pentoxyresorufin O-dependentylase (PROD) for 2B1 and naphthalene monooxygenase (NAMO) for CYP 1A1/2B1 and 2F. CYP content and activity were generally lower in pulmonary than hepatic tissues with CYP1A and soluble epoxide hydrolase marker activity particularly high in hepatic tissues. The rate of hepatic CYP1A1 marker activity was signficanlty higher than CYP2B1 activity suggesting that CYP1A isoforms may be more active in horses than those of the CYP2B subfamily.

In 2000, Schmid[232] detected the formation of 4-O-acetyl sialic acids in microsomes from equine submandibular enzymes, thus inferring the existence of equine acetyltransferases.

In 2001, Nebbia[233] studied the oxidative metabolism of monensin, a CYP3A substrate, in liver microsomes from horses, pigs, broiler chicks, cattle, and rats. The study covered also rates of erythromycin N-demethylase and triacetyloleandomycin (TAO) in liver microsomes from these species as additional makers of CYP3A activity. The results indicated that CYP3A activity in the horse is slightly lower than in the rat, which is lower than the CYP3A activity in man.

In 2003, Larsson[234] reported the metabolic activation of aflatoxin B1 in olfactory and respiratory tissues in horses. Immunohistochemistry revealed that cells expressing proteins reacting with CYP3A4 and CYP2A6/2B6 antibodies had a similar distribution to those having capacity to activate aflatoxin B1. This metabolic pathway may have significance for horses exposed to mouldy dust since activated alfatoxin is a potential carcinogen.[222]

In 2003, Nebbia[235] studied the comparative expression of liver microsome activities in horses, cattle, pigs, chicks, rabbits, and rats. The following markers were used to probe CYP activity: ethoxyresorufin O-deethylase, methoxyresorufin O-demethylase and benzo(a)pyrene hydroxylase for CYP1A, benzphetamine

N-demethylase and benzyloxyresorufin O-debenzylase for CYP2B, aniline hydroxylase, N-nitrosodimethylamine (NDMA) N-demethylase and p-nitrophenol hydroxylase for CYP2E and ethylmorphine N-demehtylase, erythromycin N-demethylase and TAO N-demethylase for CYP3A isofroms. Western blotting of electrophoretically separated proteins was also performed using antibodies directed against rat CYP1A1, CYP2B1/2, CYP2E1, and CYP3A1/2.

The level of CYP1A activity inferred from ethoxyresorufin O-deethylase was markedly higher than any other species studied, but particularly the rat. In order to relate this high activity to man, it is necessary to find a study comparing the rate of ethoxyresorufin O-deethylase between human and rat tissues. Steinberg[236] has compared the rate ethoxyresorufin O-deethylase between human and rat hepatocytes in suspension and found that the rates were roughly comparable. Therefore the high rate of ethoxyresorufin O-deethylase in horses compared to rats reported by Nebbia infers that CYP1A activity may also be higher in horses than in man. However, it is possible that ethoxyresorufin is not a specific marker for CYP1A isoforms in horses and that the high activity of ethoxyresorufin O-deethylase might be at least partially attributable to additional enzymes. This theory is supported by the lower western blot band intensity of CYP1A directed antibodies in horses compared to rats; the longer half-life of caffeine and theophylline in horses than man; and the relatively lower rate of phenacetin O-deethylase in comparison to man. An alternative explanation is that CYP1A isoforms in horses are divergent from those in humans in such a way that ethoxyresorufin is still a specific marker but that catalytic activity towards caffeine, theophylline, and phenacetin is reduced. More studies are therefore required to clarify the relative expression of CYP1A in horses.

CYP2B marker activity and western blot band intensities were somewhat lower in horses compared to rats and this correlates with the low activity of CYP2B observed by Lakritz in 2000[231] for pentoxyresorufin.

CYP3A western blot band intensity was similar in the two species, but marker activity was much lower in horse in comparison to rat microsomes.

CYP2E western blot band intensities were similar in rats and horses. CYP2E marker activity varied depending on the substrate; *p*-nitrophenol hydroxylase activity was similar in rats and horses, while aniline hydroxylase and NDMA N-demethylase activity were much higher in rats. Since Court showed in 1997[230] that the rate of chlorzoxazone 6-hydroxylation was much higher in horses than in rats, the low relative rates of aniline hydroxylase and NDMA N-demethylase observed by Nebbia may indicate that the horse CYP2E1 has altered substrate specificity from rats. Alternatively, it may be that rats have additional enzymes contributing to aniline hydroxylase and NDMA N-demethylase. More work is required to resolve the disparity.

In 2004 Nebbia *et al*.[237] studied the development of hepatic oxidative and conjugative drug metabolizing enzymes in 50 female horses ranging from less than 12 months to more than 12 years old. The following markers were used to probe microsomal CYP activity: ethoxyresorufin O-deethylase, methoxyresorufin O-demethylase and Benzo(a)pyrene hydroxylase for CYP1A, benzphetamine N-demethylase and benzyloxyresorufin O-debenzylase for CYP2B, aniline hydroxylase and N-nitrosodimethylamine (NDMA) N-demethylase for CYP2E and ethylmorphine N-demehtylase, erythromycin N-demethylase and TAO N-demethylase for CYP3A isofroms. The following markers were used to probe cytosolic phase 2 enzyme activity: the glucuronidation of 1-napthol for UGTs and the glutathione conjugation of CDNB, ethacrynic acid and butylene oxide for GSTs.

The results of the CYP experiments show a general increase in the activity of CYP2B, CYP2E, and CYP3A related enzymes with increasing age of the animal. These results were complimented by western blot analysis that showed increasing band intensities for CYP2B- and CYP3A-related enzymes with age. The results of the phase 2 enzyme experiments show that while UGT activity also increases with age, the activity of GSTs showed either no significant change or a decline with age depending on the particular reaction monitored. The authors of the paper also noted that there was a positive correlation between increasing CYP activity and cadmium content in the horse.

In 2008, 2009, and 2010 several equine cytochrome P450 (CYP) enzyme isoforms, named CYP2D50, CYP2C92, CYP3A89, CYP396 and CYP3A97 have for the first time been sequenced and their activities compared to human CYP2C, 2D and 3A isoforms.[49–51] 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. However, more sequencing studies are required before it can be determined whether these are the most abundant phase 1 drug metabolizing enzymes in horse liver or if there are other more important isoforms. The sequencing of equine CYP enzymes is of significance as it greatly enhances our understanding of the mechanisms of metabolism in this species. It also opens up the possibility in the future of making recombinantly produced versions of the enzymes commercially available in order to allow *in vitro* assessments of drug-drug interactions and routes of metabolism to be determined early during the veterinary drug development process.

Most recently, Scarth *et al*.[21] used selective enzyme inhibition studies to infer (based on extrapolation from known enzyme inhibitors against human CYPs) that the major 16-hydroxylation pathway for stanozolol in horses might be mediated by an enzyme related to human CYP2C8. These results are consistent with those described above, where the existence of a CYP2C isoform with the ability to 16-hydroxylate testosterone in horses was reported.[226] Again, more detailed sequencing studies would be required to confirm these results.

Induction and inhibition of drug metabolism in the equine

Induction or inhibition of drug metabolism in horses can be inferred from certain drug-drug interactions that have been reported in the veterinary literature.

The pharmacokinetics of phenobarbital in horses after single and repeated oral administration has been studied.[129] The plasma half-life after a single dose of 26 mg/kg of body weight was 24.2 and after 42 days treatment with 13 mg/kg body weight each day for 42 days the half-life had reduced to 11.2 h. This is clear evidence for CYP enzyme induction in horses, but due to the fact that phenobarbital is known to induce several different CYP isoforms in other species,[238] no firm conclusions can be drawn regarding the particular enzymes involved.

The pharmacokinetics of phenylbutazone after rifampicin treatment has been reported.[239] The plasma half-life of phenylbutazone before rifampicin treatment was 4 h but after 4 days of rifampicin at 10 mg/kg body weight each day, the half-life was reduced to 2.7 h. This is clear evidence for CYP enzyme induction in the horse, but due to the fact that rifampicin is known to induce several CYP isoforms in other species,[241] no firm conclusions can be drawn regarding the particular enzymes involved.

Quinidine has been shown to decrease the renal clearance of digoxin in horses.[242] Since quinidine is a selective inhibitor of human CYP2D6,[243] this infers the existence of a CYP2D isoform/s in horses. However, changes in the renal digoxin elimination rate by quinidine cannot be ruled out.[242]

Fluoroquinolones, such as ciprofloxacin, inhibit the metabolism of xanthines in horses.[242] Since many fluoroquinolones are inhibitors of CYP1A isoforms in man, this provides further anecdotal evidence for an involvement of a CYP1A isoform/s in xanthine metabolism in horses.

N-methyltryptamine is a monoamine oxidase inhibitor that has been shown to reduce the rate of oxidative metabolism of adrenaline and N-methylamphetamine itself in horses[244] and therefore provides evidence for the existence of monoamine oxidase/s in horses.

Other studies

In a review of clinical pharmacokinetics in veterinary medicine in 1992, Baggot[245] concluded that herbivorous species tend to metabolize lipid soluble drugs faster than carnivores and that humans typically show slower metabolism than most animals. In particular, this study concluded that horses generally have a higher capacity to clear drugs than humans.

In a German review article in 1994,[246] the existence of several other phase 1 and two biotransformations in horses was reported. Some of the phase 1 reactions reported included the aromatization of ketamine and reserpine, the reduction of chloral hydrate to trichloroethanol and the reduction of organic nitro compounds such as chloramphenicol and nitro-furan derivatives to reactive hydroxylamine or amino derivatives respectively. The authors stated that of the phase 2 conjugations in horses, glucuronic acid is quantitatively the most important while sulfation is restricted to phenols, aromatic amines, and steroids. The formation of thio-methyl and glutathione derivatives is also reported and suggests the presence of a thio-S-methyltransferase/s and a glutathione-S-transferase/s in horses.

Summary of equine drug metabolism

A full review of the *in vivo* equine drug metabolism literature would not have been possible in a single review paper, but it is hoped that the selected examples used highlight some of the interesting and important pathways in horses. A summary of all the published equine *in vitro* drug metabolism literature has been attempted and this data can begin to explain trends apparent in *in vivo* drug metabolism studies. Tables 2 and 3 summarize the evidence for the inference of phase 1 and 2 enzymes respectively in horses and where possible provide some comparison to man.

Overall, phase 1 equine metabolic pathways are generally better understood than those of phase 2. A significant factor contributing to the lack of phase 2 data is the non-specific *Helix pomatia* extract often used for hydrolyzing drug conjugates.

There are some reports of sexual dimorphism of drug metabolism in horses and there is also evidence for increased activity of many of the phase 1 and 2 enzymes with age.

Although drug-drug interaction literature for the equine is relatively sparse, the horse appears similar to man in its liability to enzyme induction by several compounds including barbiturates and rifampicin, and also to enzyme inhibition by compounds such as fluoroquinolones and quinidine.

The true extent of any inter-breed drug metabolism differences in the horse is unknown and the effect of physiological differences between horse and man, such as the horse's more flexible spleen, has not been fully determined. The fundamentally diverse digestive tracts between the two species are likely to have some effect on drug metabolism but little data is available. The equine caecum is capable of producing significant quantities of monoamines. These monoamines are absorbed into the body and their likely presence over many thousands of generations may have directed the evolution of certain drug metabolizing systems (i.e. levels of monoamine oxidases and certain CYPs/flavin mono-oxygenases[FMOs]). The concentrations of monoamines detected in the blood of horses vary with their diet according to the season.[247] It is therefore possible that any enzymes responsible for their metabolism may also show seasonal fluctuations through adaptive induction.

Activity indicative of many phase 1 drug metabolizing enzymes has been detected in horses and on the whole oxidative drug metabolism appears more extensive in horses than in man. Aromatic ring hydroxylation is notably increased in horses relative to man and N- and O-demethylation are also major pathways. The activity of the possible CYP subfamilies present in horses can be inferred using the characteristic substrate specificities of known CYPs. However, this can only act as a guide and further characterization of horse enzymes is necessary to validate the findings. All the major mammalian steroid biosynthetic pathways appear to be present in the horse and it is likely that a novel CYP is responsible for the formation of the B-ring unsaturated steroids. Lower activity of CYP1A, CYP2A and CYP3A isoforms has been detected in the horse relative to man although more work on CYP1A in the horse is necessary as not all the data are in agreement. The levels of CYP2B isoforms appear low in both species so it is difficult to make a quantitative comparison. The horse appears to have relatively high CYP2C and CYP2E activity in comparison to man, although further studies on CYP2E enzymes in the horse are necessary as there is again some conflicting data in the literature, such as observation that the catalytic activity of recombinantly expressed equine CYP2C92 was lower or equivalent to human CYP2C9.[50] Perhaps the most significant difference between the two species is the massively increased apparent activity of CYP2D in horses (as inferred from the rate of dextromethorphan O-demethylation). However, this increased activity could also originate from the presence of a different equine enzyme isoform; a theory supported by the lower relative activity of recombinantly expressed equine CYP2D50 compared to human CYP2D6.[49]

The presence of a range of phase 2 drugs conjugates in equine samples indicates that most of the phase 2 enzyme classes found in humans may also have homologs in the equine. As with humans, glucuronidation appears to be a major pathway. However, a notable difference between the species is that while horses tend to favour the sulfation of steroids (with the exception of those with a 17α-hydroxy group), glucuronidation predominated in man.[19]

Future Perspectives

The future direction of equine metabolism research for competitive sports will be influenced by several different factors, especially in the area of sports drug detection. The overall necessity for carrying out drug metabolism studies will depend on the matrices that are chosen for analysis. Although urine is currently the most popular matrix due to its ease of its post-race collection and generally higher drug concentrations, other matrices such as

Table 2A. Summary of the evidence inferring the presence of phase 1 enzymes in horses and comparison to man

Class of enzyme or type of reaction catalyzed	Evidence to infer its existence in horses	Comparison to man
CYP1A isoforms	Caffeine metabolism *in-vivo*. Fluoroquinolone inhibiting drug metabolism *in-vivo*. Phenacetin, ethoxyresorufin, methoxyresorufin and Benzo(a)pyrene metabolism *in-vitro*.	Caffeine, theophylline and phenacetin metabolism suggest lower activity in horse, but ethoxy- and methoxyresorufin suggest higher activity.
CYP2A isoforms	Coumarin metabolism *in-vitro*.	Coumarin metabolism suggests much lower activity in horse (roughly 4-fold).
CYP2B isoforms	Pentoxyresorufin, benzphetamine and benzyloxyresorufin metabolism *in-vitro*.	No evidence to compare directly but levels appear relatively low in both horse and man.
CYP2C isoforms	Meloxicam, omeprazole and warfarin metabolism *in-vivo*. P450h-1 characterisation, tolbutamide and mephenytoin metabolism *in-vitro*. Recently sequenced CYP2C92 in the horse.	Tolbutamide metabolism significantly higher in horse than man (roughly1.5-fold). No significant difference for mephenytoin.
CYP2D isoforms	Tramadol metabolism *in-vivo*. Quinidine inhibiting drug metabolism *in-vivo*. Dextromethorphan metabolism *in-vitro*. Recently sequenced CYP2D50 in the horse.	Tramadol metabolism more extensive in the horse. Dextromethorphan metabolism significantly higher in horse than man (roughly 20-fold).
CYP2E isoforms	Western blot localisation by anti-rat CYP2E1 antibodies. Chlorzoxazone, aniline, N-nitrosodimethylamine (NDMA) and p-nitrophenol metabolism *in-vitro*.	Chlorzoxazone metabolism significantly higher in horse than man (roughly 2-fold), although low rate of aniline and NDMA metabolism compared to rat.
CYP3A isoforms	Testosterone erythromycin, monensin, ethylmorphine and triacetyloleandomycin (TAO) metabolism *in-vitro*. Recently sequenced CYP3A89, CYP3A96 and CYP3A97 in the horse.	Testosterone 6β-hydroxylation significantly lower in horse than man (roughly 7-fold). Monensin, ethylmorphine, TAO and erythromycin metabolism lower in the horse than rat - therefore also lower than man.
CYP4A isoforms	Insufficient equine literature.	Insufficient equine data to make a full comparison.

Table 2B. Summary of the evidence inferring the presence of phase 1 enzymes in horses and comparison to man

Class of enzyme or type of reaction catalyzed	Evidence to infer its existence in horses	Comparison to man
FMOs	Caffeine and amphetamine metabolism *in-vitro*.	Insufficient equine data to make a full comparison.
Monoamine oxidases	N-Methytryptamine inhibition of adrenaline metabolism *in-vivo*.	Insufficient equine data to make a full comparison.
Alcohol dehydrogenases	*In-vitro* cDNA sequencing, cloning and studies of expressed proteins.	Equine isoform E active on ethanol, equine isoform S active on ethanol and steroids. Human gamma isoform displays altered steroid selectivity from equine S form.
Steroid CYPs	Some enzyme cDNAs sequenced/cloned and other enzymes inferred from production of metabolites.	All human classes are present in horse although some have different substrates specificities i.e. CYP17. The existence of the β-ring unsaturated steroids in the horse infers the presence of a novel CYP/s.
Steroid oxidoreductases	Some enzyme cDNAs sequenced/cloned and other enzymes inferred from production of metabolites.	All classes are present in both species although different levels of expression are apparent i.e. more 3β-hydroxysteroid dehydrogenase in horse liver.
Other reductive metabolism	Omeprazole, chloral hydrate, chloramphenicol *in-vivo*.	Insufficient equine data to make a full comparison.
Esterases/amidases	Local anaesthetic metabolism i.e. Lidocaine *in-vivo*.	Equine esterase data not reviewed in depth in this survey. Anecdotal comments in several papers state relatively high esterase activity exists, but no frames of reference are given.
Epoxide hydrolase	Enzyme isolation and partial characterisation *in-vitro*. Trans stilbene oxide metabolism *in-vitro*.	Insufficient equine data to make a full comparison.

Table 3. Summary of the evidence inferring the presence of phase 2 enzymes in horses and comparison to man

Class of enzyme or type of reaction catalyzed	Evidence to infer its existence in horses	Comparison to man
UGTs	Steroids, amphetamine, promazine, fenclofenac, benzoic acid and 2-napthyl-acetic acid metabolism *in-vivo*. 1-napthol metabolism *in-vitro*.	Glucuronic acid the most important quantitatively on the whole in man. Schmid[246] states that this is also the case for the equine, but does not give precise details. Glucuronic acid generally more important quantitatively for human steroid conjugation than the horse.
SULTs	Steroids, phenol and aromatic amines.	Schmid[246] states that steroids, phenols and aromatic amines can be sulfate conjugated in the horse, but does not cite many examples, therefore insufficient data to make a full comparison. Sulfate generally more important quantitatively for equine steroid conjugation than human.
Methyltransferases	Isoproterenol metabolism (catechol O-methyltransferase) *in-vivo*. Thiomethyl drug derivatives (thio S-methyltransferases) as reported by Schmid[246].	Insufficient equine data to make a full comparison.
Acetyltransferases	Sialic acids *in-vitro*, 5-aminosalicylic acid *in-vivo*.	Insufficient equine data to make a full comparison.
GSTs	Chlordinitrobenzene (CDNB) and ethacrynic acid *in-vitro*.	Insufficient equine data to make a full comparison.
Amino acid conjugation	Phenylacetic, benzoic and 2-napthyl-acetic acid with glycine and 2-napthyl-acetic acid and isoxepac with taurine (all *in-vivo*).	Insufficient equine data to make a full comparison.
Other conjugation pathways	Addition of a 2-carbon fragment to benzoic acid – possibly related to fatty acid elongation pathways.	Insufficient equine data to make a full comparison.

blood and hair are gaining popularity. Hair has the advantage of ease of collection and the ability to monitor concentrations of parent drug, such as steroid esters, for prolonged periods of time (previously unpublished equine studies from our laboratory). Blood is also a convenient matrix to collect, typically requires less sample preparation than urine, is more suitable for analysis of macromolecules and there is an argument that the circulating drug concentrations more accurately reflect the pharmacological activity.[248] Some countries, such as the USA, already rely heavily on blood testing[40] and others are using it more and more,[249] particularly for testing in training. A move to hair or plasma arguably reduces the need for metabolism studies since parent drug is generally sufficient for monitoring abuse in these matrices. However, urine is likely to remain an important matrix for a considerable period of time, especially since many of the internationally agreed thresholds (i.e. for many endogenous steroids and natural feed components) are based on urine.[1]

The increasing availability of 'designer' drugs and 'herbal' based nutraceuticals on the Internet[23] pose a potential threat to equine sports since trainers may seek to circumvent drug detection procedures by using these 'alternative' products. *In vitro* approaches to studying the metabolism of Internet-available designer drugs may be useful since these can be carried out quickly and conveniently and do not suffer from the ethical problems that may often prevent *in vitro* studies from being conducted.[23] The detection of herbal-based nutraceuticals is more challenging since many of the constituents are 'natural' products and hence may require threshold or metabolic biomarker approaches in order to discern any true cases of 'abuse'.

Although *in vivo* studies reflect the true metabolic disposition in any species, the recent developments in equine *in vitro* technologies offer many advantages and are therefore likely to be increasingly used in the future. These assays provide an approach to reduce and refine the number of *in vivo* studies. They may also assist in the identification of drug metabolites *in vivo* (producing a more concentrated, cleaner extract), in studying the fate of designer drugs, in developing a rapid response to potential new threats, in studying the detailed mechanisms of metabolism, and in generating metabolite reference standards for use as an alternative to *in vivo* post-administration urine samples in accordance with the 2009 ILAC-G7 guidelines.[59] The production of phase 2 reference standards is of particular interest as it offers the possibility of screening drugs in urine based on monitoring the intact glucuronide or sulfate conjugates, thus permitting LC-MS/MS detection and much simpler sample preparation protocols.

The recent availability of robust liquid-chromatography/high-resolution mass spectrometry (LC-HRMS) instrumentation is a significant development for drug metabolism research.[250] The acquisition of high-resolution/accurate-mass full scan data allows the simultaneous monitoring of all metabolites (at least those that will ionise using atmospheric pressure ionization) through the presence of $[M+H]^+$ or $[M-H]^-$ species without the need for targeted MS/MS experiments.[27] More detailed structural information can then be obtained through high–resolution monitoring of fragments induced in the ion source, in an ion trap or in a collision chamber.[23] Overall, the use of LC-HRMS allows for a faster and more robust assessment of the metabolite profile, although MS/MS still has its place for more targeted, high sensitivity analyses.

The recent sequencing of CYP2D50, CYP2C92, CYP3A89, CYP3A96, and CYP3A97 isoforms in the equine is of significance as it greatly enhances our understanding of the mechanisms of

metabolism in this species.[49,50,51] It also opens up the possibility in the future of making recombinantly produced versions of the enzymes commercially available in order to allow *in vitro* assessments of drug-drug interactions and routes of metabolism to be determined early during the veterinary drug development process. However, more sequencing studies are required before it can be determined whether these are the most abundant phase 1 drug metabolizing enzymes in horse liver or if there are also other important isoforms.

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