Characterisation of Equine Cytochrome P450s

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Abstract

Cytochrome P450s (CYPs) are a superfamily of enzymes involved in the phase I metabolism of endogenous and exogenous substances. They are present in almost all forms of life and have been studied extensively, particularly in relation to human medicine, where knowledge of their activities is essential for predicting drug-drug interactions. In the horse, little is currently known about CYP-specific drug metabolism, which holds importance for animal welfare and for doping control within the horseracing industry where drug-specific metabolites are tested for on race days. Recently the first recombinant equine CYPs have been produced, allowing specific data on equine P450 activity to be gathered for the first time. During the current study,46 full-length P450 sequences were identified from the equine genome. RT-PCR analysis was then carried out on equine liver in order to detect hepatic expression of P450s across various families. After this, cold-induction (pCold) *E. coli* were used for production of recombinant P450 proteins for subsquent functional testing. Four recombinant equine P450s were successfully expressed (CYP1A1, CYP2A13, CYP2C92 and CYP2D50). Due to being the isoforms most likely to be involved in drug metabolism, rCYP2D50 and rCYP2C92 were selected to be screened against ten of the most commonly used horse drugs to identify potential substrates. rCYP2C92 appeared to metabolise all four NSAIDs tested (flunixin, ketoprofen, phenylbutazone and diclofenac), however presence of the known hydroxylated metabolites of diclofenac and phenylbutazone (4-hydroxydiclofenac and oxyphenbutazone, respectively) could not be confirmed despite being present within equine liver microsome and human recombinant CYP2C9 samples. In spite of the apparant acivity displayed by rCYP2C92 towards all four NSAIDs, no

conclussions can be made about this enzyme's role in NSAID metabolism due to a

lack of known hydroxylated metabolite production.

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Contents

Chapter 1: Introduction

1.1: Overview

The study of what happens to exogenous substances when they enter the body can be traced back as far as the middle ages (Brater and Daly, 2000), however the field of pharmacology and evidence-based medicine did not fully develop until the 19th century, with the establishment of the first pharmacology department and, subsequently, the theory that drugs interact with receptors within the body (Rang, 2006). From here, the field grew exponentially, with the establishment of pharmacology departments throughout the world along with a focus on the actions of drugs on organs (Scheindlin, 2001).

Today, pharmacology can be separated into two main branches – pharmacodynamics and pharmacokinetics (PD and PK, respectively). Pharmacodynamics is the study of how a drug affects the body while pharmacokinetics relates to how the body affects the drug (Lees *et al*, 2004). Combined PK/PD models are used in clinical pharmacology as a critical part of the drug development process, allowing clinical effects and optimal dosing regimens to be determined (Derendorf *et al*, 2000).

PK can be further sub-divided using the acronym ADME – absorption, disposition, metabolism and excretion (Balani *et al*, 2005). ADME is an integral part of the drug development process and improvements in pharmacokinetic methods have resulted in less than 10% of New Chemical Entities (NCEs) failing at this stage by the 2000s (Kola and Landis, 2004). This is in contrast to almost 40% of NCEs failing due to ADME problems at this stage in the 1980s (Prentis *et al*, 1988).

1.2: The liver

The majority of drug metabolism takes place in the liver and as such it plays a central role in the study of drug metabolism (Sahi *et al*, 2010). As the largest internal organ, the liver carries out many vital roles within the body. Synthesis, metabolism and excretion are all essential liver functions, some examples of which are the storage of glycogen, the synthesis of various essential biological compounds such as cholesterol and glucose, breakdown of both ingested and exogenous substances and biliary excretion (Dias *et al*, 2009).

1.2.1: Liver architecture

Figure 1.2.1 displays an outline of liver anatomy. The liver is divided into lobes and has blood supply from the hepatic artery and portal vein, which provide the organ with nutrients and oxygen (Ishibashi *et al*, 2009). The liver can be further sub-divided into lobules, and figure 1.2.2 displays a schematic representation of a lobule while figure 1.2.3 shows a section of a lobule, highlighting the anatomical features. Lobules are the smallest structural component of the liver and within them hepatocytes line the capillaries in order to absorb materials in the blood supply via the sinusoids, which travels towards the central vein (Weibel *et al*, 1969).
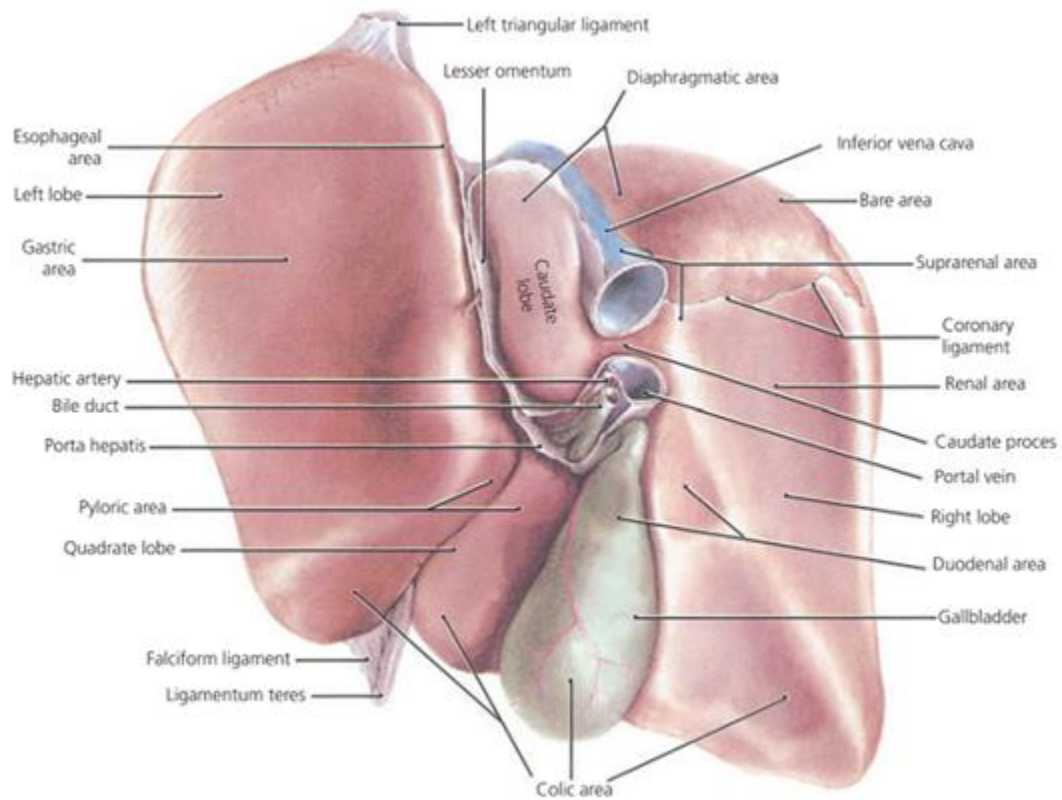
Figure 1.2.1: Anatomy of the liver. Lobes, blood supply and other anatomical features are highlighted. Adapted from "Textbook of Gastroenterology: Liver: Anatomy, Microscopic Structure, and Cell Types". By Kanel, G.C, 2009.
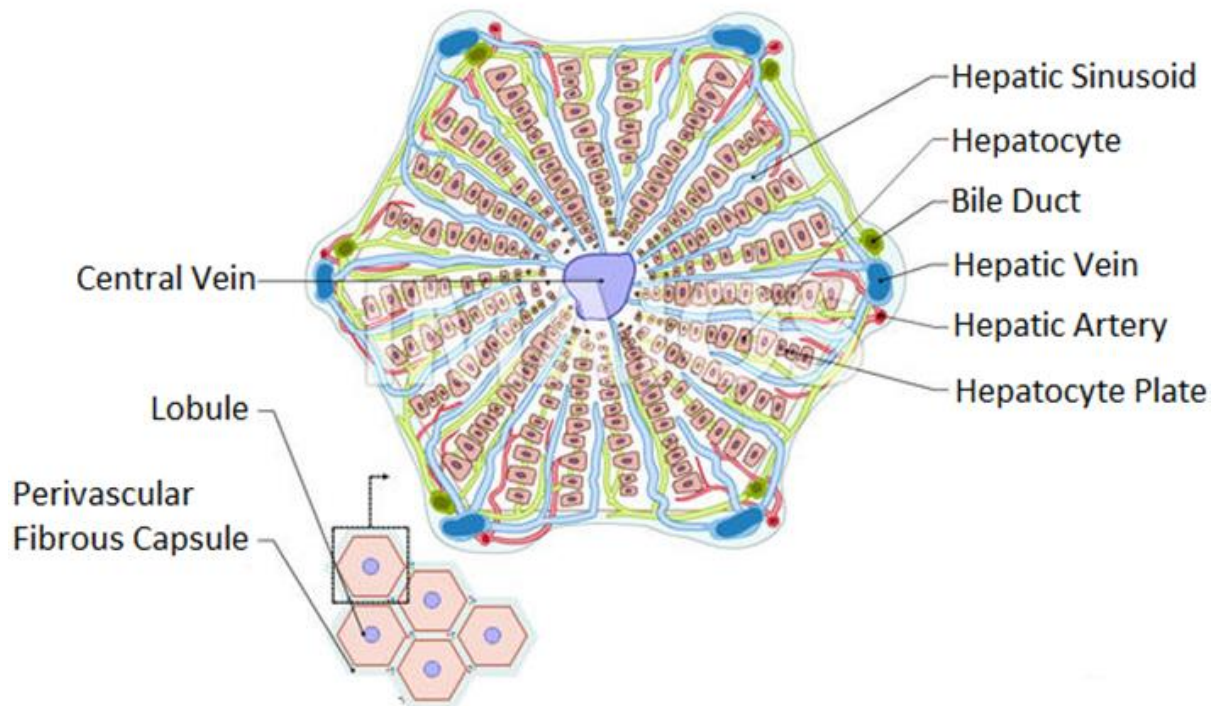
Figure 1.2.2: Schematic representation of a liver lobule. Hepatocytes line the capillaries of the lobule in order to absorb material present in the blood. © IMAIOS 2012.
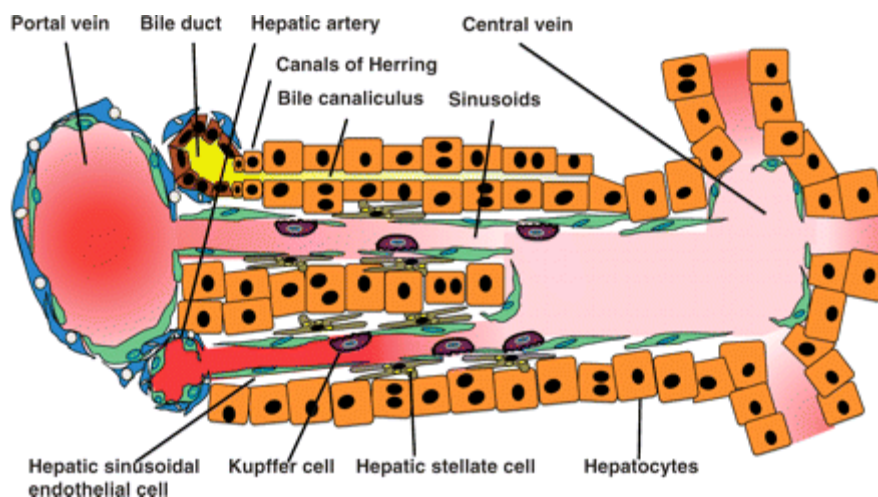
Figure 1.2.3: Schematic representation of a section of liver lobule. Blood floods towards the central vein through the portal vein and sinusoids. Adapted from "Liver stem/progenitor cells: their characteristics and regulatory mechanisms" by Tanaka, M, Itoh, T, Tanimizu, N and Miyajima, A, 2011, *Journal of Biochemistry*: 149(3):231-9.

Hepatocytes are the most important cells in the liver for its synthesis and metabolic functions and they make up around 80% of its volume (Godoy *et al*, 2013). The rest of the liver is composed of non-parenchymal cells - stellate cells, Kupffer cells and sinusoidal endothelial cells (Malik et al, 2002). Sinusoidal endothelial cells are involved in transport and filtration of macromolecules within the liver as well as some metabolic processes (De Leeuw *et al*, 1990), Kupffer cells mediate the immune response within the liver (e.g. phagocytosis and antigen presentation (Bilzer *et al*, 2006), and stellate cells (also known as fat-storing cells) play an essential role in regulation and storage of retinoids (Senoo *et al*, 2004) however when 'activated' they can contribute to liver fibrosis (Krizhanovsky *et al*, 2011). Making up most of the mass of the liver, hepatocytes are responsible for the majority of the liver's metabolic processes, from the synthesis of macromolecules to the metabolism of xenobiotics (Godoy *et al*, 2013).

1.3: Xenobiotic metabolism

When material enters the digestive tract, it may be toxic and consequentially a method of detoxification has evolved. The liver provides this detoxification method and can be seen as the first line of defence against potentially dangerous toxins after the digestive tract, consequentially meaning it is the major organ involved in drug metabolism (Gonzalez and Lee, 1996), since most drugs are administered orally (Mcginnity *et al*, 2004). It is a rich source of the enzymes involved in drug metabolism and various factors can affect the speed and efficacy that substances are processed here, such as age (Kinirons and O'Mahony, 2004), diet (Walter-Sack and Klotz, 1996) and gender (Tanaka, 1999). The large surface area of the liver means it is able to accommodate a higher level of drug metabolising enzymes

compared with other organs. The endoplasmic reticulum of hepatocytes provides a large surface area for drug metabolising enzymes and consequentially this is where such enzymes are accommodated (Stier, 1976). There are two distinct types of drug metabolising enzyme – phase I and phase II. Phase I can also be referred to as the modification phase and generally results in an active metabolite being produced (Omiecinski *et al*, 2011). Cytochrome P450s (or CYPs) are the major phase I metabolism enzyme family (see figure 1.3 for a chart of the major human phase I metabolism enzymes). Phase II (or conjugation) involves a diverse group of enzymes which generally take an 'active' substrate and deactivate it while making it water soluble and ready for excretion in urine or bile (Jakoby and Ziegler, 1990).
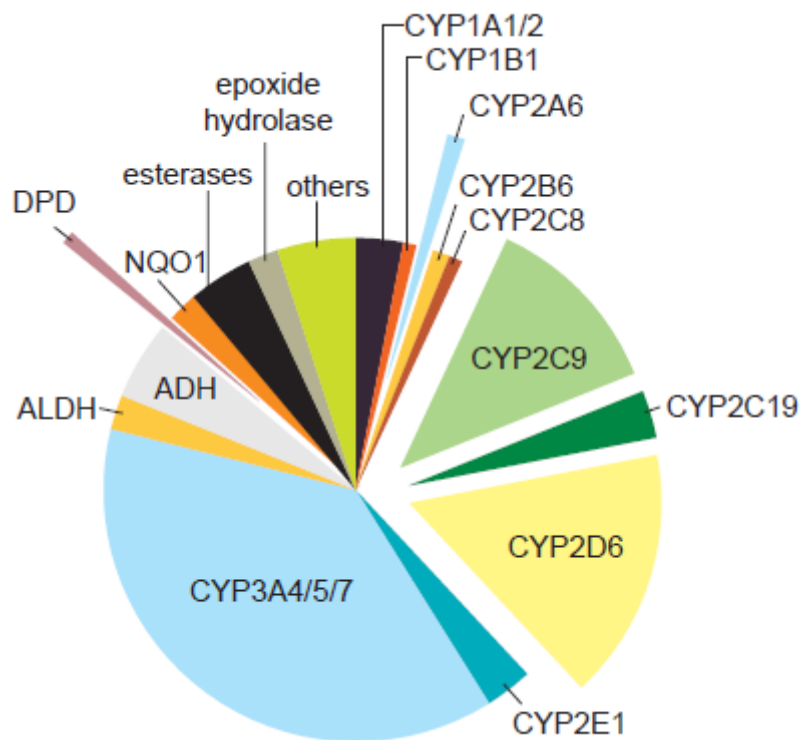
Figure 1.3: Contribution of phase I enzymes towards human drug metabolism. ALDH = aldehyde dehydrogenase, ADH, alcohol dehydrogenase, NQO1 = NAD(P)H dehydrogenase, quinone 1. Adapted from "Pharmacogenomics : Translating Functional Genomics into Rational Therapeutics" by Evans, W and Relling, M, 2009, *Science*: 286: 487

1.4: Cytochrome P450s

CYPs were first discovered by Klingenberg (1958) in pig liver microsomes and their presence was confirmed by Omura and Sato (1964) when it was noted that they showed an unusual absorbance spectrum of 450 nm when bound with carbon monoxide, hence the name P450. Like other cytochromes, they are haemproteins, containing a haem cofactor in their reaction site, and are involved in electron transfer reactions (Gray and Winkler, 1996). They are distinct however in that they are primarily involved in detoxification and steroidogenesis, unlike other cytochromes which are involved in cellular energy generation (Reedy and Gibney, 2004). They are classed as mixed function oxidases, or monooxygenases, due to the fact they act as catalysts for the introduction of a single molecular oxygen atom (with a simultaneous reduction of the other oxygen atom into H_2O) into their substrates (Sono *et al*, 1996).

CYPs are present in both eukaryotic and prokaryotic organisms, although it is important to note that not all living things contain them – *Escherichia coli* (*E. coli*), for example, lacks any P450 genes (Werck-reichhart and Feyereisen, 2000). There are two distinct classes of P450 – class I and class II, with class I being found predominantly in prokaryotes and class II in eukaryotes (Roberts *et al*, 2002). Class I P450s are found in the mitochondria (Omura, 2006). In bacteria, they often have roles in the metabolism of antimicrobial compounds while in eukaryotes they have thus-far only been attributed to endogenous metabolism, specifically relating to adrenal and sex hormones (Omura and Morohashi, 1995). Class II P450s (otherwise known as microsomal P450s) are solely a eukaryotic class of P450 and are responsible for the vast majority of xenobiotic metabolism in eukaryotes as well as metabolism of endogenous compounds (Nebert and Gonzalez, 1986).

1.4.1: Mechanism of CYP-based metabolism

As phase I metabolism enzymes, P450s can be seen as the first line of defence against potential toxins after digestion. Together with nicotinamide adenine dinucleotide (NADPH), cofactors (cytochrome b_5 and cytochrome P450 reductase) and oxygen they generally create an active metabolite (see figure 1.4.1). It is possible to summarise CYP-based metabolism based on the following four main stages (Meunier *et al*, 2004):

1. Substrate binding: this occurs near the haem cofactor, which is located in the active site. The spin-state is altered (low to high) and consequentially the spectral properties of the enzyme change, from ~420nm to 390nm (Schenkman and Jansson, 2006).

2. Reduction of the haem: an electron is transferred from NADPH and the haem cofactor is reduced to the ferrous state (Fe^{3+} to Fe^{2+}).

3. Binding of molecular oxygen: molecular oxygen binds to the ferrous haem iron to form a dioxygen complex.

4. Second reduction and subsequent protonation: the dioxygen complex is reduced and becomes negatively charged. This forms a peroxo group which is protonated twice and P450 compound I is released (Rittle and Green, 2010).

From here, the metabolites, which are most often active, move onto phase II of the metabolism process where they are generally detoxified and excreted.

(1st electron)

AH   P450^{3+} AH   e$^-$ ← CPR ← NADPH

P450^{3+} P450^{2+} AH

A.OH ← H$_2$O   O$_2$

P450^{2+} AH / O$_2$

e$^-$ ← b$_5$ ← b5R ← NADH

P450^{2+} AH / O$_2$ (2nd electron)
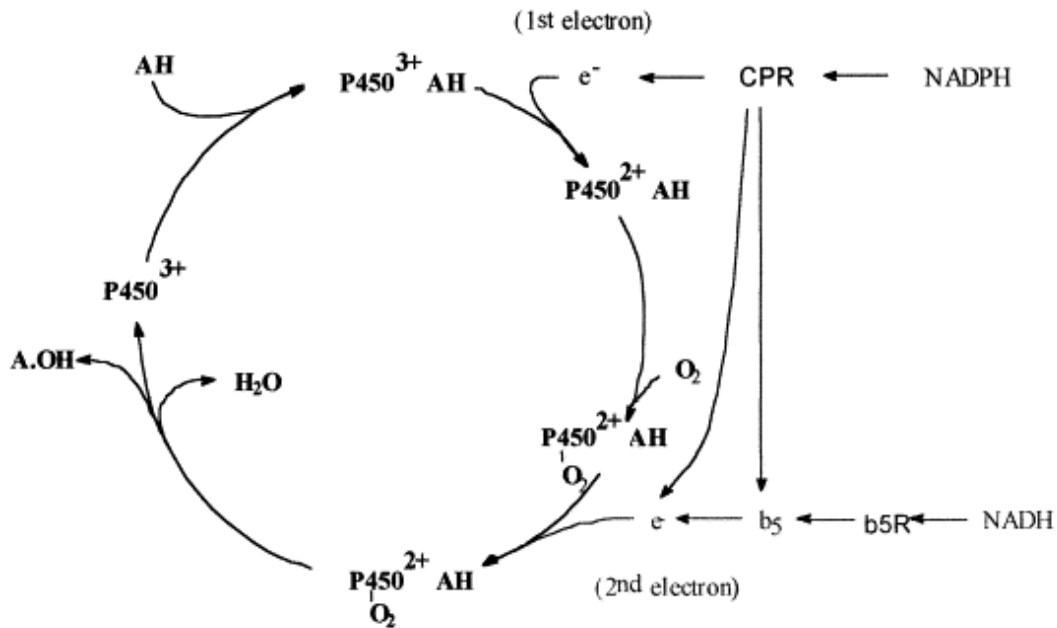
Figure 1.4.1: The P450 cycle. AH represents the substrate and CPR represents cytochrome P450 reductase. B5 represents cytochrome b5 and b5R represents cytochrome b5 reductase, which are involved in the electron transport cycle for some – but not all – P450s. Adapted from "Biodiversity of the P450 catalytic cycle: yeast cytochrome b5/NADH cytochrome b5 reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction" by Lamb, D.C., Kelly, D.E., Manning, N.J., Kaderbhai, M.A., Kelly, S.L., 1999, *FEBS Letters*: 462(3):283-8

1.5: Other phase I metabolism enzymes

Although P450s catalyse most of the phase I metabolic processes, there are numerous other enzymes, which are not related to them, involved in phase I metabolism. Most metabolise endogenous substances however a notable few are essential in drug metabolism, although some have the same substrates as CYPs. The most significant non-P450 phase I enzymes are flavin monooxygenases, monoamine oxidase, alcohol dehydrogenase, aldehyde dehydrogenase, aldehyde oxidase and xanthine oxidase (Beedham, 1997).

1.5.1: Flavin monoxygenase

Flavin Monoxygenases (FMOs) are found in microsomes along with the more abundant P450s and, like P450s, use NADPH as a cofactor (Zeigler, 1993). Many of the drug metabolites produced by FMOs are the same as for P450s thus it can be difficult to differentiate between them *in vivo* or in microsomal studies, however some substrate specificities are known (Cashman, 2000).They are involved in the metabolism of various xenobiotics ranging from pesticides to some drugs such as nicotine and tamoxifen (Damani *et al*, 1988, Parte and Kupfer, 2005). FMOs have also been implicated in trimethylaminuria ('fish odour syndrome') due to a lack of conversion of trimethylamine by FMO3 into the odourless metabolite trimethylamine-N-oxide (Messenger *et al*, 2013).

1.5.2: Alcohol dehydrogenases

Alcohol dehydrogenases are the major enzymes involved in alcohol metabolism in naive individuals (Edenberg, 2007), although ethanol is also a substrate of CYP2E1 (see later section). They are found predominantly in the liver and stomach however they are expressed in various other tissues at lower levels (Estonius *et al*, 1996). Alcohol dehydrogenase converts ethanol into acetaldehyde, the compound thought to be the major cause of hangovers (Kim *et al, 1994*). There are seven alcohol dehydrogenase genes in human (Jörnvall *et al*, 2000) and they also metabolise other alcohols in addition to ethanol (Pocker *et al*,1985, Cotton and Goldman, 1988). Alcohol dehydrogenases have also been implicated in the metabolism of the endogenous alcohol retinol (Hellgren *et al*, 2007).

1.5.3: Aldehyde dehydrogenase

Aldehyde dehydrogenase is the second enzyme involved in ethanol detoxification, where it oxidises acetaldehyde into a non-toxic metabolite (Crabb *et al*, 2004). There are 19 known aldehyde dehydrogenase genes in human (Marchitti *et al*, 2008). Variations in the aldehyde dehydrogenase genes have been linked to reduced prevalence of alcoholism (Crabb *et al*, 2004) as well as the alcohol flush phenomenon (Thomasson *et al*, 1991). The major site of aldehyde dehydrogenase expression is the liver although it is found in other tissues such as lung, ovary and stomach (Alnouti and Klaassen, 2008). Aldehyde dehydrogenases also metabolise other aldehydes such as formaldehyde and acrolein, which are found in cigarette smoke and car exhaust fumes, and dietary aldehydes such as benzaldehyde, which is used as a food flavouring (Vasiliou *et al*, 2004).

1.5.4: Monoamine oxidases

Monoamine oxidases are found in the outer membrane of mitochondria and metabolise biogenic amines and certain neurotransmitters (Tipton *et al*, 2004). The neurotransmitters they metabolise are monoaminergic neurotransmitters such as serotonin, noradrenaline and dopamine (Edmondson *et al*, 2004) and high/low levels of activity have been associated with mental illnesses such as schizophrenia and depression (Simpson *et al*, 1999, Meyer *et al*, 2006). Monoamine oxidase inhibitors are frequently used in psychiatry in order to increase the availability of the neurotransmitters they metabolise (Liebowitz *et al*, 1990). Monoamine oxidases are most abundant in certain types of neurons as well as placenta and platelets; however they appear to be expressed in most cell types (Chen, 2004). In addition to their link with mental illness, they have also been implicated in the development of

some neurodegenerative disorders via oxidative damage to mitochondria (Hauptmann *et al*, 1996).

1.5.5: Xanthine oxidase

Xanthine oxidase has received particular research attention due to its role in the development of conditions such as gout as well as its potential involvement in cardiovascular disease (Pacher *et al*, 2006). Its physiological role is not fully understood although some studies have suggested it has a bactericidal function (Silanikove *et al*, 2005), and others imply it may have a role in the production of antioxidants (Frederiks and Bosch, 1995). Its most well-studied role relates to its involvement in purine and pyramidine metabolism however it is also important in the metabolism of some drugs, such as caffeine (Rashidi and Pashaei-asl, 2009).

1.6: Phase II metabolism (conjugation)

Phase II metabolism is often also termed conjugation due to the fact that in this stage of drug metabolism, the (generally) active metabolites from phase I are conjugated with compounds which make them less active and ready for excretion (Jakoby and Zeigler, 1990). Like with phase I enzymes, phase II enzymes are ubiquotous throughout the body, but are most highly expressed in the liver (McCarver and Hines, 2002). Phase II reactions have received less research attention that phase I enzymes, mostly because drug-drug interactions involving them are very rare (but not absent). Adverse drug reactions due to phase II metabolism can have substantial clinical significance (Bjornsson *et al*, 2003). One of the most well-known examples involves cats and their relative lack of the phase II enzyme UDP-glucuronosyltransferase – low expression levels here means the

enzyme is unable to convert in sufficient quantities the toxic metabolite of CYP-based metabolism into its inactive form, which can result in severe hepatotoxicity (Allen, 2003). The main phase II enzymes are UDP-glucoronosyltransferases (UGTs), sufotransferases (SULTs), n-acetyltransferases (NATs) and glutathione S-transferases (GSTs), Jancova *et al* (2010).

UGTs catalyse the most abundant conjugation reaction – conjugating the substrate with α-D-glucuronic acid (Cashman *et al*, 1996). UGTs are highly expressed and between 40 and 75% of drugs are metabolised by them (Wells *et al*, 2004) and they are located on the luminal side of the endoplasmic reticulum, giving them direct access to phase I metabolism products (Tukey and Strassburg, 2000). At least 117 UGTs have been identified in mammals (Mackenzie *et al*, 2005).

SULTs conjugate various compounds but are particularly important in phenol, amine and alcohol metabolism (Glatt and Meinl, 2004). There are four families of SULT in human (SULT1, SULT2, SULT4 and SULT6) with there being 13 total known (Lindsay *et al*, 2008). NATs are primarily involved in the metabolism of compounds such as aromatic amines and hydrazine (Butcher *et al*, 2002). Two forms of the enzyme are found in humans – NAT1 and NAT2 (Hein *et al*, 2000). GSTs are mostly found in the cytosol and are involved in many important xenobiotic reactions as well as playing a role in protection from oxidative stress and metabolism of prostaglandins and steroids (Sheehan *et al*, 2006).

1.7: Evolution of the P450 superfamily

The cytochrome P450 superfamily can be traced back to an ancestral gene which arose around 3.5 billion years ago (Nelson *et al*, 1993). When oxygen levels increased on earth as a result of photosynthetic organisms, systems using oxygen

were able to develop at a much faster rate with the result that the eukarya expanded rapidly (Knoll, 1992). The Devonian period – when land was colonised by plants and animals – is when the biggest explosion in P450s took place, and also when the largest rise in environmental oxygen occurred (Cloud, 1976).

When multicellular organisms started to evolve, many more endogenous chemicals arose which P450s were able to evolve to cope with (Lewis, 1997). By around 1000 million years ago sexual reproduction had developed with the consequence of steroid hormones requiring biosynthesis (Close *et al*, 2010). As life forms grew in size, they developed more complex and diverse metabolic processes – from here P450s branched out to allow for these new substrates to be metabolised efficiently (Nebert *et al*, 1989). This resulted in the arrival of the CYP3 and CYP4 families (McArthur, 2003, Nelson, 1998). The CYP1 and CYP2 families diverged next, followed by CYP17 and CYP21, with the end result being that separate CYPs had evolved to deal with mostly endogenous or mostly exogenous substrates (Lewis and Sheridan, 2001).

During the Devonian period, P450 systems were expanded on - from their origins as enzymes purely involved in the metabolism of endogenous substrates, to being used to detoxify potentially dangerous plant materials (which had evolved as a method to cope with predators), sometimes known as 'animal-plant warfare' (Gonzalez and Nebert,1990). Likewise, the evolution of plants and insects is also tied together in this way. Many insects eat plants that contain compounds toxic to most species, such as tobacco hornworm consuming the leaves of tobacco plants which contain nicotine (Snyder *et al*, 1993) and black swallowtail being able to resist the toxicity of its host plant (Ma *et al*, 1994). P450s have developed to cope with this insect-plant warfare much like they have with animal-plant warfare (Futuyma and Agrawal, 2009).

The most ancient P450, sterol 14-demethylase (P45014DM or CYP51 for the fungal form), is thought to be responsible for the viability of eukaryotic cells; playing a vital role in the biosynthesis of the sterols that make up an essential component of eukaryotic cell membranes (Yoshida *et al*, 2000) and is the only CYP which is spread throughout eukarya as well as being found in some bacteria such as *Mycobacterium tuberculosis*, although no role in steroid metabolism here is apparent (Lepesheva and Waterman, 2004). Despite the major role this CYP appears to have played in evolution, no conclusive evidence has been drawn to suggest its exact origin – suggestions have been made for both a bacterial (Yoshida *et al*, 1997) and plant (Renzen *et al*, 2004) origin.

P450s can be found in most, but not all, forms of life. Eubacteria such as *E. coli*, for example, contain no CYP genes, nor do archaea (Kelly and Kelly, 2013). There are currently over 21,000 P450 genes known, across all kingdoms of life (Nelson, 2009) with the highest number- ~5100 - being in plants (Nelson and Werck-Reichhart, 2011). In addition to the role CYP51 has played in eukaryotic evolution, numerous other P450s have contributed towards the evolution of life as we know it, such as the ability of plants to produce pollen (Hamberger and Bak, 2013), the survival of insect hatchlings (Qui *et al*, 2012) and the viability of life moving from the sea to dry land (Omura, 2013).

1.8: P450 structure, function and conservation

CYPs generally range in size from 42KDa to 62KDa and the first crystal structure of a CYP was generated in 1987 by Poulos *et al* (P450cam from *Pseudomonas putida*). From the start of the 21st century, many more crystal structures have become available. The first mammalian crystal structure – rabbit CYP2C5 - was deduced by

Williams *et al* (2000). Three years later, the same group elucidated the first human CYP crystal structure (CYP2C9), Williams *et al* (2003). In the 11 years since then, the number of structures of P450s available has exploded and there are now hundreds of unique structures on the protein data bank (www.rcsb.org, Berman *et al* (2000)).

Structural determination of P450 enzymes is of particular interest to the pharmaceutical industry as this could allow for inexpensive and efficient means to screen drug candidates against P450s – determining whether a compound is likely to interact with a P450, either as an inhibitor or as a substrate (Lewis and Ito, 2009). Although structures now exist for various P450s, there are other approaches to estimating drug interactions with a particular CYP. Ligand models and homology modelling are two alternative approaches (Lui *et al*, 2013). Whereas X-ray crystallography and NMR spectroscopy can pose a challenge due to difficulties with crystallisation, solubility or size (De Groot, 2006), homology modelling in particular can be a useful substitute (Vyas *et al*, 2012). Homology modelling involves taking an amino acid sequence of a protein and generating a 3D model of its structure based on a related protein – it relies on the general observation that amino acid sequences generally correlate with tertiary structural features (Kaczanowski and Zielenkiewicz, 2010). Homology modelling has been used for various P450s based on those crystal structures that are available (Lewis, 1999). Ligand modelling can take the form of either pharmacophore or 3D-QSAR modelling (Quantative structure-activity relationship, which is a type of modelling that involves defining the mathematical relationship between structure and function) and has been used for various P450s thus far (De Groot and Ekins, 2002).

The general structure of a CYP involves a conserved C-terminal containing the haem-binding domain as well as conserved k-helix and PERF domains (Denisov *et al*, 2005). Microsomal CYPs also contain a conserved N-terminal for anchoring to the ER membrane (Poulos, 2005).

The primary structure of CYPs varies significantly across families; however protein folding is highly conserved, in both microsomal and mitochondrial enzymes (Graham and Peterson, 1999). The most conserved area is the haem-binding region and consequentially it provides a method to quickly determine whether a sequence might belong to a P450 as the motif FxxGxxxCxG is found in all P450 sequences (Ranasinghe and Hobbs, 1998). As far as substrate specificity is concerned, it seems there are specific residues within the primary structure that are linked with the substrate-binding region (Zharkova *et al*, 2013). The most thorough map of this was compiled by Zawaira *et al* (2011) as an expansion to the work of Gotoh (1992) where residues involved in substrate recognition within the CYP2C family were deduced. Zawaira *et al* used ten different CYP isoforms across multiple mammalian species and found 33% of the sequence was involved in substrate recognition. Furthermore, numerous studies have been carried out in order to ascertain the specific residues involved in recognition of specific substrates. Melet *et al* (2003) found that the residues Phe114, Ser356 and Phe 479 were essential for the binding of two different drug substrates (sulfaphenazol and diclofenac). Likewise, a study carried out by Van Waterschoot *et al* in 2006 found that Phe120, Glu216 and Thr309 were involved in substrate binding in CYP2D6. Many of the major CYPs have been studied this way, including 2E1 (Collom *et al*, 2006), 2B4 (Sulc *et al*, 2008), 2A6, 2A13 (Devore *et al*, 2011), 2B6 (Shah *et al*, 2011), 3A4 (Roussel *et al*, 2000) and 1B1 (Wang *et al*, 2011).

1.9: P450 nomenclature convention

Originally, P450s were named based on certain phenotypic characteristics such as substrate specificity and enzymatic activity however as increasing numbers of CYPs were isolated it became apparent that this system was not wholly efficient, largely because overlaps of enzymatic activities between different families were possible (Nelson, 2004). In 1987, a new system was devised to combat this issue whereby CYPs were named based on amino acid sequence identity (Gonzalez, 1990a). This system meant that P450 names would all start with 'CYP' followed by a number denoting their family (determined based on whether sequence similarity is >40%), a letter for their subfamily (>55% sequence identity) and a number to denote the specific gene - e.g. in CYP3A4, '3' represents the family, 'A' is the subfamily and '4' refers specifically to the gene itself. A sequence identity variation of >3% is needed for a gene to be classified as unique (Nelson *et al*, 1996). P450 pseudogenes also have a nomenclature system, with naming being based on how complete the gene is - e.g. the suffix 'P' is used if the gene is near full-length and if it is so short that it cannot be assigned to a family, the suffix 'un' is used (Nelson *et al*, 2004a). For polymorphisms, an asterisk is used after the gene name followed by a number to denote the allele, e.g. CYP2C9*3 represents the CYP2C9 polymorphism Leu359 (Sullivan-Klose *et al*, 1996). Before a new P450 can be named, it must be submitted to a P450 nomenclature committee for approval in order to ensure names are not repeated and rules are adhered to (Nelson, 2004).

1.10: Expression and tissue distribution of P450 enzymes

Cytochrome P450s are expressed to the largest degree in the liver however they are present in almost all tissues, ranging from the brain to kidney, lung, spleen,

leukocytes, heart and kidneys amongst others, and their expression patterns vary in each tissue (Seliskar and Rozman, 2007). Although it is the major source of P450s, not all isoforms are expressed in the liver. P450s can play important endogenous roles when expressed differentially within specific tissues (Nebert and Dalton, 2006). For example, some have been shown to be involved in maintaining vascular homeostasis in the cardiovascular system (Flemming, 2001), involved in regulation of cerebral blood flow (Harder *et al*, 1998) and essential for the determination of germ cell fate in mice (Bowles *et al*, 2006). Outside of the liver, the intestines, kidneys and lung have relatively high P450 content (Preissner *et al*, 2013).

On a subceullar level, eukaryotic P450s are all membrane-bound and are primarily found in microsomes, anchored to the endoplasmic reticulum via the N-terminal (Sakaguchi *et al*, 1984). Eukaryotes also possess mitochondrial P450s, which make up around 10% of known human CYPs (Nelson *et al*, 2004a). Mitochondrial P450s are involved in the biosynthesis of steroids (Omura and Morohashi, 1995) and Vitamin D3 metabolism (Masumoto *et al*, 1988). There have been some suggestions of involvement in xenobiotic metabolism (Honkakoski *et al*, 1988, Niranjan *et al*, 1984) as well as microsomal-type P450s being found in the mitochondria (Shayig and Avadhani, 1989). Mitochondrial xenobiotic-metabolising P450s may also play a more significant role in pathophysiology than originally thought (Knockaert *et al*, 2011), although more research is needed in the area before conclusions can be drawn. Outside of microsomes and mitochondria, P450s have been found in the nucleus of ovarian cancer patients (Leung *et al*, 2005), the cytosol in mice with haem impairment (Meyer *et al*, 2005) and on the plasma membrane in patients with autoimmune hepatitis (Loeper *et al*, 1993).

CYP expression levels can vary across different people and within an individual can be influenced by various factors. The biggest influencing factors on P450 expression are sex, age, diet and lifestyle (Kramer and Testa, 2009). Sex differences have had adverse clinical consequences particularly for women due to their historical underrepresentation in clinical trials (Schmucker and Vesell, 1993). Hormonal changes have been attributed to differences in P450 expression in women (Kashuba and Nafziger, 1998) and some specific P450 isoforms seem to have variable activity according to gender, such as CYP1A2 being more active in men and the metabolism of some CYP3A4 substrates being faster in women (Parkinson *et al*, 2004). Aging has been studied significantly in regards to drug metabolism as it can affect drug disposition to a significant extent, particularly since individuals are more likely to be receiving treatments with multiple drugs when they get older (Jörgensen *et al*, 2001). Reasons for aging-related changes in drug metabolism are numerous, such as decreased gastric motility altering oral bioavailability (Orr and Chen, 2002) and changes in body composition (Beaufrère and Morio, 2000) altering the volume of distribution of some drugs. However, as far as P450 expression levels are concerned, in humans it does not seem expression decreases with age (Parkinson *et al*, 2004) although in rats there are significant differences with age (Vieira-Brock *et al*, 2013). Diet and lifestyle can affect P450 metabolism as many substances consumed through the diet have the potential to either inhibit or induce P450 enzymes (Boullata and Hudson, 2012). Lifestyle factors play a similar role, with smoking status (Rasmussen *et al*, 2002) and alcohol consumption (Girre *et al*, 1994) altering drug metabolism to some extent. Another lifestyle factor that may affect P450-based metabolism is exercise, with some evidence suggesting certain P450

enzymes are upregulated during exercise (Vistisen *et al*, 1991, Frenk *et al,* 1980), although this has been disputed (Michaud *et al*, 1994).

1.11: Human P450s

P450s have been studied in humans more than in any other species, largely due to the fact they hold much importance therapeutically. There are 57 P450 genes in the human genome and about 75% of all drugs are metabolised by CYPs in humans (Guengerich, 2008), and they have become a central issue in clinical science; affecting treatment regimens and drug development to a great extent (Ingelman-Sundberg, 2004). Drug-drug interactions are a particular concern, since some drugs can inhibit or induce P450s (Rendic, 2002). Consequently the major research focus has been around characterising potential drug-drug interactions in order to negate harmful interactions occurring in patients treated with multiple drugs at once (e.g. Overholser and Foster, 2011, and Zhou *et al*, 2003).

The major xenobiotic metabolising CYPs in humans are CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP2E1 (see figure 1.3). Others of important clinical importance are CYP2C8, CYP2B6, CYP2A6, CYP1B1, CYP1A1 and CYP1A2 (Evans and Relling, 1999). Importantly, these CYPs also have significant roles outside of drug metabolism (Ding and Kaminsky, 2003).

1.11.1: The CYP1 family

The CYP1 family includes CYP1A1, CYP1A2 and CYP1B1 and research attention around them has had particular focus on their ability to bioactivate polycyclic aromatic hydrocarbons (PAHs) and other procarcinogens to generate carcinogenic metabolites (Baird *et al* , 2005). Compared with other CYPs they do not contribute

heavily towards drug metabolism due to their largely extrahepatic nature (although CYP1A2 metabolises some important drugs) but nevertheless have high clinical importance due to their role in carcinogenesis (Cui and Li, 2014), Go *et al*, 2015).

1.11.1.1: CYP1A1

There is very little CYP1A1 expression in the adult liver; however it is expressed in the foetal liver (Kitada *et al*, 1991) and evidence suggests it can be induced in adult liver by PAHs such as benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene and benzo[k]fluoranthene (Galván *et al*, 2005). It is primarily expressed in the lung (Shimada *et al*, 1992) although can also be found in the placenta (Lucier *et al* 1987) as well as lymphocytes and monocytes (Robie-Suh *et al*, 1980). When discovered, CYP1A1 was named aryl hydrocarbon hydroxylase due to its role in the 3-hydroxylation of benzo[a]pyrene (Nebert and Gelboin, 1968).

Induction of CYP1A1 has been studied extensively due to the enzyme's link to lung cancer in smokers as smoking induces lung CYP1A1 expression (McLemore *et al*, 1990). Other substances which induce CYP1A1 expression are heterocyclic amines and polychlorinated biphenyls (Lucier *et al*, 1987) as well as the drug omeprazole, which acts as an aryl hydrocarbon-like inducer for the CYP1A subfamily (Diaz *et al*, 1990).

1.11.1.2: CYP1A2

Unlike CYP1A1 and CYP1B1, CYP1A2 is largely expressed in the liver rather than lung and consequentially is the only 1A P450 involved significantly in drug metabolism (Shimada *et al*, 1996). There is some evidence that CYP1A2 is at least present at the mRNA level in the lower digestive tract (Mercurio *et al*, 1995) although

to date no expression in the lung has been documented despite much searching. Around 10-15% of total hepatic P450 content is CYP1A2 (Breimer and Schellens, 1990). CYP1A2 has a large list of drug substrates, with some examples being paracetamol (which is also a substrate of CYP2E1 and CYP3A4, Patten *et al*, 1993), caffeine, clozapine, melatonin and bufaralol (Wang and Zhou, 2009). In addition to being a substrate, caffeine is also a potent inducer of CYP1A2 (Chen *et al*, 1996). Other inducers are charbroiled food, tobacco smoking, omeprazole, cruciferous vegetables and even exercise (Vistisen *et al*, 1992). CYP1A2 induction can have profound effects on treatment regimens involving drugs metabolised by the enzyme (Gunes and Dahl, 2008). One of the most well-known examples involves treatment using certain antipsychotics in smokers, since drugs such as clozapine, haloperidol and olanzapine are metabolised by CYP1A2 and thus smokers require higher dosages to achieve adequate effects (de Leon, 2004). Similarly, smokers also consume more caffeine on average than non-smokers due to CYP1A2 induction (Swanson *et al*, 1994).

CYP1A2 has the ability to bioactivate certain carcinogens, especially heterocyclic and aromatic amines, although it has other carcinogenic substrates such as polycyclic and nitropolycyclic hydrocarbons (Nebert and Dalton, 2006). High CYP1A2 activity has been associated with colorectal cancer via food-borne heterocyclic amines (Lang *et al*, 1994). Inhibitors of CYP1A2 include polycyclic acetylenes (Shimada *et al*, 1998), furafylline (Racha *et al*, 1998) and fluvoxamine (Brøsen, 1995).

1.11.1.3: CYP1B1

CYP1B1 is most highly expressed in the kidneys and is also expressed in the spleen, prostate, thymus, lung, small intestine, ovaries, uterus, large intestine and mammary glands (Shimada *et al*, 1996a). CYP1B1 expression in these tissues has particular significance as it has been associated with malignant tumour development (Murray *et al*, 1997). Additionally, CYP1B1 mutations have been associated primary congenital glaucoma therefore is thought to play a role in foetal development (Stoilov *et al*, 1998).

Like with CYP1A1 and CYP1A2, CYP1B1 has the ability to bioactivate procarcinogens such as polycyclic hydrocarbons, heterocyclic and aromatic amines and nitropolycylic hydrocarbons (Shimada *et al*, 2001). It has also been implicated in oestrogen-related cancers where it produces carcinogenic metabolites from the metabolism of 17-beta-estradiol (Hayes *et al*, 1996) and oestrone (Shimada *et al*, 1999).

Various polycyclic compounds are strong inhibitors for CYP1B1 (Shimada *et al*, 1998). There are a number of other known inhibitors, one of the most interesting of which is a compound called resveratrol found in red grapes which is known to inhibit the development of cancer (Chang *et al*, 2000). Importantly, it seems the anti-cancer effect of this compound is also aided by the fact CYP1B1 metabolises it into piceatannol which is known to have anti-cancer properties (Potter *et al*, 2002). Other anticancer compounds also seem to have inhibitory effects on CYP1B1 (Rochat *et al*, 2001).

1.11.2: The CYP2 family

The CYP2 enzymes are much more diverse than the CYP1 enzymes, catalysing a huge variety of reactions (Rendic, 2002). CYP2 enzymes are responsible for as much as 50% of all drug metabolism, the majority of which is carried out by CYP2C9, CYP2C19, CYP2D6 and CYP2E1 (Lewis, 1998). P450s of the CYP2 family tend to, in comparison to other CYPs, have substrates that are of small to medium molecular weight, and despite the variety of substrate specificities amongst members of the subfamilies, there is some overlap; particularly between CYP2A and CYP2E (Rendic and Di Carlo, 1997).

1.11.2.1: CYP2A

There are three CYP2A P450s in human – CYP2A6, CYP2A7 and CYP2A13 (Su *et al*, 2000). Although they are not major drug-metabolising enzymes, they have received research attention due to CYP2A6 and its role in metabolising nicotine as well as the bioactivation of many carcinogens (Fernandez-Salguero and Gonzalez, 1995, Messina *et al*, 1997). CYP2A6 accounts for around 1-10% of total hepatic P450 content and is also expressed in the respiratory tract (Su *et al*, 2000). CYP2A7 meanwhile appears to be liver-specific and has no documented catalytic activity while CYP2A13 has low hepatic expression and is predominantly expressed in the respiratory tract (Su *et al*, 2003). Although little is known about its activity, CYP2A13 seems to have some overlap in substrate specificity with CYP2A6, metabolising substrates such as nicotine, cotinine and coumarin (Fukami *et al*, 2007). Drugs metabolised by CYP2A6 include the anti-epileptic drug losigamone and the anticonvulsant valproic acid (Raunio *et al*, 2001).

1.11.2.2: CYP2C9

CYP2C9 is one of four known human CYP2C enzymes (the others being CYP2C8,
CYP2C18 and CYP2C19) and is a major drug metabolising enzyme, contributing to
the metabolism of around 20% of drugs in humans (Breimer *et al*, 1990). Despite
sharing high sequence similarity, there is little overlap in substrate specificities
across the CYP2C enzymes (Goldstein and de Morias, 1994). It is the second most
highly-expressed P450 in the liver (Shimada *et al*, 1994) and has also been found in
the intestine (Obach *et al*, 2001). One of the first major CYP2C9 substrates
discovered was the anti-epileptic phenytoin (Shimada *et al*, 1986) while drugs such
as warfarin and various non-steroidal anti-inflammatory drugs (NSAIDs) have
received much research attention due to their clinical significance relating to drug-
drug interactions and CYP2C9 polymorphisms (Rettie and Jones, 2009). The extent
of polymorphisms in CYP2C9 was first highlighted when two variations (Cys144 and
Leu359) of the gene were discovered within a Caucasian population (Stubbins *et al*,
1996). Subsequent studies have suggested around 35% of Caucasians possess one
of six CYP2C9 allelic variations, although the rate of polymorphisms is substantially
lower in other ethnic groups (Lee *et al*, 2002). Significantly, polymorphisms have
been associated with various severe adverse drug reactions, such as prolonged
bleeding times during warfarin therapy (Aithal *et al*, 1999) and overdose with
phenytoin (Ninomiya *et al*, 2000). They have also been associated with reduced
clearance time of drugs, with some polymorphisms generating as little as half the
clearance rate of the wildtype for a variety of clinically important drugs (Kirchheiner
and Brockmöller, 2005).

In addition to xenobiotics, CYP2C9 metabolises some endogenous substances such as vitamin A (McSorley and Daly, 2000) and linoleic acid (Draper and Hammock, 2000). It also has various well-characterised inhibitors, including the anti-fungals sulfaphenazole (Veronese *et al*, 1990), miconazole and fluconazole (Iwa *et al*, 2005), and tienilic acid as a mechanism-based inhibitor via s-oxygenation (Dansette *et al*, 1992).

1.11.2.3: CYP2C19

CYP2C19 is mostly expressed in the liver, accounting for around 5% of total hepatic P450 content (Breimer and Schellens, 1990), although it has also been detected in the intestines (Lapple *et al*, 2003) and brain (Booth Depaz *et al*, 2015). CYP2C19 does not metabolise as many drugs as CYP2C9 although it does have some significant clinical importance, particularly relating to a polymorphism that was first discovered in mephenytoin metabolism, noting a genetic deficiency of the hydroxylation of the drug (Kupfer and Preisig, 1984). In total there are eight CYP2C19 polymorphisms that can result in poor metabolism and have been estimated to be present within 12-23% of Asian populations, 1-6% of Caucasian populations and 1-1.75% of black African populations (Desta *et al*, 2002). Substrates primarily metabolised by CYP2C19 include mephenytoin, omeprazole (Karam *et al*, 1996), thalidomide (Ando et al, 2002) and clopidogrel (Hulot *et al*, 2006). CYP2C19 has some involvement with steroids such as progesterone and testosterone, playing a relatively important role in their oxidation (Yamazaki and Shimada, 1997).

1.11.2.4: CYP2D6

CYP2D6 is the second most important drug metabolising enzyme, with involvement in around 25% of xenobiotic metabolism (Evans and Relling, 1999). Despite this,

hepatic expression levels are relatively low, accounting for around 5% of total liver

P450 content, although this figure can be as low as 1% in some individuals

(Shimada *et al*, 1994). CYP2D6 expression has also been detected in lung (Guidice

et al, 1997), and brain (Siegle *et al*, 2001) where higher expression levels have been

associated with alcoholism (Miksys *et al*, 2002). The first discovered CYP2D6

substrate was debrisoquine, and this was also the first xeonobiotic found to be

subject to polymorphic metabolism (Mahgoub *et al*, 1997). CYP2D6 metabolises a

range of highly prescribed drugs such as antidepressants, beta-blockers, opioids and

antiarrhythmics (Ingelman-Sundberg, 2005). Consequentially CYP2D6

polymorphisms are the most clinically significant of the P450 polymorphisms and can

cause a range of adverse reactions. Although a few endogenous substrates have

been found relatively recently (Yu *et al*,2003, Yu *et al*, 2003a), it seems CYP2D6

does not play any major physiological role, since individuals lacking an active gene

appear to have no major negative consequences, although psychological studies

have suggested that personality may be affected in CYP2D6 poor metabolisers due

to lack of metabolism of certain neurotransmitters (Llerena *et al*, 1993, Roberts *et al*,

2004). Additionally, it has been suggested that patients possessing certain

polymorphisms are more likely to develop schizophrenia (Llerena *et al*, 2007)

although this finding conflicted with previous reports of no association (Daniels *et al*,

1995). Unlike many other CYPs, CYP2D6 does not appear to be inducible (Zanger *et

al*, 2004) although inhibitors include various cardiovascular drugs (Otton *et al*, 1984),

bupropion (Kotlyar *et al*, 2005) and some antidepressants (Ereshefsky *et al*, 1995).

There are at least 74 alleles of CYP2D6 (Zhou, 2009), giving phenotypes that can

either generate no metabolism, poor metabolism, 'normal' metabolism or ultra-rapid

metabolism – and of these 74 alleles, 15 are known to code non-functional proteins

(Wolf and Smith, 1999). Understandably, the vast range of phenotypes an individual may possess can create many different clinical outcomes, from those that are undetected to those that are fatal (Zanger *et al*, 2004). Examples of some adverse drug reactions that can occur due to CYP2D6 polymorphisms are excessive morphine concentrations during codeine treatment of ultra-rapid metabolisers (Crews *et al*, 2012), an increased risk of peripheral neuropathy amongst poor metabolisers treated with the antianginal drug perhexiline (Shah *et al*, 1982), an increased risk of central nervous system side effects for poor metabolisers treated with the antiarrythmic propafenone (Siddoway *et al*, 1987) and a longer duration of pulmonary effects in poor metabolisers treated for bronchoconstriction with maprotiline (Firkusny and Gleiter, 1994).

1.11.2.5: CYP2E1

CYP2E1 is not one of the major drug metabolising enzymes however it has received most research attention due to its role in ethanol metabolism (Kessova and Cederbaum, 2003, Leung and Nieto, 2012). Mixed function oxidase of ethanol was discovered almost 50 years ago (Orme-Johnson and Ziegler, 1965). Since then the role of CYP2E1 in ethanol metabolism has been debated, with the consensus eventually becoming that although alcohol-dehydrogenase is the main enzyme responsible for ethanol metabolism, CYP2E1 is inducible by ethanol (Song *et al*, 1986) and with prolonged ethanol exposure it appears to play a more important role in ethanol metabolism (Lu and Cederbaum, 2008). CYP2E1 ethanol induction is associated with increased liver injury due to the reactive metabolites produced by this pathway which otherwise would be detoxified via the alcohol dehydrogenase/aldehyde dehydrogenase pathway (Lu *et al*, 2010).

CYP2E1 is most highly expressed in the liver, making up ~7% of total hepatic P450 content, and it appears in infant livers a few hours after birth (Vieira *et al*, 1996). It is also expressed in the lungs (Hukkanen *et al*, 2002), gastrointestinal tract (Ding and Kaminsky, 2003), nasal mucosa (Zhang *et al*, 2005), pancreas (Foster *et al*, 1993) and the brain (Farin and Omiecinski, 1993). It is important with regard to endogenous substances such as acetone (Bondoc *et al*, 1999) and ketones (O'Shea *et al*, 1994) as well as some xenobiotic compounds of clinical significance (Anzenbacher and Anzenbacherová, 2001). Drugs metabolised by CYP2E1 include the muscle relaxant chlorzoxazone (Kim and Peter, 1996), paracetamol (Lee *et al*, 1996) and some anaesthetics (Spracklin *et al*, 1997, Kharasch *et al*, 1999). It has significant roles relating to various carcinogenic compounds, such as N-nitrosamines (Wrighton *et al*, 1986), benzene, styrene and various other low molecular weight suspected carcinogens (Guengerich *et al*, 1991). Inhibitors include some that also inhibit alcohol dehydrogenase and aldehyde dehydrogenase such as 4-methylpyrazole (Pernecky *et al*, 1990) and 3-amino-1,2,4-triazole (Koop, 1990).

1.11.3: The CYP3 family

In humans, the CYP3 family includes CYP3A4, CYP3A5, CYP3A43 and CYP3A7, although only CYP3A4 and CYP3A5 make any significant contribution towards drug metabolism (Williams *et al*, 2002). CYP3A7 is mostly expressed in foetal liver and has only occasionally been detected in adult liver while CYP3A43 is most highly expressed in the prostate with low expression in adult liver (<5% that of CYP3A4), Williams *et al* (2002). CYP3A enzymes are involved in the metabolism around 50% of all drugs (Guengerich, 1999) and as such are considered the most important therapeutically, although they also contribute to the metabolism of many endogenous

substances (Nakamura *et al*, 2002). Of the CYP3A enzymes, CYP3A4 has the most substrates attributed to it and is the most highly expressed, making it the most important of the CYP3A family (Thummel and Wilkinson, 1998).

1.11.3.1: CYP3A4

CYP3A4 makes up, on average, 25-30% of total liver P450 volume (Shimada *et al*, 1994), although its highest relative expression level is in the small intestine – up to 60% of total P450 volume (Guengerich, 1990). It can also be found expressed in the large intestine, stomach (Ding and Kaminsky, 2003) and lung (Kelly *et al*, 1997).

The list of CYP3A4 substrates is long (Rendic, 2002) but some important examples include statins (Wang *et al*, 1991), protease inhibitors for HIV treatment (Huang *et al*, 2001), various anticancer agents (Harmsen *et al*, 2007) and calcium channel blockers (Yoshida *et al*, 2008). In addition to xenobiotics, CYP3A metabolises the steroid hormones testosterone (Waxman et al, 1988), cortisol (Abel and Back, 1993), progesterone (Yamazaki and Shimada, 1997) and estradiol (Kerlan *et al*, 1992). It also seems to play a role in cholesterol metabolism (Lütjohann et al, 2009).

Inhibitors of CYP3A4 can cause severe clinical consequences and have caused some drugs metabolised by the enzyme to be withdrawn, such as the antihistamine terfenadine, which was withdrawn after drug interactions were shown to cause cardiac arrhythmias (Kivistö *et al*, 1994). The most well-known inhibitor of CYP3A4 is grapefruit juice, which has had such a profound effect on CYP3A4-mediated metabolism that many drugs metabolised by this P450 now contain warning labels about consumption of grapefruit juice during treatment (Greenblatt *et al*, 2001). Various furanocoumarins are thought to be the main substance responsible for this inhibitory effect (Guo *et al*, 2000), however only orally administered drugs appear to

be inhibited by grapefruit juice (Ducharme *et al*, 1995). As well as being substrates of CYP3A4, many protease inhibitors used in HIV treatment are also strong inhibitors, which can be particularly problematic for avoiding drug-drug interactions due to HIV patients being on variable treatment regimens with multiple protease inhibitors (van Heeswijk *et al*, 2001). Other clinically important examples of inhibitors include oral contraceptives, which cause mechanism-based inactivation (Lin *et al*, 2002), macrolide antibiotics (Westphal, 2000) and the antifungal ketoconazole (Lorusso *et al*, 2008).

As far as inducers are concerned, St John's wort is a potent inducer and is associated with many drug-drug interactions (Zhou *et al*, 2004). It can cause the failure of oral contraceptives, due to overly rapid elimination of 17-alpha-ethynylestradiol (Schwarz *et al*, 2003) and it has been associated with organ rejection in patients treated with the immunosuppressant cyclosporine (Mai *et al*, 2003) as well as lowered efficacy of antidepressants metabolised by CYP3A4 (Johne *et al*, 2002). Similarly, rifampicin is a CYP3A4 inducer and has been associated with organ rejection (Capone *et al,* 1996) as well as potential sub-therapeutic levels of protease inhibitor in HIV patients where it is often co-administered (Grub *et al*, 2001). Barbiturates are another clinically important CYP3A4 inducer (Guengerich, 1988).

1.11.3.2: CYP3A5

Although CYP3A5 does not hold the same level of importance clinically as CYP3A4, it does have many important metabolic properties (Emoto and Iwasaki, 2006). CYP3A5 expression levels vary significantly across ethnicities (Kuehl *et al*, 2001) and CYP3A5 substrates tend to be the same as those for CYP3A4 (Williams *et al*,

2002). It is the main CYP3A enzyme expressed outside the liver and intestines, in tissues such as kidney, lung and leukocytes (Kuehl *et al*, 2001).

1.13: CYPs in non-mammals

In non-mammals, P450s have often been studied due to their roles in insecticide and drug resistance. For example, roles relating to drug susceptibility have been found for CYPs with the pathogenic fungus Aspergillis (Mellado *et al*, 2011) as well as *M. tuberculosis*, the causative agent of tuberculosis (McLean *et al*, 2007). Similarly, CYPs have been found to have a role in insecticide resistance – which is especially important with regards to mosquitos that can transmit malaria (Djouaka *et al*, 2011). Another role for CYPs in infectious agents can be exemplified via the protozoan parasite *Leishmania donnovani*, where CYP5122A1 has been found to be involved in the infection process (Verma *et al*, 2011).

Plants have more identified P450s than any other organism, with a total of 246 being found in Arabidopsis and 356 in rice (Nelson *et al*, 2004). In fact, some plants contain so many P450 genes that they make up as much as 1% of the genome of rice, Arabadopsis, grape, papaya, moss and poplar (Nelson *et al*, 2008). Fish have also received a relatively high amount of research attention and 137 fish CYP genes have been identified thus far (Uno *et al*, 2012). Zebrafish and fugu have been studied most and have 81 and 54 CYPs respectively (Nelson, 2003). Fish CYPs also have the intriguing use of being used as biomarkers for water pollution (Fent, 2003).

1.14: CYPs in mammals

Mice and rats, as key model organisms, have had many CYPs identified and studied and as such much is known about their activities. 102 CYP genes have been found

in the mouse genome (Nelson *et al*, 2004a) and 89 have been found in the rat

(compared to the 57 known CYP genes in human (Nelson, 2009).

Comparative studies between species are common. The advantages of this type of

research are that it both allows for potential model species to be identified (Sharer *et

al*, 1995, Bogaards *et al*, 2000) and it is useful for highlighting important species

differences in drug metabolism (Martignoni *et al*, 2006, Eberhart *et al*, 1991,

Yasumori *et al*, 1993).

P450s in many animals which are not so easily available for research purposes have

been studied, such as monkeys, chimps and dogs (Emoto *et al*, 2013,Williams *et al*,

2007, Zhou *et al*, 2010). Monkeys and chimps are seen as being good models for

humans (Uno *et al*, 2001) while dogs are occasionally used as an alternative to

rodent studies – although their status as companion animals also fuels research into

the therapeutic role of CYPs (Shou *et al*, 2003). In the dog, CYPs from many of the

major families in human have been identified, with nine in total being sequenced and

seven being recombinantly produced using a bacmid insect cell system and later an

E. coli expression system (Locuson *et al*, 2009). Other domestic animals where

P450s have been studied include cow, pig, chicken and horse (Giantin *et al*, 2008,

Anzenbacher *et al*, 2002, Yang *et al*, 2014, Scarth *et al*, 2011).

1.15: Equine drug metabolism

Horses are important both socially as companion animals throughout history and as

athletic animals, with particular significance economically especially within the

horseracing industry. In the UK, the horseracing industry is estimated to be worth

around £3.9 billion per year (British Horseracing Authority, 2014). When compared

with that of many species, knowledge of equine P450s is very limited. Despite this,

studies into equine P450s hold a high level of importance for numerous reasons. As popular companion and athletic animals, horses are frequently administered multiple drugs at once with the consequence that drug-drug interactions are possible, many examples of which can be found in the literature. Examples of reported adverse drug interactions in the horse include increased clearance of barbiturates after repeated dosing, faster metabolism of phenylbutazone when prescribed along with rifampin, slower clearance of quinidine when dosed along with digoxin and inhibited metabolism of xanthines by fluroquinolones (Scarth *et al*, 2011, Brumbaugh, 2001).

Considering the central importance drug-drug interactions have in human medicine, it is logical that they should also be given this status in equine medicine. Another area where equine P450s may have vast importance is within the horseracing industry. Doping control is a huge concern in horse racing and many substances, such as anabolic steroids, are banned within racehorses due to potential performance-enhancing effects (Scarth *et al*, 2010). Recent years have seen doping scandals in the horseracing industry highlighted by the media, such as the scandal involving the dosing of an anabolic steroid to horses in the Godolphin stables (Wood, 2013). Like with human athletes, doping control in racehorses involves searching for certain metabolites in the plasma and urine; however unlike the human situation much of the data regarding horse-dosing and potential drug-drug interactions is largely based on other species as details of the specific enzymes involved in the horse are sparse. Furthermore, many studies involving comparisons of drug metabolism between species suggest that it may not be ideal to extrapolate metabolism data between species (e.g. Chauret *et al*, 1997, Martignoni *et al*, 2006, Mössner *et al*, 2011). Drug metabolism studies in the horse are often much more difficult to conduct than in other animals, due to their size; expense; difficulty

obtaining large sample numbers and the associated regulations around large mammal research (Animals in Science Regulation Unit, 2014). With the elaboration of *in vitro* research techniques the area of equine drug research has expanded in recent years. Standards for approval of veterinary drugs are lower than for those used in human medicine therefore it is arguably understandable that much less is known about drug metabolism in the horse. From a horse racing angle, it is worth noting that many more substances are banned than in human sport (BHA Rules of Racing, The World Anti-Doping Code 2014) so an expanded knowledge of metabolite production is all the more crucial to keep on top of doping control.

1.15.1: Equine P450s

The first equine CYP to be discovered was found by Komori *et al* (1993) and was named P450-(h-1). Although the full sequence for it was never generated, the assumption was that it belonged to the CYP2C family due to the cross-reactivity of an antibody generated against it with rat 2C P450s. P450 research in the horse at this time was scarce and the next equine P450 study was a comparative one between horse, dog, human and cat. This involved the use of microsomes and the finding was that troleandomycin, an inhibitor of CYP3A4 in humans, did not affect the metabolism of testosterone in the horse although inhibitors of other CYPs did affect the metabolism of specific compounds (Chauret *et al*, 1997).

Although microsome studies do not necessarily give specific information about P450-based metabolism they do act as good starting points for hypothesising how P450s operate in a species. Other comparative microsome studies include Nebbia *et al* (2003) using probe substrates for CYP1A, 2B, 2E, and 3A, Mössner *et al* (2011) using inhibitors of CYP3A4, CYP2A6, CYP2C19, CYP2B6 and CYP2C9 to look into

ketamine metabolism in the horse and Darwish *et al* (2010) using an antibody against rat CYP1A1 to demonstrate that metabolism of certain compounds was CYP1A-dependent. More recently, testosterone metabolism in horse, dog and human was compared using known inhibitors against various human P450s (Zielinska and Mevissen, 2015), finding small but significant species differences, such as CYP2B6 appearing to be involved in testosterone metabolism in horse and human but not dog and sulphenazole inhibiting metabolism in horse less than in human.

As far as specific studies in the horse go, Lakritz *et al* (2000) attempted to determine activities of various P450s in horses of different ages. This involved using known human substrates of different P450 isoforms. Although this study was again limited in that it used microsomes, it did give some interesting insight into drug metabolism in the equine; particularly that pulmonary xenobiotic metabolism was significantly lower in young than adult horses while hepatic metabolism did not vary significantly. A subsequent study by Nebbia *et al* (2004) further corroborated this finding.

1.15.2: Equine P450 expression levels

Some research focus has been given to equine P450 expression levels in recent years. Schmitz *et al* (2010) identified seven potential CYP3A genes in the horse and subsequent analysis of expression of these P450s has been performed. Members of the CYP3A subfamily have been found in the liver, intestine (Tyden *et al*, 2012) and airway (Tyden *et al*, 2012a). The finding of these studies was that CYP3A93 and CYP3A96 were highly expressed in the intestines, CYP3A89, CYP3A94, CYP3A96 and CYP3A97 were highly expressed in the liver and CYP3A95 was only minimally expressed in the liver and lung but not in the intestine. Furthermore, Tyden *et al*

(2014) expanded upon this by examining more P450 isozymes in the horse liver and intestines, this time the focus was on CYPs 1A, 2A, 2C, 2D and 2E. CYP1A and CYP2C were expressed most highly in the intestines but also had high liver expression. CYP2E was expressed moderately in the intestines and liver while CYP2D was low in both and CYP2A was almost undetectable.

1.15.3: *In vivo* equine studies

The vast majority of data regarding equine drug metabolism has been generated through *in vivo* studies, although this has significant ethical and financial implications, since it often involves the sacrifice of the research animals. The most detailed method of *in vivo* study involves injecting a radiolabelled drug and monitoring its fate and excretion. This gives the most thorough analysis although it is becoming the less-favoured option compared to other *in vivo* approaches (Scarth *et al*, 2010). As a consequence of doping control and routine drug testing within the horseracing industry, the vast majority of equine drug metabolism data comes from within this industry. Urine analysis is the most popular method for doing this although hair (Dunnet, 2005), saliva (Horner, 1976), blood (Kwok *et al*, 2010) and faeces (Popot *et al*, 2006) can also be used.

1.15.4: Recombinant equine P450s

Recombinant enzyme systems have become central to modern molecular biology and pharmaceutical research, providing an easy method of generating large scale proteins on demand (Schmidt *et al*, 2004, Assenberg *et al*, 2013). They offer the advantage of allowing the user to generate a protein of interest without necessarily knowing anything about its structure or function. There are many types of protein expression systems available, from bacterial systems to mammalian cell lines and

the choice of a system depends upon many variables such as the type of organism

the protein of interest comes from, the amount of protein required and expense/ease

of use (Sodoyer, 2004, Demain and Vaishnav, 2009). For P450 protein expression,

insect cell systems are the most well established although with advances in

technology bacterial systems are becoming more popular (Gonzalez, 1995,

Guengerich and Martin, 2006, Zelasko *et al*, 2013).

It is only relatively recently that recombinant equine P450s have been produced,

which contrasts heavily with the situation in humans where recombinant CYPs have

been around for many years and are now an integral part of drug development

procedures. DiMaio Knych and Stanley (2008) produced the first recombinant horse

CYP (CYP2D50, an isoform of the human CYP2D6 which was used for comparison)

using an insect cell expression system. The same authors have since used this

expression system to study the enzyme kinetics of two other equine P450s –

CYP2C92 which is the orthologue of human CYP2C9 (DiMaio Knych *et al*, 2009) and

CYP3A96 (DiMaio Knych *et al*, 2010) which is the possible orthologue of human

CYP3A4. During the 2010 study, two other equine P450s (CYP3A89 and CYP3A97)

were recombinantly expressed, however the former did not display catalytic activity

and the latter failed to show up within the characteristic 450 nm spectrum when

bound with carbon monoxide (although it did appear to be catalytically active). To

date these are the only recombinant equine P450 studies that directly compare the

activities of the equine enzymes with those of another species.

From all three of these studies, the equine enzymes displayed generally lower

activity than their human orthologues although it is important to note that only a few

substrates were used for each. For CYP2D50, dextromethorphan and debriquisone

were used as substrates with the formation of metabolites (o-demethylation for

dextromethorphan and 4-hydroxydebriquisone for debriquisone) being 180 fold and 50 fold slower than in the human enzymes, respectively (which, interestingly, conflicts with Chauret *et al* who showed higher dextromethorphan metabolism in horse than human). For CYP2C92 the probe substrates were diclofenac and warfarin. Here, formation of 4-hydroxydiclofenac was 20 fold slower than in the human enzyme, although formation of hydroxy (s)-warfarin and hydroxy tolbutamide was similar in horse and human. Knych *et al* (2010) showed testosterone and nefedipine metabolism by CYP3A96 to be 20 and 10 fold slower in horse compared to human, respectively.

Using a different expression technique, equine CYP2B6 (Peters *et al*, 2013) and CYP3A94 (Dettwiler *et al*, 2014) have also been recombinantly expressed. In these studies, V79 hamster lung fibroblasts were used along with ketamine as the potential substrate for CYP2B6 and, for CYP3A94, 7-benzyloxy-4-trifluoromethylcoumarin (a substrate of multiple CYPs which is metabolised to a flourescent product, Donato *et al*, 2004) was used to validate P450 activity. For CYP2B6, the metabolite norketamine was detected, as well as metabolites of norketamine itself (such as 5,6-dehydronorketamine). Additionally, clopidogrel was found to be an inhibitor of the metabolism of ketamine to norketamine. No comparison to other species was made in this study, however in humans, CYP3A4 is the major metaboliser of ketamine while CYP2B6 and CYP2C9 play more minor roles (Hijazi and Boulieu, 2002).

1.16: Methods used to study hepatic metabolism

As far as *in vitro* studies are concerned, there are four main ways to study hepatic metabolism – taking liver sections, isolating hepatocytes, isolating microsomes and producing recombinant drug metabolising enzymes (Brandon *et al*, 2003) - see figure

1.16 for a summary. Liver slices and hepatocyte cultures can give the most holistic view of liver processes although systems for hepatocyte culturing and cryopreservation are not available for all species. Liver slices are of particular use for studying induction of liver enzymes and provide a model that keeps liver architecture in place (Edwards *et al*, 2003). Compared to other methods however, liver slices have dropped in popularity since the 1970s (Ekins *et al*, 2001) due to handling difficulties, particularly with regard to maintaining viability (Hashemi *et al*, 2000). Hepatocytes can be fresh, cultured or cryopreserved. Fresh hepatocytes lose viability after a few hours (Bayliss *et al*, 1999). Hepatocytes can be cultured for as much as four weeks although some enzyme activities become gradually lower after a few days of culture maintenance (George *et al*, 1997). Cryopreservation is popular particularly for human hepatocytes where tissue availability is limited (Hengstler *et al*, 2000) and cryopreserved cells seem to retain good enzyme activity (Silva *et al*, 1999).

Microsomes are vesicles formed from the endoplasmic reticulum of cells and are the most straightforward and popular method for investigating liver drug metabolism function (Asha and Vidyavathi, 2010). Microsomes contain a variety of phase I and phase II drug metabolising enzymes, such as P450s and UGTs (Zhang *et al*, 2012). Microsomes are relatively easy to use for drug metabolism studies and have the extra advantage of being low cost, particularly since fresh liver is not required to produce them (Skaanild and Friis, 2000). The main disadvantage of microsomes is that they tend to give a higher rate of metabolism than would be found *in vivo* (Sidelmann *et al*, 1996).

Despite the vast usefulness of hepatocytes, liver slices and microsomes, they do not give isoform-specific information about drug metabolising enzymes. As such,

recombinant enzyme systems are now commercially available for many drug

metabolising enzymes (Fasuni *et al*, 2012). Recombinant systems offer a means of

investigating drug metabolism at the molecular level as well as allowing for the study

of drug-drug interactions (Yao *et al*, 2001). This is particularly important for

cytochrome P450-mediated metabolism, where knowledge of P450 isoform-specific

metabolism is essential (Lynch and Price, 2007).
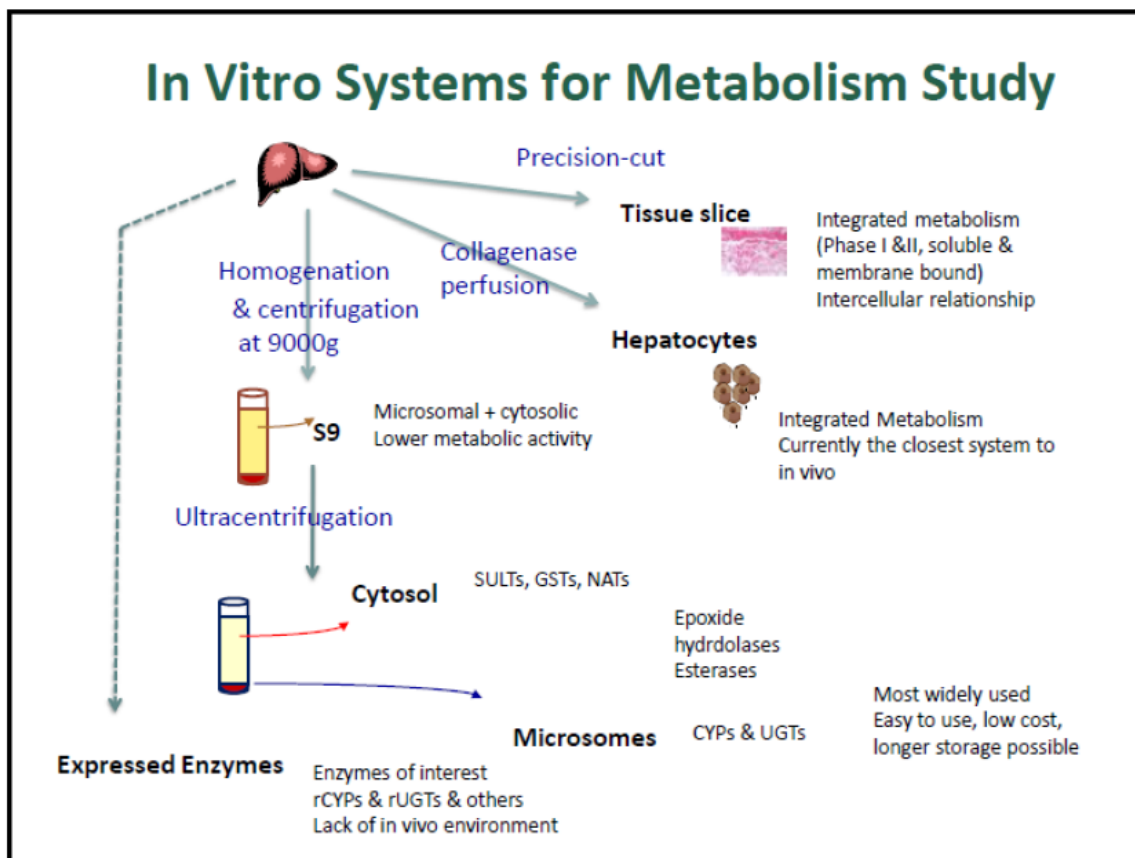
Figure 1.16: In vitro methods used to study hepatic metabolism. Liver slices and hepatocytes can

be used to study phase I and phase II metabolism together while liver fractions generated from

ultracentrifugation may be used to study specific types of enzyme. Recombinant enzymes may be

used to study specific isoforms. © The Hamner Institutes for Health Sciences, 2010.

1.17: Rational for current study

Drug metabolism in the horse may be different to that of human for various reasons. Horses are herbivores while humans are generally omnivores therefore diet, a known factor that can affect drug metabolism to a large extent (Walter-Sack and Klotz, 1996, Harris *et al*, 2003), may vary greatly between the two species. In addition to this, there is some evidence to suggest that exercise may affect the expression of certain P450 isoforms (Vistisen *et al*, 1991, Frenk *et al,* 1980), and, therefore, equines used for sport may have particularly divergent metabolism of certain substances when compared with other horses or humans. This, in addition to the differences illustrated by comparative studies between the two species, highlights the crucial need for more horse-specific data to be generated. With the equine genome now being available (Wade *et al*, 2009), it is possible to generate a full picture of the genes involved in equine drug metabolism. Once these have been elucidated, creation of recombinant enzymes and subsequent screening against drugs will provide vast amounts of much needed data about P450 isoform-specific drug metabolism, giving novel insight into equine pharmacokinetics.

1.17.1: Aims of study

To isolate and characterise equine P450s. P450 enzymes will be identified via searching the equine genome, isolated from equine liver, cloned and expressed before being functionally tested against a range of frequently used equine medications. This will allow for identification of novel substrates of CYP P450s in the horse.

Chapter 2: Materials and methods

2.1: Bioinformatics analysis

2.11: Alignments of known horse and human sequences

Equine sequences were collected using the NCBI database
(http://www.ncbi.nlm.nih.gov/) using the query 'P450' and filtering for *Equus caballus*.
Isoforms of human P450s were also collected via this method. ClustalW2
(http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for the alignments. Heat maps
were generated in Microsoft Excel in order to display percentage similarities in a
graphical format – two for all known equine sequences (protein and nucleotide) and
one for the equine and their human isoforms (nucleotide).

2.12: Searching of equine genome

The equine genome was probed by searching the UCSC genome browser
(http://genome.ucsc.edu/). Two searches were carried out – the first used various
P450 sequences from horse and other species, chosen as a cross-species selection
of P450s involved in drug metabolism (see table 2.1) while the second used all
known human P450 sequences on UniProt (http://www.uniprot.org/uniprot/). All
identified potential equine P450 sequences were analysed using NCBI BLAST
(http://blast.ncbi.nlm.nih.gov/) in order to quantify sequence similarities with P450s in
other species. Those with e values greater than zero were removed, as this
decreased the likelihood that the match occurred by chance (Altschul *et al*, 1997).

Species	CYP name	NCBI accession number
Dog	CYP2B11	NM_001006652.1
Horse	CYP2C92	NM_001101652.1
	CYP3A89	NM_001101651.1
	CYP3A93	NM_001190938.1
	CYP3A94	NM_001190939.1
	CYP3A95	NM_001190940.1
	CYP3A96	FJ755695.1
	CYP3A97	NM_001146164.1
Human	CYP1A2	NM_000761.3
	CYP2B6	NM_000767.4
	CYP2C9	NM_000771.3
	CYP2C19	NM_000769.1
	CYP2D6	NM_001025161.1
	CYP3A4	NM_017460.5
Mouse	CYP3A11	NM_007818.3
Rat	CYP2C11	NM_019184.2

Table 2.1: Sequences selected for first equine genome search. Sequences were selected as a cross-species selection of known drug-metbolising enzymes.

2.13: Searching for automatically annotated equine sequences

Due to the fact the direct equine genome searching generated many sequence fragments, the Ensembl ((www.ensembl.org/) and NCBI databases were searched for sequences automatically annotated as P450s in the horse. 'P450' was used as the search term in both databases and this data was aligned with that gathered from the direct genome search in order to determine the level of crossover. These 'predicted' sequences had all been named automatically based on their similarity to certain P450 isoforms (e.g., 'CYP2D6-like') and these were the names used for the purpose of the current analyses. A table was generated of the predicted sequences, ordered by chromosome number in order to more easily compare gene clusters across species.

2.14: Generation of phylogenetic trees

Phylogenetic trees were created using ClustalW2 Phylogeny (http://www.ebi.ac.uk/Tools/phylogeny/) via the neighbour joining method with distance correction turned on (Bruno *et al*, 2000), and percentage identity matrix and excluding gaps turned off. Two trees were produced – one for all predicted equine P450s (where separation into the nine vertebrate clans was highlighted) and one for all known equine P450s and their human isoforms, in order to look at predicted evolutionary divergence.

2.15: Identification of conserved haem-binding motif

The FxxGxxxCxG haem-binding motif is conserved amongst all P450s (Ranasinghe and Hobbs, 1998) therefore for final sequence validation, all predicted sequences were analysed for the presence of this domain. Protein sequences were obtained using ExPaSy Translate (http://web.expasy.org/translate) and the motif was searched manually in each sequence.

2.2: Cloning, sequencing and protein structure predictions

2.21: Isolation of RNA from equine liver

A liver was acquired from a one year old male gelding. Liver was removed immediately after death and was in a healthy condition. It was immediately packaged onto dry ice and transported to the laboratory where it was cut into 4-5cm^3 chunks and stored at -80°C in RNAlater (Life Technologies). A Qiagen RNeasy Maxi Kit was used following the manufacturer's protocol to extract total RNA from 1g of liver. Total

RNA was then quantified using a nanodrop 8000 spectrophotometer, with concentration ranging between 400-600ng/µl and a 260/280 purity value of 1.8-2.

2.22: Synthesis of cDNA

The SuperScript III First Strand Synthesis System (Life Technologies) was used for cDNA synthesis using a random hexamer priming method (reviewed in Rio, 2014). 500ng-1µg total RNA was used per reaction and cDNA was stored at -20°C.

2.23: RT-PCR analysis

Primers were used to amplify sequences obtained during the *in silico* analysis in order to verify their expression in liver. Primers were designed manually and specificity validated via NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Additionally, primers for the already known CYP3A97 were designed as a positive control (Table 2.1.1). Polymerase chain reaction comprised of 10µl of MegaMix-Blue (Microzone), 1µl of cDNA and 2µl of each primer (2µM stock). Reactions were carried out with an annealing temperature gradient of 50°C to 68°C in 33 cycles with initial denaturation at 95°C for 30 seconds followed by 33 cycles of denaturation at 95°C for 30 seconds, annealing for 1 minute and extension for 1.5 minutes at 68°C with a final extension for 5 minutes at 68°C. PCR products were run on 1% agarose gels in 1x TBE buffer with 10µl ethidium bromide/100ml at 100v for 40 minutes. Imaging was carried out using an Image Quant 300 (GE Healthcare) in UV mode.

Template sequence name	Forward primer	Reverse primer
Ecab.P450.1	5'TCGACCCCACCTTTGTCCTT3'	5'AATTACAAGCTCTGCATCAT3'
Ecab.P450.4	5'CAGTTGCCTCAGCATTTTGT3'	5'AGCCTTACCTCATCTGTGCA3'
Ecab.P450.5	5'ATGATGTTGTCCCAGCTCAG3'	5'GCACGGCTACGGTTTTCCAT3'
Ecab.P450.17	5'AGCTTCAGTGATGACAACCT3'	5'TGTCTTTGGCTTCCTGGTGA3'
Ecab.P450.18	5'GGAGGTATATCAACTGTGGT3'	5'TGCTTCAGCGGTTTCACTTG3'
P450.Equ.18	5'GCCATTGCCCCCAAGGATGA3'	5'TGGCTGAACGTGGAGCCGCT3'
P450.Equ.23	5'CCTTCTGGGACACCTCCCCT3'	5'TCGAGTTCCGGCTGGTGCCC3'
P450.Equ. 37	5'AGGCATAGCCGCTGTGCTGC3'	5'GCAAATGGCTTTGGCCCAGG3'
CYP1A1-like	5'ATGTTTTCTGTGTTTGGATT3'	5'AGGATGAAGAAACACGCTGA3'
CYP1B1-like	5'ATGGCCACTAGCCTCAGCCT3'	5'CCGAGGAAGACAGCCAGTGA3'
CYP2U1-like	5'TGATCACGTCCTTGAAGGGA3'	5'CCTGACTGGAAGATACGGTC3'
CYP3A97	5'aataagtcgacATGGAGCTGGGAAGAGCCTT3'	5'CCGTGACTGGAGCCTGACaagctttatt3'
CYP2C26-like	5'aataagtcgacAGAAGAGACAATGGATCTGGTTGTG3'	5'GCTCTGCTTTATTCCTGTGTGAaagcttttatt3'

Table 2.1.1: Primer sequences for RT-PCR of P450 sequences. Restriction sites are highlighted in yellow. Refer to appendix for regions primers were designed against.

2.24: Commercial clones

Clones were purchased from Genscript in the pUC57 vector (all constructs were cloned into vector using Xba I and BamHI). Six of these were obtained – CYP1A1, CYP2D50, CYP2E1, CYP3A96, CYP2A13, and CYP2C92. All constructs were cloned in the same direction except CYP1A1, which was cloned in the opposite direction (BamHI --> XbaI). Clones were selected based on likihood of being involved in drug metabolism (CYP2D50, CYP2E1, CYP3A96 and CYP2C92) or because they had not previously been recombinantly produced (CYP1A1, CYP2A13).

2.25: Cloning for pCold expression system

Top 10 *E. coli* cells (Life Technologies) were transformed with the pUC57 construct according to manufacturer guidelines. These were then were grown in 10ml general purpose nutrient broth (Oxoid) with ampicillin (100µg/ml) for 24hrs after which plasmid DNA was extracted using a Quiagen QuickLyse miniprep kit. DNA

concentrations were recorded using a Nanodrop 8000 spectrophotometer. See figure 2.21 for vector maps and multiple cloning sites. Plasmid DNA was digested using the strategies outlined in table 2.21. Restriction enzymes and DNA ligase were purchased from Promega and manufacturer's protocol was followed. DNA obtained from PCR reactions (for CYP3A97 and CYP2C26-like) was directly ligated into expression vector using the restriction sites in the primers (see table 2.21).
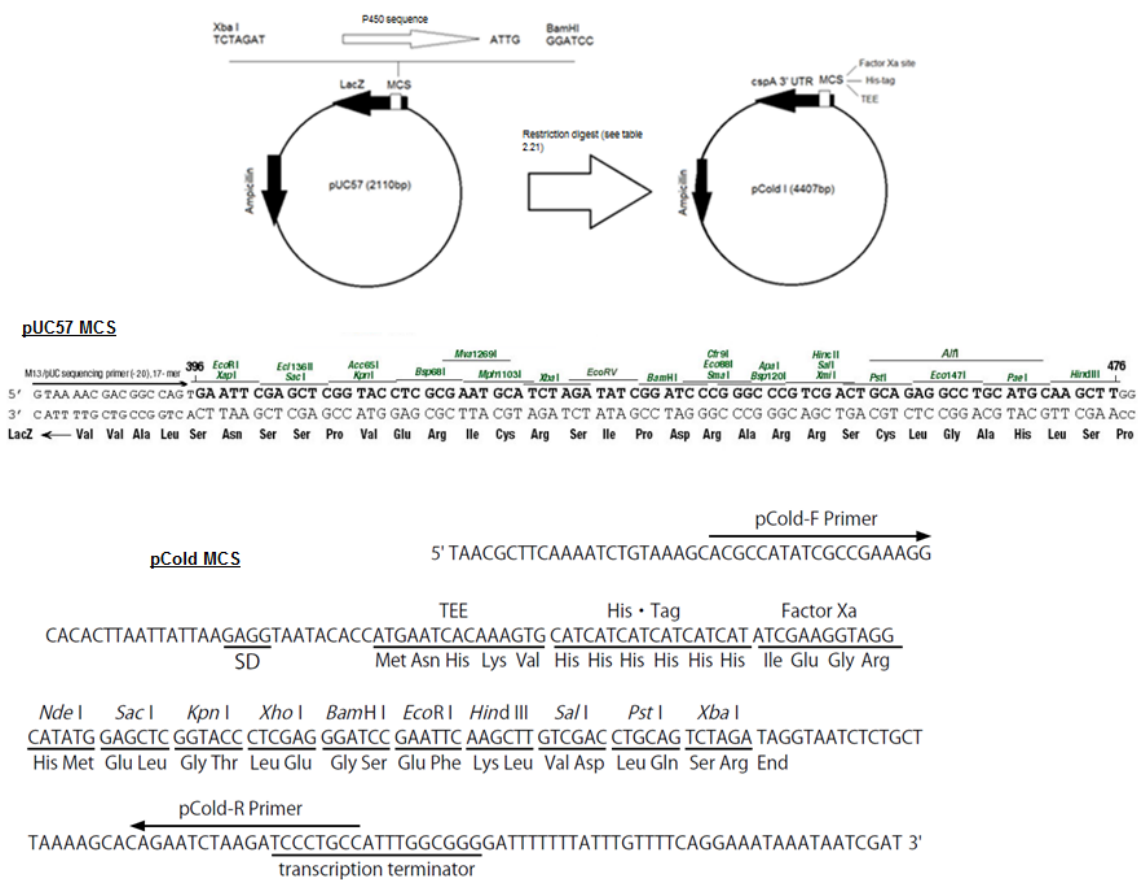
Figure 2.21: Vector maps and multiple cloning sites. Sequences were first of all cloned into the pUC57 vector using Xba I and BamHI and subsequently cloned into the pCold vector for expression using the enzymes in table 2.21. Note that CYP1A1 was inserted into the pUC57 vector in the opposite direction and so was digested using the cloning strategy of BamHI+ XbaI to correct this. Adapted from "Cold Shock Expression System pCold™ DNA manual" by Takara Inc. and "pUC57 plasmid DNA datasheet" by Genescript.

Construct	pCold cloning strategy
1A1	BamHI+ XbaI
2A13	BamHI + HindIII
3A96	SalI + XbaI
3A97	SalI + HindIII
2D50	EcoRI + HindIII
CYP2E1	BamHI + HindIII
2C92	BamHI + HindIII
2C26-like	SalI + HindIII

Table 2.21: Cloning strategies for insertion into pCold vector. Restriction enzyme combinations used for each P450 construct are highlighted.

2.26: Cloning for sf9 expression system

Top 10 cells from Life Technologies were transformed with the pUC57 constructs as per manufacturer's transformation protocol. Plasmid DNA was extracted using a Quiagen QuickLyse miniprep kit and plasmid DNA concentration was measured using a nanodrop. Restriction digests (1µl of each restriction enzyme, 1µg DNA, 5µl of buffer, total reaction volume: 50µl) were performed as outlined in table 2.22 and DNA was ligated into the pFastBac 1 expression vector (Life Technologies) as per manufacturer's protocol. See figure 2.22 for vector map.

pUC57 construct	pFastBac-1 cloning strategy
1A1	KpnI + SalI
2A13	SalI + XbaI
3A96	SalI + XbaI
3A97	SalI + HindIII
2D50	EcoRI + XbaI
CYP2E1	BamHI + HindIII
2C92	Acc65I + HindIII

Table 2.22: Cloning strategies for insertion into pFastBac-1 vector. Restriction enzyme combinations used for each P450 construct are highlighted.
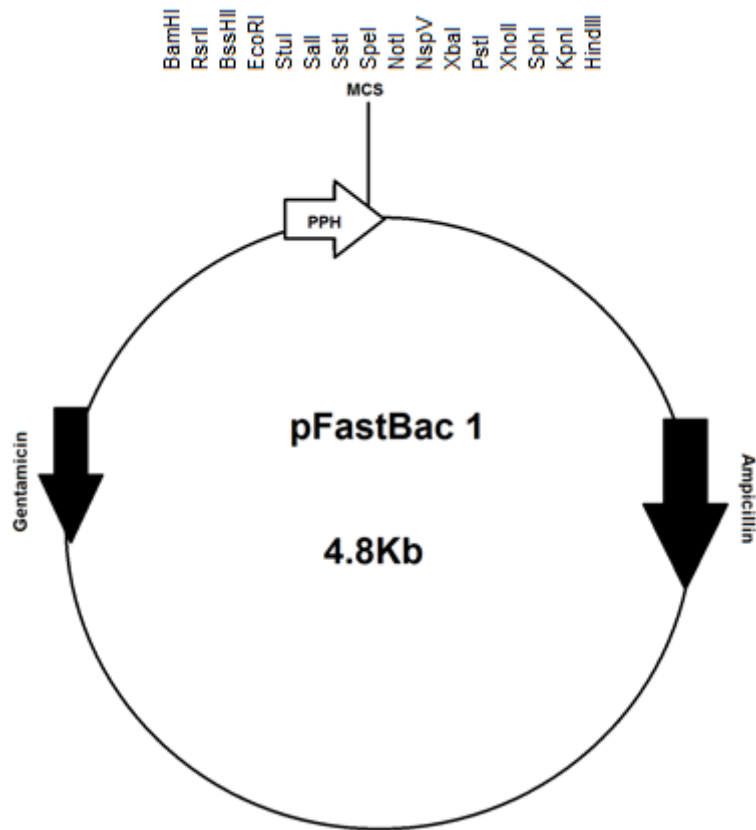
Figure 2.22: pFastBac 1 expression vector. Vector contains a polyhedron promotor (PPH) for production of high quantity of recombinant protein in baculovirus-infected insect cells as well as ampicillin and gentamycin resistance markers. Multiple cloning site (MCS) features are highlighted.

2.27: Sequence verification of cloned constructs

Successfully cloned products were sequence verified via Sanger sequencing (Source Bioscience, Nottingham, UK) using primers designed against both ends of the pCold cloning vector (forward: ACGCCATATCGCCGAAAGG, reverse: GGCAGGGATCTTAGATTCT, hightlighted in figure 2.21). M13 primers were used for the pFastBac 1 constructs. Sequencing output chromatograms were analysed using BioEdit (Ibis Biosciences).

2.28: Protein structure prediction

DNA sequencing results were translated using ExPaSy Translate with standard genetic code. Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) was used in normal mode to generate 3D structure predictions of the protein sequences. In the cases where the reference sequence varied from the expression construct, predictions were made of both sequences for comparison.

2.3: Protein expression and verification

2.31: BL21 pCold expression

BL21 *E. coli* competent cells were ordered from New England Bioscience (NEB) and were transformed with pCold I constructs from Takara Bio as per NEB protocol. Serial dilutions (1 in 10 and 1 in 100) were spread onto 90mm plates containing nutrient agar and 100µg/ml ampicillin. After overnight incubation at 37°C, individual colonies were inoculated into 10ml nutrient broth with 100µg/ml ampicillin and shaking at 200rpm/37°C overnight. Glycerol stocks were established (500µl overnight bacterial culture and 500µl of 50% glycerol (Sigma-Aldrich) with 50% sterile reverse osmosis (RO) water) for subsequent expression studies. Stocks were frozen and stored at -80°C. All cultures used for glycerol stocks were sequence verified to ensure no mutations had occurred.

To induce expression, a 1:100 dilution of overnight culture into fresh nutrient broth + ampicillin was incubated with shaking at 37°C until OD_{600} was between 0.4-0.5. Temperature was then brought down to 15°C and cultures were left to stand for at least half an hour after which induction using the optimum IPTG (isopropyl-β-d-thiogalactopyranoside, VWR International) concentration (determined by performing

titrations ranging from 0 to 1mM) was initiated and cultures were left shaking overnight at 15°C. Cells were pelleted by centrifugation for 10 minutes at 3000rpm and lysed using CelLytic B (Sigma-Aldrich) with protease inhibitor and lysozyme (Sigma-Aldich) at the concentrations recommended by the manufacturer. 20% glycerol was added for protein stability (Gekko and Timasheff, 1981). Total protein was measured using a Bradford assay (Sigma-Aldrich) according to manufacturer's protocol and a spectrophotometer with detection at 595nm. Lysate was stored at -80°C in 1ml aliquots.

Two further lysis techniques were also used to compare protein integrity – one involved using lysozyme (concentration 1mg/ml – incubated at 5°C with rotation overnight) for lysis and the other involved sonication (30 seconds on/ 30 seconds off for 5 minutes) using a standard lysis buffer (50mM Tris-HCl (Merk Millipore), 100mM NaCl, 1mM dithiothreitol (DTT), 5% glycerol) with protease inhibitor. Lysates were centrifuged at 16,000g for ten minutes.

2.32: Integration of pFastBac-1 constructs into bacmid

Incorporation of DNA into bacmid involved using the Bac-to-Bac expression system (Life Technologies), see figure 2.32 for summary. 100µl of DH10Bac cells containing the bacmid DNA were incubated on ice with 1ng of pFastBac DNA for 30 minutes after which they were heat shocked at 40°C for 45 seconds, chilled on ice for two minutes, mixed with 900µl of super optimal broth media and incubated at 37°C/225rpm for four hours. 10-fold serial dilutions were performed and cells were streaked on LB agar plates containing 50µg/ml kanamycin (Sigma-Aldrich), 7µg/ml gentamycin (Sigma-Aldrich) and 10µg/ml tetracycline (Sigma-Aldrich) along with 100µg/ml x-gal (Promega) and 40µg/ml IPTG for blue-white screening. Plates were

incubated for 48 hours at 37°C before white colonies were selected and restreaked on fresh plates for further verification. Overnight liquid cultures of 10ml were then set up using nutrient broth with 50µg/ml kanamycin, 7µg/ml gentamycin and 10µg/ml tetracycline. These cultures were used to extract DNA for the next stage using the PureLink HiPure Plasmid DNA Miniprep Kit (Life Technologies).

2.33: Insect cell maintenance, transfection and viral stock amplification

Sf9 insect cells (Life Technologies) were grown and maintained in sf-900 III SFM media (Life Technologies) at 28°C with gentamycin at 10µg/ml prior to transfection experiments. Daily cell counts were performed to ensure log phase growth (2-2.5x10^6 cells/ml) was maintained and viability was monitored using trypan blue (Life Technologies) to ensure >95% viability (Richardson, 1995). Subculturing was carried out in 75ml flasks with filter caps when cell density reached >2.5x10^6 cells/ml.

Prior to transfection, cells were counted, centrifuged at 500rpm for five minutes and resuspended in unsupplemented Grace's Insect Cell media (Life Technologies) to generate a cell density of 4x10^5 cells/ml. 2ml of cell culture was added to each well of 6-well plates and cells were left to attach for 15 minutes. 8µl of Cellfectin II reagent (Life Technologies) was diluted in 100µl of unsupplemented Grace's media while 1µl of bacmid DNA (~0.5ng/µl) was diluted in 100µl Grace's media. Both dilutions were combined and incubated at room temperature for 15-30 minutes after which the mixture was added to each well (209µl mixture per well). Cells were incubated for five hours at 27°C and subsequently the transfection mixture was removed by centrifugation of cells after which Grace's media supplemented with 10% FBS (Life Technologies) was added. Cells were then placed back into the incubator and were monitored for signs of viral infection via microscopic observation from 72hrs post-

infection (with late stage infection being shown by cell lysis). When cells reached late stage infection, virus was removed by collecting the supernatant after centrifugation at 500g for 5 minutes. Viral stocks were stored protected from light at 4°C (aliquots were also stored for long term purposes at -80°C).

P1 viral stock was added to sf9 cells in 6-well plate format (4×10^{6} cells/ml in SFM media) at volumes ranging from 10µl-100µl and cells were incubated at 27°C for 48 hours after which samples were removed and virus isolated – sample times ranged from 48 hours to 90 hours to determine optimal incubation time. P2 viral stocks were stored along with P1 stocks.

2.34: Sf9 expression attempts

For protein expression, 6×10^{5} cells were added per well to a 24 well plate and bacmid stock was added (volumes ranging between 10µl-100µl). Cells were incubated at 27°C and harvested at various time points ranging from 0 to 72hr post-transfection in order to assess optimal time for protein expression. For analysis, 1ml of cells at each time point was centrifuged and lysed in 1x lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and run on coomassie stained gels or Western blots.
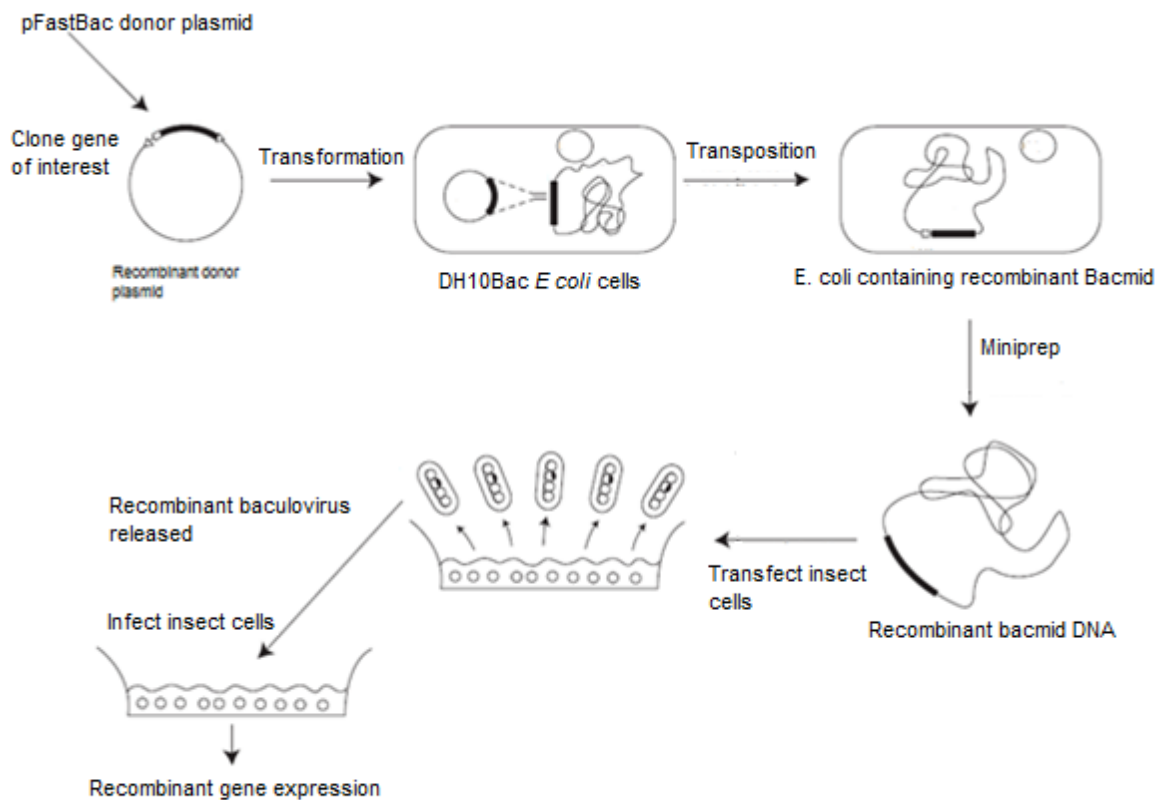
Figure 2.32: Summary of sf9 insect cell expression procedure. The gene of interest in a donor plasmid (pFastBac 1) is used to transform DH10Bac *E coli* , where the gene of interest is transposed into the bacmid present in the DH10Bac cells. Recombinant bacmid DNA is then extracted and used to transfect insect cells for recombinant protein expression. Adapted from "Bac-to-Bac Expression System User Guide" by Life technologies.

2.35: Western blotting

Lysates were prepared by mixing them in a 1:1 ratio with diluted 4x NuPage LDS sample buffer (Life Technologies) and 100mM DTT added as the reducing agent. After heating at 70°C for 10 minutes, 20-30µl of sample was loaded to 4-12% bis-tris mini gels (Life Technologies) which were run on an Xcell SureLock electrophoresis system (Life Technologies) at 200 volts for 40 minutes. Protein markers were either a 175KDa prestained protein (NEB) or MagicMark 220KDa (Life Technologies). Transfer of proteins was achieved by sandwiching the gel between filter paper and

nitrocellulose membrane with a pore size of 0.45um (Life Technologies). Transfer was carried out at 30 volts for 60 minutes in an XCell Blot Module (Life Technologies) using NuPage transfer buffer (Life Technologies) prepared as a 1x solution as per manufacturer's guidelines.The membrane sandwich was disassembled and the nitrocellulose membrane underwent blocking in 2.5% non-fat milk (Marvel) in PBS for half an hour at room temperature with rotation on a roller. All primary antibodies (rabbit anti-P450 - Enzo Life Sciences, rabbit anti-CYP2D6 - Antibodies-online and rabbit anti-his tag - Novagene) were diluted 1:1000 in PBS and incubated with membranes at 4°C overnight with rotation. Secondary (anti-rabbit conjugated to horse radish peroxidase, Source Bioscience) antibody (1:1000 dilution in PBS) was incubated at room temperature for 1.5 hours with rotation. After blocking and primary and secondary antibody incubations, three five-minute wash steps in PBS were carried out. ECL Prime (Amersham) was used according to manufacturer's instructions and a Typhoon Trio Variable Mode Imager set to detect with the blue laser at 488nm allowed visualisation of the blot.

2.36: Coomassie staining of cell lysates

Protein samples were prepared as above and run on 10% bis-tris gels for 40 minutes at 200v with the 175KDa prestained protein marker. Gels were then washed three times for five minutes in distilled water then stained in a tray with rotation for one hour at room temperture using 20ml of SimplyBlue Safe Stain (Life Technologies). Destaining took place overnight in 100ml distilled water and images of gels were taken using an Image Quant 300 (GE Healthcare) in white light mode.

2.37: Protein purification

His-Select columns (Sigma-Aldrich) and immunoprecipitation (using an anti-P450 antibody) were used to purify recombinant proteins from the total protein lysate. His-Select columns were equilibrated with buffer containing 50mM sodium phosphate and 0.3M sodium chloride. The lysate was loaded onto the column with centrifugation at 5000rpm. Two washes using buffer containing 50mM sodium phosphate, 0.3M sodium chloride (Sigma-Aldrich) and 5mM imidazole (Sigma-Aldrich) were followed by a final elution with 50mM sodium phosphate (Sigma-Aldrich), 0.3M sodium chloride and 250mM imidazole. A Dynabeads protein G kit (Life Technologies) was used for immunoprecipitation. Anti-P450 antibody (10μg) was bound to 3mg of beads in 50μl citrate-phosphate buffer, pH 5, (rotation at room temperature for 10 minutes) after which the beads with captured with a magnet and the supernatant was removed. 500μl-1ml of cell lysate (variable depending on protein concentration – refer to chapter 5) was added and incubated with rotation for ~20 minutes in order to bind the target antigen to the antibody/bead complex. Beads were separated from the supernatant using a magnet after each step and the complex was washed three times in 200μl PBS before antigen elution using 20μl 0.1M citrate.

2.38: Mass spectrometry protein identification

Purified protein concentration was measured using a Bradford assay as previously described and samples were run on a Q-TOF2 mass spectrometer using ESI and and a Waters CapLC HPLC system with a C18 column. Positive ion mode was used and a capillary voltage of 3000v was selected. Data directed analysis was used to switch between MS and MS/MS based upon charge state, intensity and mass and

cone voltage varied between 15-55v. ProteinLynxGlobalServer, MASCOT (web version), Swissprot and NCBI were used for data analysis and interpretation. Work carried out in collaboration with Dr Susan Liddell.

2.39: P450 quantification

P450 quantification involved using the carbon monoxide difference spectrum as first described by Omura and Sato (1964). Protocol followed was as per Guengerich *et al* (2009) whereby CO was bubbled into both samples before reduction by sodium dithionite (Sigma-Aldrich) of one sample. A Unicam UV4 spectrophotometer set to detect wavelength between 390-510nm was used and absorbance values at 420nm, 450nm and 490nm were recorded.

2.4: Enzyme kinetics

2.41: Recombinant P450 phenotyping

Preliminary drug assays were carried out with each CYP in order to gauge which compounds were turned over by the enzyme. Three time points were used – 0, 30 and 60 minutes. Incubation involved shaking at 500rpm in a 37°C incubator. Test compounds were dissolved in DMSO (Sigma-Aldrich) to give a stock concentration of 300µM. Each incubation contained 296µl phosphate buffer (pH 7.4), 20µl crude pCold lysate, 20µl P450 reductase (45 pmol/ml, Sigma-Aldrich), 20µl b_5 (225 pmol/ml, Sigma-Aldrich), 4µl test drug (3µM, Sigma-Aldrich) and 40µl NADPH (11.3mM, Roche). All components of incubation mixture except NADPH were preincubated at 37°C for 5 minutes in order to equilibrate. Addition of NADPH immediately started the reaction. At each time point, 50µl of the incubation was removed and quenched into 100µl of ice cold methanol and immediately stored at -

20°C in capped tubes for a minimum of four hours before being centrifuged at 500rpm for ten minutes to remove any unprecipitated material.

2.42: Enzyme kinetic analysis

When turnover with a specific drug appeared to take place in the preliminary studies, attempts were made to determine the kinetic parameters of each drug with rCYP2C92 by carrying out incubations at eight different concentrations, ranging from 1 to 150 µM. Samples were removed and quenched at 0, 2, 4, 8, 12, 16, 30 and 60 minutes. Internal standards were used at a concentration of 5µM in quenching methanol in order to ensure apparent decrease in substrate concentration was not due to non-specific processes or analysis error.

2.43: CYP2C92 inhibition assay

In order to further demonstrate whether the CYP2C92 construct had P450-like activities an inhibition assay was carried out using fluconazole (VWR International), a potent inhibitor of human CYP2C9 (Kunze *et al*, 1996). Inhibitor concentration ranged from 0 to 125 µM and diclofenac was used as the substrate at 75 µM.

2.44: Negative control assays

To ensure apparent drug metabolism was caused specifically by the rCYP2C92 construct two sets of negative control were carried out – one set involving a denatured (left at room temperature overnight) CYP2C92 construct and one set involving the other, CYP2D50, construct. All four NSAIDs were used with each at a concentration of 75 µM.

2.45: Microsome assays

To complement the recombinant enzyme drug incubations, equine liver microsomes (provided by Mr Khaled Shibany) were also tested against the NSAIDs that had apparent turnover in the recombinant system as well as with the presence of fluconazole. Microsomal P450 content was determined by the CO absorbance spectral shift to be 0.61nmol/mg of protein. NADPH and test drug concentrations used were the same as for the recombinant enzyme incubations.

2.46: Human recombinant supersomes

Commercially produced human recombinant supersomes (CYP2C9*1) were purchased from BD Biosciences. P450 concentration in incubations was 10 pmol and these were run under the same conditions as the equine incubations.

2.4.7: Liquid chromatography mass spectrometry (LCMS) method development

Methods for all compounds were developed on a Micromass Quattro Ultima mass spectrometer using electrospray positive mode with an Agilent 1100 HPLC. Stock compounds were prepared in methanol at a concentration of 10mg/L. Solvent flow rate was kept a constant 0.5 ml/min with an upper pressure limit of 400 bar. Solvent A was 10% methanol, 90% water and 0.002% formic acid and Solvent B was 100% methanol and 0.002% formic acid. See table 2.4.1 for details on methods developed for each drug.

Drug	Mass	Mass of ion	Cone voltage	Column	Gradient or isocratic?	Details
Dextromethorphan	271	272.89	50	3x150mm, 5µm	Isocratic	50:50 (A:B), 1ml/min
Diclofenac	296	296.19	35	3x150mm, 5µm	Gradient	See table 2.42 (chapter 2)
Flunixin	296	297.26	40	3x150mm, 5µm	Gradient	See table 2.42 (chapter 2)
Fluphenazine	437	438.26	35	3x150mm, 5µm	Gradient	0-100% B in 5 mins, 0.5ml/min
Glycopyrrolate	318	319.67	40	3x150mm, 5µm	Isocratic	50:50 (A:B), 1ml/min
Hydroxyzine	374	407.68	35	3x150mm, 5µm	Gradient	0-100% B in 10 mins, 0.5ml/min
Ketoprofen	254	332.63 (MW + formic acid + methanol)	15	3x150mm, 5µm	Gradient	See table 2.44 (chapter 2)
Phenylbutazone	308	309.19	50	3x150mm, 5µm	Gradient	See table 2.43 (chapter 2)
Pyrilamine	285	286.12	35	3x150mm, 5µm	Gradient	0-100% B in 4 min, 0.5ml/min
Triamcinolone	394	395.63	40	250x3.2mm, 5µm	Isocratic	50:50 (A:), 0.5ml/min

Table 2.4.1: Methods used for LCMS analysis of each drug. All methods involved ESI+ mode and were run on C18 columns. Solvent A was 10% methanol, 0.002% formic acid and 90% water while solvent B was 100% methanol with 0.002% formic acid.

Time (mins)	B%
0	50
2	100
4	100
5	50
7.5	50

Table 2.42: HPLC gradient used for diclofenac and flunixin analysis. The percentage of solvent B with time is highlighted.

Time (mins)	B%
0	50
1	50
3	100
4	50
5	50

Table 2.43: HPLC gradient used for phenylbutazone analysis. The percentage of solvent B with time is highlighted.

Time (mins)	B%
0	0
2	100
5	0
6	0

Table 2.44: HPLC gradient used for ketoprofen analysis. The percentage of solvent B with time is highlighted.

In addition to substrate depletion, diclofenac and phenylbutazone samples were monitored for the appearance of known metabolites, 4-hydroxydiclofenac and oxyphenbutazone. These ions were monitored for along with the parent drug ions and methods developed ensured retention times for drug and metabolite varied enough to distinguish between peaks.

2.48: LCMS data analysis

LCMS data was analysed via the Masslynx v4.0 software (Waters). The natural logarithm (Ln) of each chromatogram peak area (measured in arbitrary units) was calculated using Microsoft Excel. This was plotted against time using GraphPad Prism 6 in order to generate linear graphs at each substrate/inhibitor concentration. The initial rate (v_0) was calculated using the slope of the line (k_{dep}) of each individual graph via the following equation where $[S]_0$ is the initial substrate concentration:

$$v_0 = k_{dep}[S]_0$$

v_0 was used to generate a Michaelis-Menten analysis in order to estimate the Michaelis constant (K_m) and/or the maximum velocity of the reaction (V_{max}). Michaelis-Menten graphs were created in GraphPad Prism 6. When the substrate or metabolite caused inhibition of metabolism, Michaelis-Menten kinetics could not be used and so a substrate inhibition curve was fitted to the data in GraphPad Prisim 6.

In order to generate an intrinsic clearance (CL_{int}), the following equation was used:

$$CL_{int} = \frac{V_{max}}{K_m}$$

In cases where the V_{max} and K_m could not be estimated (i.e. where substrate inhibition had occurred), the initial velocity (the slope of the line where substrate

concentration is below K_m) of the reaction was used to estimate CLint (see Chapter 6).

Chapter 3: Bioinformatics analysis

3.1: Introduction

The equine genome was first sequenced in 2009 (Wade *et al*, 2009) using a single Thoroughbred mare ('Twilight'). Since then, the genome for an American Quarter horse has been sequenced (Doan *et al*, 2012) as well as draft genomes for Przewalski's horse (*Equus Przewalski*) and a donkey (*Equus asinus*), Orlando *et al* (2013). The sequencing of the equine genome means new approaches can now be taken in equine genomic research, and the *in silico* applications of this are numerous (Hert *et al*, 2008, Hobert, 2010). The choice of a Thouroughbred for the original sequencing was largely due to the high level of homozygosity this breed contains, which is a factor that can greatly influence the assembly of a genome, making the assembly process substantially easier than for more heterozygous genomes (Kajitani *et al*, 2014). A whole-genome shotgun method was used and the genome was found to be ~2.5 Gb in size, which is around the same size as mouse (Mouse Genome Sequencing Consortium, 2002), smaller than human (3 Gb, International Human Genome Sequencing Consortium, 2001), and cow (3 Gb, Bovine Genome Sequencing and Analysis Consortium, 2009) but larger than dog (2.4 Gb, Lindblad-Toh *et al*, 2005). During sequencing of the equine genome, a single nucleotide polymorphism (SNP) map was created, which has since been expanded, with 54,000 SNPs being analysed (McCue *et al*, 2012). See table 3.1 for details of gene numbers from the equine genome assembly (Ensembl build 80.2).

	Horse	Human	Mouse	Dog	Cow
Genome Size (Gb)	2.5	3	2.5	2.4	3
Coding	20,449	20,296	22,547	19,856	19,994
Non-coding	2,142	25,173	12,583	3,774	3,825
Pseudogenes	4,400	14,424	8,770	950	797

Table 3.1: Summary of predicted genes for Horse, Human, Mouse, Dog and Cow. Data was retreived from Ensembl (http://www.ensembl.org/) build 81.2 (horse), 81.38 (mouse and human), 81.31 (dog and cow).

3.1.1: Equine P450 genes

To date, no research has been carried out to ascertain how many CYP genes the equine genome may contain, although Schmitz *et al* (2010) analysed the CYP3A gene cluster, finding six potential genes, which contrasts with the four in human (Gellner *et al*, 2001). Two pseudogenes were also found, which matches the number of CYP3A pseudogenes in human (Finta and Zaphiropoulos, 2000) and P450 sequences in both species were highly homologous. The first aim of this study was to expand on this knowledge by finding all P450 genes present in the horse. This was implemented in two ways – first, the equine genome was searched using all known human CYP genes as probes. Secondly, the Ensembl and NCBI databases were searched for all equine genes automatically annotated as P450s. Duplicate genes were removed and so a full list of potential equine P450 genes was generated.

3.1.2: P450 clans

In human, there are 57 known P450 genes and 58 pseudogenes, however P450 numbers vary significantly across species – even within the Mammalia - with 102 functional genes in the mouse, 89 in the rat, 59 in cattle and pigs, and 62 in the chimpanzee (Nelson *et al*, 2003, Nelson, 2009, Sim and Ingelman-Sundberg, 2010, Puccinelli and Gervasi, 2011). P450s are divided into 'clans', which are sometimes also referred to as gene clusters (Good *et al*, 2014, Nelson, 1998). Clans/clusters are used to organise P450s based on evolution, with P450s being grouped together due to common evolutionary ancestors determined via phylogenetic analysis of sequence divergence (Kirischian *et al*, 2011, McArthur *et al*, 2003). It is possible for P450s of different families to be in one clan as they may be closely related phylogenetically (Nelson, 1998). In mammals there are nine distinct clans – CYP1, CYP2, CYP3, CYP4, CYP19, CYP20, CYP26, CYP51 and the mitochondrial P450 clan (Nelson, 2003, Omura, 2012) – see table 3.1.2.

There are currently 14 known equine P450 genes – CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96, CYP3A97 (Schmitz *et al*, 2010, DiMaio Knych *et al*, 2010), CYP2C92 (DiMaio Knych *et al*, 2009), CYP2D50 (DiMaio Knych and Stanley, 2008), CYP2B6 (Peters *et al*, 2013), CYP11A1 (Boerboom and Sirois, 2001), CYP27B1, CYP2A13, CYP2E1 (based on unpublished data by DiMaio Knych *et al*) and CYP19A1 (Seralini *et al*, 2003). Genes from the CYP1 CYP4, CYP7, CYP20, CYP26 and CYP51 clans have yet to be found in the horse (see table 3.1.2).

Clan/Cluster	Families in Clan/Cluster	Equine Genes in Clan/Cluster
CYP1	CYP1	None
CYP2	CYP2	CYP2A13, CYP2B6, CYP2C92, CYP2D50, CYP2E1
CYP3	CYP3, CYP5	CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96, CYP3A97
CYP4	CYP4, CYP5	None
CYP17	CYP17, CYP21	None
CYP19	CYP19	CYP19A1
CYP20	CYP20	None
CYP26	CYP26	None
CYP51	CYP51, CYP7, CYP8, CYP39	None
Mitochondrial	CYP11, CYP27	CYP11A1, CYP27B1

Table 3.1.2: Organisation of mammalian P450 families into clans along with those known in the horse. All mammalian P450 clans/clusters are highlighted along with which families they contain. All 14 known equine P450 genes are highlighted in order to display which clans they belong to.

3.1.3: Current study

For the current study, attempts were made to find all equine P450s by searching the equine genome. Once sequences had been gathered, homology was determined through multiple sequence alignments and phylogenetic relationships were inferred through the generation of phylogenetic trees, allowing the validation of grouping the sequences into clans. Additionally, haem-binding (conserved across all P450s, Ranasinghe and Hobbs, 1998) and substrate recognition regions were highlighted in order to validate their P450 status and to postulate potential substrate similarities with human isoforms.

3.2: Results

3.21: Alignment of known equine P450s shows high homology

A heat map was generated based on the DNA sequence similarities of all known equine CYPs (fig 3.1) The mitrochondrial P450s have the lowest overall similarity with other CYPs as well as having relatively low similarity to each other. The lowest score was between CYP27B1 and the CYP3A subfamily, ranging from a 45.9-52.63%. The highest degree of similarity was between the 3A P450s (ranging from 88.23-91.6%). A heat map of an alignment of the protein sequences of these enzymes was also generated (fig 3.2). Protein sequence scores had a much larger range, from the lowest value of 16.99% for the alignment between CYP2D50 and CYP11A1 to the highest for CYP3A89 and CYP3A93 (88.47%), indicating that the protein sequences vary more between isoforms. Overall the protein alignment scores are much lower than for the DNA alignments. For those of the same subfamily (i.e. 3A), protein alignment scores are similar to DNA alignment scores, indicating high homology within subfamilies.

	CYP2A13	CYP2B6	CYP2C92	CYP2D50	CYP2E1	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97	CYP11A1	CYP19A1	CYP27B1
CYP2A13	100													
CYP2B6	67.07	100												
CYP2C92	64.44	60.94	100											
CYP2D50	59.19	57.51	56.75	100										
CYP2E1	63.16	62.96	65.17	58.75	100									
CYP3A89	59.39	57.71	55.82	53.36	56.36	100								
CYP3A93	49.49	53.13	57.41	46.91	54.3	91.6	100							
CYP3A94	52.26	54.34	59.39	47.17	55.29	91.73	91.01	100						
CYP3A95	53	54.07	56.75	46.17	54.89	89.68	88.89	88.89	100					
CYP3A96	53.13	53.67	56.3	47.84	52.78	88.52	88.43	88.82	88.62	100				
CYP3A97	57.78	58.25	52.65	52.3	53.79	87.98	89.68	89.62	88.23	87.53	100			
CYP11A1	52.93	53.54	52.53	53.43	52.91	54.7	52.12	50.33	50.07	50.07	51.12	100		
CYP19A1	64.11	65.52	60.11	62.48	62.37	56.78	63.23	63.76	63.23	61.6	62.26	63.34	100	
CYP27B1	53.94	54.61	49.68	55.89	51.61	52.63	45.97	49.07	45.9	49.26	51.61	53.28	60.93	100

Figure 3.1: Heat map of an alignment between all known equine P450s. All known equine CYPs were aligned using ClustalW and a heat map was produced to highlight levels of percentage similarity, with the highest in green and the lowest in red (colour changes occur every five percentage points, e.g. 95-100 is darkest green etc.).

	CYP2A13	CYP2B6	CYP2C92	CYP2D50	CYP2E1	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97	CYP11A1	CYP19A1	CYP27B1
CYP21A13	100													
CYP2B6	53.77	100												
CYP2C92	51.63	51.53	100											
CYP2D50	37.8	41.1	41.19	100										
CYP2E1	48.58	47.55	55.21	36.12	100									
CYP3A89	24.57	24.78	26.3	24.15	24.14	100								
CYP3A93	25.22	25.65	26.74	24.15	23.92	88.47	100							
CYP3A94	25	25.86	25.43	24.36	23.92	88.07	86.48	100						
CYP3A95	24.57	25.65	25.22	23.5	22.84	85.09	83.3	84.29	100					
CYP3A96	20.69	22.41	22.17	21.15	21.12	78.93	76.74	78.33	77.53	100				
CYP3A97	24.78	25.22	25.87	22.86	23.71	85.29	84.1	84.69	81.31	76.34	100			
CYP11A1	18.5	18.72	19.73	16.99	18.76	23.97	23.33	22.46	23.97	19.44	23.54	100		
CYP19A1	20.52	19.56	20.26	17.67	21.23	21.27	22.85	22.17	20.36	18.33	22.62	17.08	100	
CYP27B1	21.4	22.2	22.47	20.91	22.81	24.68	24.68	22.73	24.24	21	24.03	31.25	18.69	100

Figure 3.2: Protein sequence alignment heat map of known equine P450s. All known equine CYPs were aligned using ClustalW and a heat map was produced to highlight levels of percentage similarity, with the highest in green and the lowest in red.

A third heat map was generated from an alignment of all known equine P450s with their human isoforms (fig 3.3). The highest scores were within subfamilies of the same species (e.g between the equine CYP3A sequences) and the highest overall

score was between human CYP3A7 and CYP3A4 (94.18). The highest interspecies

score was between human CYP3A4 and equine CYP3A93 (85.19%). The 19, 11 and

27 subfamilies had consistently low (<45%) scores with other P450s and between

each other, however lowest score overall was between human CYP3A43 and equine

CYP2A13 (29.22%).

Figure 3.3: Heat map of horse vs human alignment. All known equine P450s were aligned with their human isoforms using ClustalW and a heat map was generated based on percentage identity alignment scores. Human isoforms are highlighted in blue while the highest, lowest and highest interspecies scores are circled.

	CYP19A1	CYP19A1	CYP27B1	CYP27B1	CYP1A1	CYP3A96	CYP3A97	CYP3A95	CYP3A94	CYP3A89	CYP3A93	CYP3A43	CYP3A5	CYP3A4	CYP3A7	CYP2D6	CYP2D50	CYP2A13	CYP2B6	CYP2B6	CYP2C9	CYP2C92	CYP2E1	CYP2E1
CYP19A1	100																							
CYP19A1	82.4	100																						
CYP27B1	35.88	35.29	100																					
CYP27B1	33.66	33.16	88.3	100																				
CYP1A1	31.5	32.96	46.04	45.89	100																			
CYP1A1	30.84	31.95	45.53	45.68	84.07	100																		
CYP3A96	34.38	35.34	33.29	32.23	32.44	31.58	100																	
CYP3A97	34.57	35.88	34.26	33.16	32.44	32.07	87.07	100																
CYP3A95	34.57	35.3	33.08	32.18	30.76	31.39	88.09	87.96	100															
CYP3A94	33.5	34.48	33.64	33.42	32.74	32.43	88.42	89.55	88.82	100														
CYP3A89	33.57	37.31	34.91	33.36	35.24	31.81	88.14	85.91	89.48	91.6	100													
CYP3A93	33.89	34.68	33.29	33.16	32.02	30.87	88.02	89.48	88.82	91.01	91.6	100												
CYP3A43	33.3	34.48	32.73	31.72	32.22	31.8	79.48	79.7	81.35	80.89	81.68	80.69	100											
CYP3A5	33.85	34.8	34.07	33.28	31.69	31.43	80.83	79.75	82.37	83.37	81.07	83.76	83.23	100										
CYP3A4	33.5	34.27	33.08	32.91	31.19	30.66	83.26	83.2	83.53	85.05	85.19	85.19	83.23	89.4	100									
CYP3A7	33.7	34.9	34.11	33.55	31.95	31.68	80.92	81.34	81.94	83.73	82.88	83.8	82.14	84.19	94.18	100								
CYP2D6	29.69	31.6	35.78	35.45	34.3		34.82	35.51	34.9	36.45	36.33	36.15	36.01	36.19	35.73	35.26	100							
CYP2D50	30.06	32.03	35.07	36.09	34.08	32.96	35.75	35.98	35.24	37	36.5	36.15	36.15	37.01	36.5	35.77	83.7	100						
CYP2A13	34.36	33.54	33.35	35.5	30.89	30.64	37.76	37.63	38.76	38.76	38.83	39.25	38.48	38.39	38.24	36.59	48.9	49.73	100					
CYP2B6	33.73	33.15	32.96	34.34	32.4	32.08	38.27	37.95	38.59	38.8	38.38	38.87	38.24	38.39	37.15	36.59	50.03	49.83	80.08	100				
CYP2B6	34.62	34.09	33.88	34.98	32.76	31.25	37.78	38.27	38.78	38.82	38.92	38.8	38.36	37.95	37.95	49.41	49.8	60.66	61.23	100				
CYP2C9	33.93	33.87	34.78	34.17	32.71	39.53	39.61	38.9	39.61	39.62	40.52	38.9	39.96	39.62	49.29	49.18	60.54	60.77	83.5	100				
CYP2C92	34.62	34.75	33.87	33.59	33.09	38.03	38.49	38.55	38.83	39.74	38.9	38.71	37.76	37.41	47.06	47.81	53.99	57.37	55.13	80.16	100			
CYP2E1	35.03	34.54	35.88	35.65	34.89	37.05	36.76	37.23	37.44	37.17	37.91	37.47	37.58	37.04	48.06	49.23	54.95	57.56	55.94	56.21	61.04	100		
CYP2E1	34.72	33.77	35.65	38.06	33.9	36.48	36.81	37.89	38.18	38.2	39.02	39.93	37.09	36.55	48.18	48.13	55.58	58.28	56.61	56.55	60.95	59.33	79.38	100

81

3.22: Equine genome and annotated sequence search, 59 potential sequences

Two approaches were taken to identify novel equine P450 sequences – searching the UCSC genome browser via BLAT and searching the Ensembl and NCBI databases for automatically annotated sequences.

From the BLAT search 59 potential sequences were extracted, although most appeared to be partial sequences due to being less than 1kb in length. Using NCBI BLAST, the sequences were compared against the full NCBI database and those that matched non-P450 genes at this stage were removed. For the second method, the Ensembl and NCBI sequence databases were searched for equine sequences automatically annotated with 'P450' by searching within the *Equus Caballus* build for this term. These sequences were aligned with the sequences from the equine genome BLAT search in order to detect overlaps and duplicates. All sequences from the equine genome BLAT search are displayed in table 3.2, highlighting which P450s they match and whether they are an exact match for an annotated sequence. Sequence lengths are illustrated as one parameter to differentiate full from partial sequences, since most P450s are around 1.5kb in length.

Given Name	Chromosome	Strongest BLAT Match	Clustal % Horse	Clustal % Human	Length (bp)
Ecab.P450.1	**chr1**	**CYP2E1**	**100%**		**1179**
Ecab.P450.2	*chr1*	*CYP2C26*	*100% predicted*		*675*
Ecab.P450.3	*chr1*	*CYP2C26*	*100% predicted*		*645*
Ecab.P450.4	chr1	CYP2R1		*90%*	681
Ecab.P450.5	*Chr1*	*CYP1A2*	*100% predicted*		*1545*
Ecab.P450.6	*chr1*	*CYP1A1*	*100% predicted*		*1524*
Ecab.P450.7	**chr10**	**CYP2B6**	**97%**		**648**
Ecab.P450.9	chr13	CYP2W1		86%	615
Ecab.P450.10	**chr13**	**CYP3A97**	**100%**		**1509**
Ecab.P450.11	*chr13*	*CYP3A12*	*100% predicted*		*1506*
Ecab.P450.12	*chr13*	*CYP2G1*	*91% predicted*		*717*
Ecab.P450.13	*chr15*	*CYP1B1*	*100% predicted*		*1311*
Ecab.P450.14	*chr2*	*CYP2J2*	*100% predicted*		*624*
Ecab.P450.15	*chr2*	*CYP2U1*	*100% predicted*		*780*
Ecab.P450.17	*chr28*	*CYP2D14*	*99% predicted*		*639*
Ecab.P450.18	chr7	CYP2R1.	100% predicted *E. przewalskii*		1197
Ecab.P450.19	*unmapped*	*CYP2D14*	*100% predicted*		*897*
P450.Equ.1	*Chr1*	*CYP1A1*	*100% predicted*		*519*
P450.Equ.2	**Chr10**	**CYP2B6**	**99%**		**666**
P450.Equ.6	*Chr13*	*CYP2G1*	*92% predicted*		*480*
P450.Equ.12	*Chr15*	*CYP1B1*	*100% predicted*		*1311*
P450.Equ.16	*Chr18*	*CYP20A 1*	*100% predicted*		*1119*
P450.Equ.17	*Chr2*	*CYP2U1*	*100% predicted*		*636*
P450.Equ.18	*Chr21*	*CYP4F22*	*100% predicted*		*1212*
P450.Equ.19	*Chr21*	*CYP4F3*	*100% predicted*		*1557*
P450.Equ.23	*Chr24*	*CYP46A1*	*100% predicted*		*1395*
P450.Equ.30	*Chr28*	*CYP2D14*	*97% predicted*		*507*
P450.Equ.37	**Chr6**	**CYP27B1**	**100%**		**837**

Table 3.2: Sequences generated from equine genome search. The UCSC genome browser was searched using probe sequences from other species. Those highlighted in bold match previously known equine P450s while those in italics match sequences predicted as P450s on the NCBI database. The strongest match for each sequence is shown and % similarity (horse or human, depending on strongest match) is displayed.

The annotated P450 sequences are listed in table 3.3, ordered by chromosome number. All major P450 families (1A, 2B, 2C, 2D, 2E, 3A) are represented and in total there were 46 full-length sequences, verified by BLAST database searches against P450s in other species.

Chromosome	P450 genes
1	CYP2E1, CYP17A1-like, CYP2C21-like, CYP2C92, CYP2C19-like, CYP2C18-like, CYP26A1-like, CYP26C1-like , CYP1A2-like, CYP1A1 CYP11A1-like, CYP19A1
2	CYP2J2-like, CYP4X1-like, CYP4B1-like, CYP4A11-like, CYP4A7-like, CYP2U1-like
6	CYP27B1
9	CYP11B1-like
10	CYP2F5-like, CYP2A13, CYP2B6, CYP2G1-like, CYP2B4-like, CYP2S1-like
13	CYP3A93, CYP3A89, CYP3A94, CYP3A95, CYP3A96, CYP3A97, CYP3A12-like
15	CYP26B1-like, CYP1B1-like
18	CYP27C1-like, CYP20A1-like
21	CYP4F22-like, CYP4F6-like, CYP4F3-like
24	CYP46A1-like
27	CYP4V2-like
28	CYP2D14-like, CYP2D50

Table 3.3: Summary of predicted and known equine sequences on each chromosome.

Sequences were sorted by chromosome and order within chromosome. In total there are 46

sequences (14 known and 32 novel).

Only three sequences (E.cab.P450.4, Ecab.P450.9 and Ecab.P450.18) of the 59

from the original UCSC genome search using human probe sequences were not

matches with the 46 annotated (known and predicted) equine sequences – and of

these none appear to be full length sequences, although at 1197bp Ecab.P450.18 is

near the expected length for a P450 gene. E.cab.P450.4 does not possess the

FxxGxxxCxG haem-binding motif (which is conserved across all P450s) however

Ecab.P450.18 and Ecab.P450.9 do, meaning they appear to at least be partial P450

sequences.

All other sequences were identical to NCBI/Ensembl annotated equine sequences.

Ecab.P450.18 was aligned with its closest protein BLAST match in order to

demonstrate the level of sequence conservation and to point out the haem-binding

region (fig 3.4). The haem-binding region is also highlighted for Ecab.P450.9 (fig

3.5). Additionally, the protein sequences of all annotated sequences were analysed for the presence of haem-binding motif (refer to appendix for sequences). CYP3A12-like, CYP2S1-like and CYP20A1-like all lack this motif while the rest were confirmed to possess it. Additionally, some sequences (CYP2C, CYP3A, CYP2D, CYP2E1 and CYP1A2) which are most likely involved in drug metabolism (due to being matches with human isoforms involved in drug metabolism) were aligned with their human isoforms with substrate recognition sites highlighted in order to demonstrate whether substrate specificities may be the same (figures 3.6-3.10). For downstream studies (chapter 4), sequences were selected based on their likihood of being involved in drug metabolism (Ecab.P450.1, Ecab.P450.5, P450.Equ.30, CYP3A97, CYP2C26-like) or because expression levels/substrate specificities in other species are not well characterised (Ecab.P450.5, Ecab.P450.18, P450.Equ.18, P450.Equ.23, P450.Equ.37, CYP2U1-like). Additionally, CYP1B1-like and CYP1A1-like were selected as P450s not expected to be significantly expressed in liver.

```
Ecab.P450.18    ------------------------------------------------------------ 0
CYP2R1          MWDPRGAEACAAALGGALLLLLFALGVRQLLKQRRPAGFPPGPSGLPFIGNIYSLAASAE 60


Ecab.P450.18    ---------------------------------------------------------MTK 3
CYP2R1          LPHVYMRKQSQAYGEIFSLDLGGISTVVLNGYDVVKECLVHQSEIFADRPCLPLFKKMTK 120
                                                                            ***

Ecab.P450.18    MGGLLNARYGRGWVDHRRLAVNSFRYFGYGQKSFESKILEETKFFIDAVETYKGRPFDFK 63
CYP2R1          MGGLLNARYGRGWVDHRRLAVNSFRYFGYGQKSFESKILEETKFFIDAVETYKGRPFDFK 180
                ************************************************************

Ecab.P450.18    QLITNAVSNITNLIIFGERFTYEDTDFQHMIELFSENVELAASASVFLYNAFPWIGILPF 123
CYP2R1          QLITNAVSNITNLIIFGERFTYEDTDFQHMIELFSENVELAASASVFLYNAFPWIGILPF 240
                ************************************************************

Ecab.P450.18    GKLQQLFRNADVVYEFLSRLIEKVSVNRKPQLPQHFVDAYLDEMDQGQNDPSSTFSKENL 183
CYP2R1          GKLQQLFRNADVVYEFLSRLIEKVSVNRKPQLPQHFVDAYLDEMDQGQNDPSSTFSKENL 300
                ************************************************************

Ecab.P450.18    IFSVGELIIAGTETTTNVLRWAILFMALYPNIQGQVQKEIDLIMGPSGKPSWDDKCKMPY 243
CYP2R1          IFSVGELIIAGTETTTNVLRWAILFMALYPNIQGQVQKEIDLIMGPSGKPSWDDKCKMPY 360
                ************************************************************

Ecab.P450.18    TEAVLHEVLRFCNIVPLGIFHATSEDTVVRGYSIPKGTTVITNLYSVHFDEKYWRDPEIF 303
CYP2R1          TEAVLHEVLRFCNIVPLGIFHATSEDTVVRGYSIPKGTTVITNLYSVHFDEKYWRDPEIF 420
                ************************************************************

Ecab.P450.18    YPERFLDSSGYFAKKEAFIPFSLGRRHCLGEQLARMEMFLFFTALLQRFHL--------- 354
CYP2R1          YPERFLDSSGYFAKKEAFIPFSLGRRHCLGEQLARMEMFLFFTALLQRFHLHFPHELVPN 480
                **************************************************

Ecab.P450.18    -------------------- 354
CYP2R1          LKPRLGMTLQPQAYLICAERR 501
```

Figure 3.4: Clustal Omega alignment of sequence (Ecab.P450.18) from equine genome with CYP2R1 from *Equus przewalskii*. Protein sequences were aligned with 100% identity and haem binding region is highlighted (red box).

```
> Ecab.P450.9

MVACALDMVMAGTETTSATLQWAALLMGKHPSVQGRVQEELDRVLGPERLPRLEDQRSL
PYTNAVLHEVQRFITLLPHVARCTAADIQLCGYQLPKGTPVVPLLSSVLLDKTQWATPHQF
NPGHFLDADGRFVKPAAFLRFSAGRRVCVGRAAGQKLFLLFAGLLQRYRLAARLSPATLDT
TPAPAFTMRPPAQALCAGP
```

Figure 3.5: Protein sequence of Ecab.P450.9 with haem-binding region highlighted.

Figure 3.6: Annotated protein alignment of human CYP2D6 and equine CYP2D50 (% identity 77.26). Alignment was carried out with Clustal Omega. Substrate recognition regions (SRSs) as proposed by Ito *et al* (2008) are highlighted on the human isoform in order to observe similarity with the horse isoform. The residues determined to be critical for substrate recognition are highlighted in yellow (Ito *et al*, 2008).

Figure 3.7: Annotated protein alignment of human CYP2C9 with all predicted equine CYP2C isoforms. Alignment was carried out with Clustal Omega. Substrate recognition regions (SRSs) as proposed by Zawaira *et al* (2011) and Lewis and Ito (2009) are highlighted on the human isoform in order to observe similarity with the horse isoforms. The residues determined to be critical for substrate recognition are highlighted in yellow.

```
                                                                        SRS1A        SRS1B
CYP3A96   MDLIPSFSVETWVLLATSLALLYLGIYTHGLFKKLGIPGPKPLPFLGTLLGYRKGFGGF  60
CYP3A4    MALIPDLAMETWLLLAVSLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKGFCMF  60
CYP3A95   MDLIPSLSMETWVLLATSLVLLYLYGTYSHGHFKKLGIPGPTPLPFFGTVLGYIKGVLAF  60
CYP3A97   MDLIPNFSTETWVLLATSLVFLYLFGTYTHGLFKKLGIPGPTPLPFFGNILSYRKGIWDF  60
CYP3A94   MDLIPNFSMETWVLLATSLVLLYLYGTYTHGLFKKLGIPGPTPLPFLGTVLGYLKGFWDF  60
CYP3A89   MDLIPSFSMETWVLLATSLVLLYLYGTYTHGLFKKLGIPGPTPLPFFGNVLSYHKGIWDF  60
CYP3A93   MDLIPTFSMETWVLLATSLVLVYLYGTYTHGLFKKLGIPGPTPLPFFGNVLSYRKGISNF  60
          * *** :: ***:***.**.::**:* ::** *********.****:*.:*.* **.  *

                                                              SRS1A        SRS1B
CYP3A96   DTECLKKYRKMWGFYDGRQPVLAITDPDLIKTVLVKECYSVFTNRRSFGPVGFMKNAISI  120
CYP3A4    DMECHKKYGKVWGFYDGQQPVLAITDPDMIKTVLVKECYSVFTNRRPFGPVGFMKSAISI  120
CYP3A95   DKKCFKKYGKMWGFYDGRQPVLAITDPDMIKTVLVKECYSVFTNRRSFGPMGLMKNAITI  120
CYP3A97   DKKCFKKYGKTWGFYEGRLPVLAITDPDMIKTVLVKECYSVFTNRRTFGPEGFMKNAITR  120
CYP3A94   DKKCFKKYGSMWGIYDGPQPVLAITDPDMIKTVLVKECYSVFTNRRSLGPVGFMKNAISL  120
CYP3A89   DKKCFEKYGKMWGTYHGTKPVLAITDPDMIKTVLVKECYSVFTNRRPFGPFGFMKSAISL  120
CYP3A93   DRKCFKKYGKMWGVYDGRRPVLAITDPDMIKTVLVKECYSVFTNRRPFRPVGFMKSAISL  120
          * :* :** . ** *.* *********:****************: * *.**.**:

CYP3A96   SEDEQWKRIRTLLSPTFTSGKLKEMFPIIGQYGDVLVRNLKKEAEKGKPIALKDIFGAYS  180
CYP3A4    AEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAETGKPVTLKDVFGAYS  180
CYP3A95   AEDEKWKRIRTLLSPTFTSGKLKEMFPIIGQYGDMLVRNLRKEAEKGKPITMKDIFGAYS  180
CYP3A97   SEDEQWKRIRTLLTPTFTSGKLKEMFPIIGHYGDVLVRNLRNEAEKGKPVTLKNIFGAYS  180
CYP3A94   SEDEQWKRIRTLLSPTFTSGKLKEMFPIIGQYGDVLVRNLRKETEKGKPVTLKNIFGAYS  180
CYP3A89   SEDEQWKRIRTLLSPTFTSGKLKEMFPILGQYGDVLVRNLKKEAEKGKPITLKDIFGAYS  180
CYP3A93   SEDEWKRIRTLLSPTFTSGKLKEMFPIIGQYGDVLVRNLKKEAEKGKPVTLKDIFGAYS  180
          :***:***:*:**:***********.**:.:***:****:.*:*.***:::*:*****

                              SRS2          SRS3
CYP3A96   MDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFDFLDPFILSITIFPFLNPVFELLNIFL  240
CYP3A4    MDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFDFLDPFFLSITVFPFLIPILEVLNICV  240
CYP3A95   MDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFDFLDPFFLSILFFPFLIPVYEALNIFL  240
CYP3A97   MDVITSTSFGVNIDSLNNPQDPFVDNAKRLLRLDFLDPLILSITLFPFLRPVYEALNISV  240
CYP3A94   MDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFNFLDPFFLSITLFPFLKAVFEVIHIYM  240
CYP3A89   MDVITSTSFGVNIDSLNNPQDPFVENTKKLFSFDFLDPLILSITLFPFLNAVFEVLNVFV  240
CYP3A93   MDVITSTSFGVNIDSLNNPQDPFVENSNKLLRFDFLNPLILLMVLFPFLQPIFEVLNISL  240
          **************************:*::**:**::*:*  :.**** .* *:.:  :

                                                                  SRS4
CYP3A96   FPKSVTDFFTKSVKGIKESRLKDKEKQRVDFLQLMINSQNSKEMDTHKALSDLELVAQSI  300
CYP3A4    FPREVTNFLRKSVKRMKESRLEDTQKHRVDFLQLMIDSQNSKETESHKALSDLELVAQSI  300
CYP3A95   FPKCVTDFFIKSVKKMKESRLKDKAKQRVDFLQLMINSQNSKEIDNLKVLSDLELVAQSI  300
CYP3A97   FPKSVTDFFIKSVKRMKESRLKNKETNRVDFLQLMINSQNSKEMDTHKALSDLELVAQSI  300
CYP3A94   FPKRVTDFFTKSVKRMKESRLKDKQKHRVDFLQLMINSQNSKEMDTHKALSDLELVAQSI  300
CYP3A89   FPKSVTDFFIKSVKRMKESRLKDKEKHRVDFLQLMINSQNSKELDTHKALSDLELVAQSI  300
CYP3A93   FPKSAIDFFTKSVKRMKESRLKDKDKHRVDFLQLMINSQNSKELDTHKGLSDLELVAQSI  300
          **:. .:*: **** :*****::. ..**********:***** :. * ***********

CYP3A96   IFIFAGYEPSAVLFFLFCIFWPLTLMSSRSCRRRLMQLPPIWHLPTYDALVQMEYLDMVL  360
CYP3A4    IFIFAGYETSSVLSFIMYELATHPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVL  360
CYP3A95   TFIFAGYEPVSTSLSFLLYLLATHPDVQQKLQEEIDTFPNKAPPTYDALVQMEYLDMVL  360
CYP3A97   VFIFAGYETTSTSLSFLMYLLATHPDVQQKLQKEIDVTFPNKVPPTYDALLQMDYLDMVL  360
CYP3A94   IFIFAGYETTSTSLSFLMYLLATHPDVQQKLQDEIDVTFPNKALPTYDTLLQMEYLDMVL  360
CYP3A89   IFIFAGYETTSSSLSFLMYFLATHPDVQQKLQEEIDATFPNKAPPTYDALVQMEYLDMVL  360
CYP3A93   VFIFAGYETTSTSLSFLLYLLATHPDVQQKLQEEIDAIFPNKAPPTYDALVQMDYLDMVL  360
          *******  :  ::            .:. : .:    *    ****::**:*****:

               SRS5
CYP3A96   NESLRLFPVAGRIERTCKKDVELGGVFIPKGTVVMVPSFVLHRDTELWPQPEEFHPERFS  420
CYP3A4    NETLRLFPIAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKYWTEPEKFLPERFS  420
CYP3A95   NETLRLFPIADRLERVCKKDVEINGVFIPKGSTVMMPIFVLHQHPEFWSEPEEFRPERFS  420
CYP3A97   NESLRLFPVAGRLQRICKKDVELNGVFIPKRTLVTVPTFVLHRASEFWPEPEEFRPERFS  420
CYP3A94   NESLRLFPIAGRLERVCKKDVEINGVLIPKGTVVMVPTFILHRASEFWPEPEEFCPERFS  420
CYP3A89   NESLRLFPIAVRLERVCKKDVEINGVFIPKGTVVMVPTFALHKHPEFWPEPEEFRPERFS  420
CYP3A93   NESLRLFPVAVRLERVCKKDAEINGVFIPKGTVVMVPTFSLHRASEFWPEPEEFRPERFS  420
          **:*****:* *::* ****.*: *::*** *  * :* : **:   : * :**:* *****

                                                                  SRS6
CYP3A96   KENKDSINLYIYMPFGNGPRNCIGMRFVLMNMKVAVVRVLQNFSFNPCKETQIPLKLVTY  480
CYP3A4    KKNKDNIDPYIYTPFGSGPRNCIGMRFALMNMKLALIRVLQNFSFKPCKETQIPLKLSLG  480
CYP3A95   KKNKDSINPYTYLPFGTGPRNCIGMRFAIVNIKLAVVRVLQNFSFKPCKETQIPLKLVSQ  480
CYP3A97   KENKDSINPYIYLPFGTGPRNCIGMRFALMNMKLAVVRVLQNFSFKPCKETEIPIKLGTE  480
CYP3A94   KNNKDNINPYIYLPFGTGPRNCIGMRFAIMNMKLAIVRVLQNFSFKPCKETQIPLKIGNQ  480
          *:***.*: ** *** ***********.::*:*:*:***********:****:*:**:

CYP3A96   GFFHPEKPIVLKVESRAGTVSGA   503
CYP3A4    GLLQPEKPVVLKVESRDGTVSGA   503
CYP3A95   GLIRPEKPIVLKVESRDGTMSGA   503
CYP3A97   AIVKPQKPIVLKVEPRDGTVTGA   503
CYP3A94   GLIQPQKPIVLKVESRDGTWNGA   503
CYP3A89   GLLQPQKPIVLKVESRDGTVSGA   503
CYP3A93   GLLQPQKPIVLKVESRDGTVNGA   503
          .::*:*:**:***** * ** .**
```

Figure 3.8: Annotated protein alignment of human CYP3A4 with all known equine CYP3A isoforms. Alignment was carried out with Clustal Omega. Substrate recognition regions (SRSs) are highlighted on the human isoform (Lewis and Ito, 2009) in order to observe similarity with the horse isoforms. The residues determined to be critical for substrate recognition are highlighted in yellow.

```
CYP1A2-like   MMLSQLSPFSATELLLASTIFCLVFWVVRAWQPQIPKGLKSPPGPWGWPFLGHVLTLGKN   60
Human_CYP1A2  MALSQSVPFSATELLLASAIFCLVFWVLKGLRPRVPKGLKSPPEPWGWPLLGHVLTLGKN   60
              * *** ***********:********:. :*::******* ***** **********

                                                             SRS1A
CYP1A2-like   PHLALSRLSQRYGDVMQIRIGSTPVLVLSGLDTIRQALVRQGDDFKGRPDLYSFTLITNG  120
Human_CYP1A2  PHLALSRMSQRYGDVLQIRIGSTPVLVLSRLDTIRQALVRQGDDFKGRPDLYTSTLITDG  120
              *******:*******:******************************* : ****:*

              SRS1B
CYP1A2-like   QSMTFNPDSGPVWAARRRLAQNALNTFSIASDPASMSSCYLEEHVSKEAEALLSRLQKLM  180
Human_CYP1A2  QSLTFSTDSGPVWAARRRLAQNALNTFSIASDPASSSSCYLEEHVSKEAKALISRLQELM  180
              **:**. ************************** ******** **:**** :**

                                                   SRS2        SRS3
CYP1A2-like   SVAGRFDPSSQVVASVANVIGAMCFGQHFPHSSEEMISLLRSSHEFVQTASSGNPVDFFP  240
Human_CYP1A2  AGPGHFDPYNQVVVSVANVIGAMCFGQHFPESSDEMLSLVKNTHEFVETASSGNPLDFFP  240
              :  *:*** .*** .***.*************.**:**:**::.:****:*******:****

CYP1A2-like   ILRYLPNPPLQRFKSFNQRFLRFLQKIIQEHYRDFDKNSIQDITGALFKHREKSSRASGV  300
Human_CYP1A2  ILRYLPNPALQRFKAFNQRFLWFLQKTVQEHYQDFDKNSVRDITGALFKHSKKGPRASGN  300
              ******** *****:****** **** :****:******:.********* :*. ****

                            SRS4
CYP1A2-like   LIPQEKIINIINDIFGAGFDTVTTAITWSLTYLVTNPKIQRKIQEELDTVVGRARQPRLS  360
Human_CYP1A2  LIPQEKIVNLVNDIFGAGFDTVTTAISWSLMYLVTKPEIQRKIQKELDTVIGRERRPRLS  360
              *******:*:::.*************:**** ****:* :*****:.****:** ** *:****

                            SRS5
CYP1A2-like   DRPQLPYMEAFILETFRHSSFVPFTIPHSTVRDTTLNGFYIPKERCVFINQWHVNHDEEL  420
Human_CYP1A2  DRPQLPYLEAFILETFRHSSFLPFTIPHSTTRDTTLNGFYIPKKCCVFVNQWQVNHDPEL  420
              *******:************* :********* *************: *** *** :**** **

CYP1A2-like   WENPFEFRPERFLSADGTTINKTLSEKVMLFGMGKRRCIGEVLAKWEVFLFLAILLQRLE  480
Human_CYP1A2  WEDPSEFRPERFLTADGTAINKPLSEKMMLFGMGKRRCIGEVLAKWEIFLFLAILLQQLE  480
              **:* ********:****:***.*****:************************:*********:**

              SRS6
CYP1A2-like   FSVPPGVKLDLTPIYGLTMKHASCEHVQARLRFSIK   516
Human_CYP1A2  FSVPPGVKVDLTPIYGLTMKHARCEHVQARLRFSIN   516
              ********:************** ************:
```

Figure 3.9: Annotated protein alignment of human CYP31A2 with the predicted equine CYP1A2 ('CYP1A2-like'), % identity 83.53. Alignment was carried out with Clustal Omega. Substrate recognition regions (SRSs) are highlighted on the human isoform (Lewis and Ito, 2009) in order to observe similarity with the horse isoforms. The residues determined to be critical for substrate recognition are highlighted in yellow.

```
Horse_CYP2E1   MAALGITVALLVWVATLLPISIWKQIYSSWNLPPGPFPLPIIGNLFHLDLKNIPKSFTRL   60
Human_CYP2E1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL   60
               *:***:*******.* ** :*:*:*;:*************************:*********

                                                  SRS1A          SRS1B
Horse_CYP2E1   AERYGPVFTLYLGSQRVVVMHGYKAVKEVLLNYKNELSGRGEIAVFQAHKDNGVIFNNGP   120
Human_CYP2E1   AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP   120
               *:*:*******:****:********** .**;**:*:****:: .*:**:*.*:******

Horse_CYP2E1   SWKDTRRLSLTILRDYGMGKQRNEERIQRETHFLLEALRKTQGQPFDPTFVLGGGPFNVI   180
Human_CYP2E1   TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI   180
               :*** **:*** **:****** **.*****:*********************::* .* ***

                            SRS2          SRS3
Horse_CYP2E1   ADILFHKHFDYEDKTCQRLMHLFNENFYLLSTPWLQAYNYFSTYLRYLPGSHRKVMKNVS   240
Human_CYP2E1   ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA   240
               *****:*****:.  ***:****** ******** ** * ::*:*********:***:

                                                              SRS4
Horse_CYP2E1   EIKEFTSERVKEHHKSLDPNCPRDFTDNLLMEMEKEKHSAEPLFTLENITVTTADMFFAG   300
Human_CYP2E1   EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG   300
               *:**:.*******:********:**.**:********:**** *:*:: **** .**:****

                                                                   SRS5
Horse_CYP2E1   TETTSTTLRYGLLILLKHPEVEEKLHKEIDSVIGPSRIPAFKDRLEMPYMDAVVHEIQRF   360
Human_CYP2E1   TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF   360
               ***********:**:.**:**:*** ********** :*:*** **** **********

Horse_CYP2E1   INLVPSNLPHVATQDTAFRGYVIPKGTVVIPTLDSLLYDNQEFPDAEKFKPEHFLNEDGK   420
Human_CYP2E1   ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK   420
               *.********* **:** ****:*****;******:*********:******:****:**

                                                              SRS6
Horse_CYP2E1   FKYSDHFKAFSAGKRVCVGEGLARMELFLFLTAILQHFNLKSLVDPKDIDLSPVTIGFGN   480
Human_CYP2E1   FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC   480
               *****:** **:*****.*********** :* ********* ***********: ****

Horse_CYP2E1   IPPNYKLCIIPRS  493
Human_CYP2E1   IPPRYKLCVIPRS  493
               ***.****:****
```

Figure 3.10: Annotated protein alignment of human CYP2E1 with equine CYP2E1 (% identity 83.53 (% identity 79.3). Alignment was carried out with Clustal Omega. Substrate recognition regions (SRSs) are highlighted on the human isoform (Lewis and Ito, 2009) in order to observe similarity with the horse isoforms. The residues determined to be critical for substrate recognition are highlighted in yellow.

3.23: Phylogenetic analysis

Once equine P450 sequences had been gathered phylogenetic trees were generated using ClustalW2 Phylogeny. Firstly, only the predicted equine sequences were used (figure 3.7) and then the previously known equine and their corresponding human orthologs (figure 3.8). Distance values (the number of nucleotide changes divided by the length of the sequence) are presented next to the gene names and aid in comparison of divergence between similar isoforms. From these phylogenetic

trees, a clear separation into clans (shown in bold in figure) is evident. All clans are represented in these predictions. The only equine P450 that could not be grouped into a clan is CYP46A1.
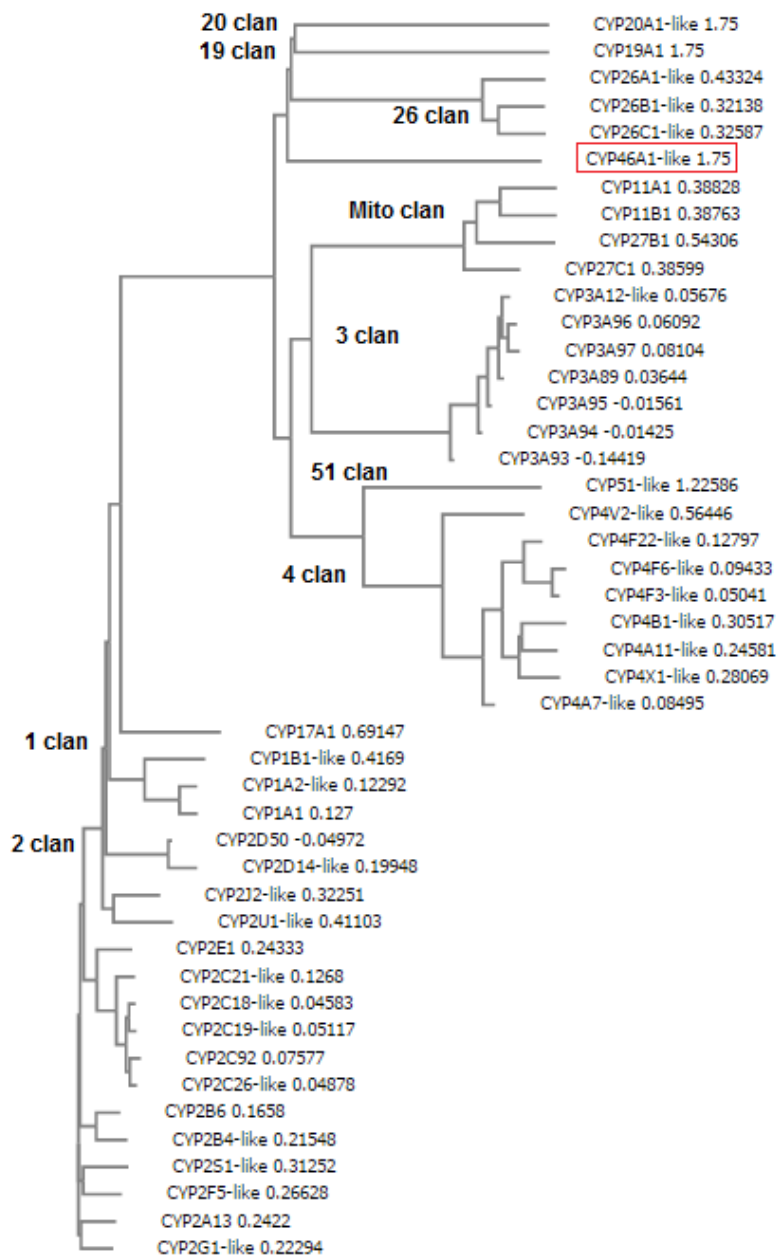
Figure 3.7: Phylogenetic tree of all known and predicted *Equus caballus* sequences. Tree was created using the neighbour joining method (Saitou and Nei, 1987) in Clustal Omega. Distance values are given next to the gene names and signify the predicted evolutionary divergence ((calculated as the number of nucleotide changes divided by the length of the sequence (Kimura, 1980)) All clans are highlighted. CYP46A1, which does not fit into any current clan, is outlined in red.
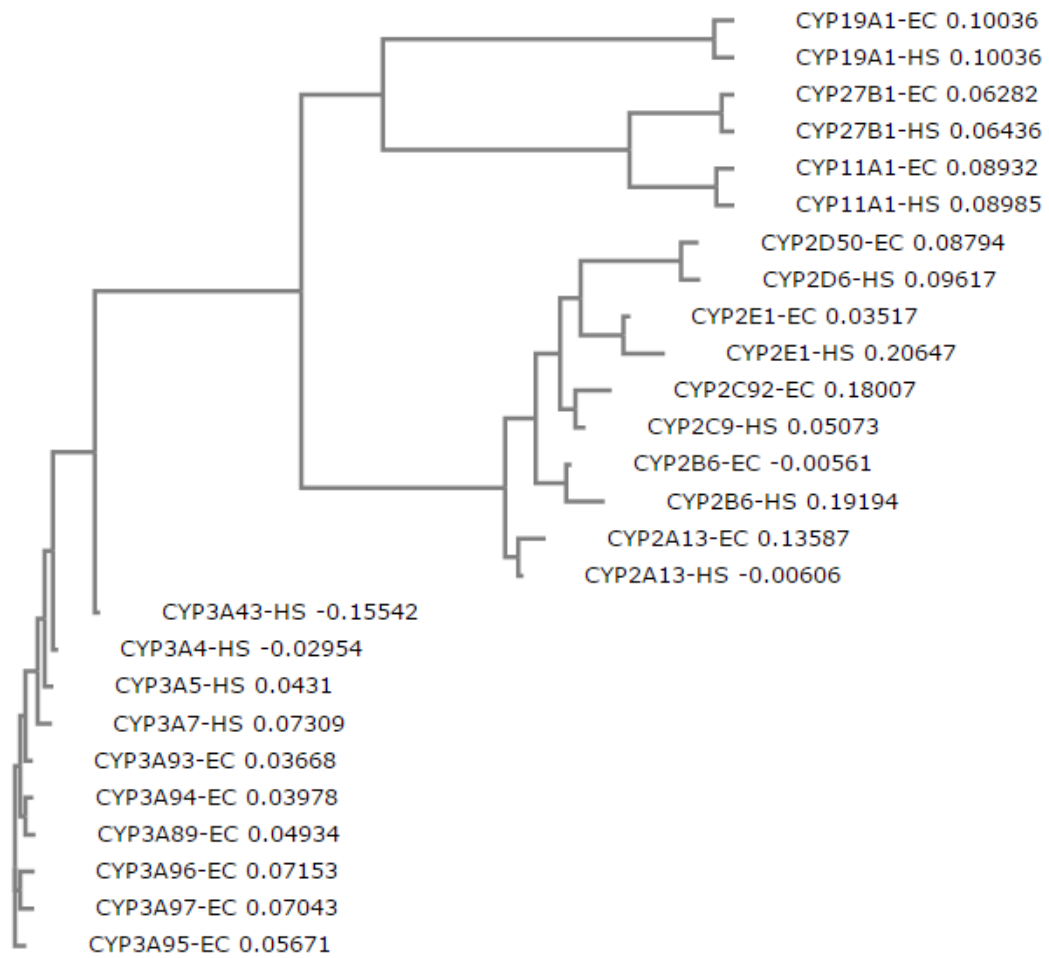
Figure 3.8: Phylogenetic tree of known horse P450s and their human orthologs. Genes with suffix '-HS' are the human isoforms and those with suffix '-EC' are the equine isoforms. Method used to create tree was the same as for figure 3.4.

Along with the construction of phylogenetic trees, the equine predicted P450s were analysed for synteny with all 57 human P450s. Figure 3.9 shows the result of this analysis, with regions of conserved order highlighted. The most conserved chromosome was chromosome 1, which showed synteny with two human chromosomes (10 and 15).

Equine chromosome	Order on chromosome	Human chromosome	Order on chromosome	Equine chromosome	Order on chromosome	Human chromosome	Order on chromosome
1		10		10		19	
	CYP2E1		CYP2E1		CYP2F5-like		CYP2A6
	CYP17A1-like		CYP17A1		CYP2A13		CYP2A7
	CYP2C21-like		CYP2C8		CYP2B6		CYP2B6
	CYP2C92		CYP2C9		CYP2G1-like		CYP2A13
	CYP2C19-like		CYP2C19		CYP2B4-like		CYP2F1
	CYP2C18-like		CYP2C18		CYP2S1-like		CYP2S1
	CYP26A1-like		CYP26A1	21			CYP4F22
	CYP26C1-like		CYP26C1		CYP4F22-like		CYP4F8
		15			CYP4F6-like		CYP4F3
	CYP1A2-like		CYP1A2		CYP4F3-like		CYP4F12
	CYP1A1		CYP1A1				CYP4F2
	CYP11A1-like		CYP11A1				CYP4F11
	CYP19A1		CYP19A1				
2		1		13		7	
	CYP2J2-like		CYP2J2		CYP3A93		CYP2W1
	CYP4X1-like		CYP4A22		CYP3A89		CYP51A1
	CYP4B1-like		CYP4Z1		CYP3A94		CYP3A5
	CYP4A11-like		CYP4X1		CYP3A95		CYP3A7
	CYP4A7-like		CYP4A11		CYP3A96		CYP3A4
		4	CYP4B1		CYP3A97		CYP3A43
	CYP2U1-like		CYP2U1		CYP3A12-like		
27	CYP4V2		CYP4V2				
6		12		15		2	
	CYP27B1		CYP27B1		CYP26B1-like		CYP1B1
					CYP1B1-like		CYP26B1
				18	CYP27C1-like		CYP27C1
					CYP20A1-like		CYP20A1
							CYP27A1
9	CYP11B1	8	CYP7A1	24	CYP46A1	14	CYP46A1
			CYP7B1				
			CYP11B1				
			CYP11B2				
				28	CYP2D14-like	22	CYP2D6
					CYP2D50		CYP2D7

Figure 3.9: synteny between equine and human P450 genes. NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) was used to determine the order of P450 genes in each chromosome of human and horse. When order was conserved, genes names were highlighted via colour coding while lines were used to illustrate homologues.

3.3: Discussion

The data presented in this chapter are the first attempts to fully ascertain the number of cytochrome P450 genes present in the equine genome. The first approach taken was to probe the equine genome using P450 sequences from other species - this generated 59 sequences however most were only fragments. The second approach involved searching the NCBI RefSeq and Ensembl databases for sequences that

had been automatically predicted as being P450s using their computational genome annotation software (Pruitt *et al*, 2002, Curwen *et al*, 2004). A list of predicted equine P450s was compiled and compared with those manually obtained from the equine genome. As expected, there was a large degree of overlap between the two datasets, with almost all manually-obtained sequences matching a predicted sequence 100%. Only three manually-obtained sequences did not match a predicted sequence; however none were the ~1.5kb expected for P450s (Nelson *et al*, 1996). Two (Ecab.P450.4 and Ecab.P450.18) matched CYP2R1 genes in *Homo sapiens* and *Equus przewalskii,* respectively, while one (Ecab.P450.9) matched human CYP2W1. Ecab.P450.18 and Ecab.P450.9 possess the characteristic FxxGxxxCxG motif of the P450 haem-binding region (see figures 3.4 and 3.5) while Ecab.P450.4 does not. These may be gene fragments or pseudogenes as full length sequences could not be obtained in either the UCSC genome browser or via annotated genome searching.

The aim of these two approaches was to assemble a list of equine P450s across all nine vertebrate clans. A phylogenetic tree was created using ClustalW2 Phylogeny to group sequences into clans; this served as further validation of the predicted P450 sequences, showing that they did indeed cluster together. All sequences clustered on the tree as expected (figure 3.8). CYP40A1 was the only sequence that could not be grouped into a clan, which is as expected when compared to phylogenetic analysis of other species, where it also did not cluster with other sequences (Nelson, 2003, Kawashima and Satta, 2014). The known horse CYP450s and their human isoforms were also used to generate a ClustalW2 phylogenetic tree, which is useful for observing how closely-related the enzymes are in the two species. The CYP2 enzymes appear to be the most diverged, particularly CYP2A13, CYP2B6 and

CYP2C92/CYP2C9. The CYP19A1 and CYP11A1 isoforms are the least diverged. For the CYP3As, equine CYP3A89 appears to be most similar to human CYP3A5 while equine CYP3A96 and CYP3A97 are most similar to CYP3A7. In human, CYP3A5 is not majorly expressed in the liver in most people and only contributes a minor role towards drug metabolism (Westlind et al, 2001). CYP3A7 is only highly expressed in faetal liver and therefore does not seem to play any significant role in drug metabolism (Leeder *et al*, 2005). CYP3A93 was the closest match to the major human drug-metabolising P450, CYP3A4, which is responsible for around 50% of drug metabolism (Evans and Relling, 2009). As an additional analysis of evolutionary divergence, synety between equine and human P450s was analysed. Although chromosome 1 showed a high conservation of gene order, others (e.g. chromosome 10) did not. Not all equine P450s identified during this research have homologues in human, however since some subfamilies appear to be more expanded in human and horse (e.g. CYP4F and CYP11), this may suggest either evolutionary divergence or that not all equine sequences were identified during this research.

When contrasted with some other mammalian species, the number of P450s in the horse appears to be relatively small (for example, there are 57 P450 genes in human, 59 in cattle and 89 in rat (Nelson *et al*, 2003, Nelson, 2009, Sim and Ingelman-Sundberg, 2010). There may therefore be some that have not been found within this study, however it is important to point out that P450 numbers vary substantially across species, even within vertebrates – from 41 in chicken to 102 in the mouse (Nelson *et al*, 2013, Hrycay and Bandiera, 2009).

The only previous study looking at P450 gene clusters in the horse was by Schmitz *et al* (2010) whereby the CYP3A gene cluster was characterised. The present study agrees with this data, since the 'CYP3A12-like' enzyme in the current study appears

to be a pseudogene, due to the fact it lacks the haem-binding motif and therefore cannot be an active P450. Two other sequences also lack the haem-binding motif – CYP2S1-like and CYP20A1-like. Lacking this motif is by no means the only signifier of a P450 pseudogene (Wen *et al*, 2001) but it does mean a functional P450 protein cannot be produced, since the haem-binding region is required for P450 function (Guengerich, 2007, Ranasinghe and Hobbs, 1998). As another method of predicting function, some sequences were aligned with their human isoforms in order to compare substrate recognition regions (SRSs). The CYPs looked at here were CYP2D and CYP2C. Both had some key differences between the human and equine isoforms. A lab-based approach will be required to validate functionality further.

Taken together, this data gives an insight into the number of P450 genes in the horse; however expression of each P450 sequence and functional analyses are needed before conclusions can be drawn about its significance. Using equine liver samples to look for gene expression will provide evidence that these genes exist in vivo, and once completed this will be followed by isolation, expression and functional testing of each gene.

Chapter 4: Cloning and sequence verification

4.1: Introduction

4.1.1: cDNA synthesis and RT-PCR

Generation of complementary DNA (cDNA) has allowed for the development of recombinant DNA technology while also enabling the user to gain an insight into which messenger RNAs (mRNA) are expressed within a tissue (Okayama, 2012). Reverse transcription polymerase chain reaction (RT-PCR) is a highly sensitive technique used to study gene expression and is often the method of choice for this type of analysis (however for high throughput studies, DNA microarrays and RNA sequencing are more efficient techniques), Costa *et al* (2013).

RT-PCR has been widely used to study expression of P450 genes within various species such as mouse, human and zebrafish (Sarikaya *et al*, 2007, Goldstone *et al*, 2014, Graves *et al*, 2013). In the horse, RT-PCR has been used to detect P450 expression in the liver, digestive tract and airway (Schmitz *et al*, 2010, Tyden *et al*, 2012, Larrson *et al*, 2012). Six CYP3A isoforms have been detected in equine liver (CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96 and CYP3A96, Schmitz *et al*, 2010). Additionally, equine liver expression of CYP1A, CYP2A, CYP2C, CYP2D and CYP2E1 have been detected (Tyden *et al*, 2014, DiMaio Knych *et al*, 2009, DiMaio Knych *et al*, 2009, Peters *et al*, 2013). For cloning and subsquent functional protein expression (in sf9 insect cells and V79 hamster fibroblasts), RT-PCR amplification of CYP3A96, CYP2D50, CYP2C92, CYP2B6 and CYP3A94 from

equine liver has been carried out (DiMaio Knych *et al*, 2008, DiMaio Knych *et al,* 2009, DiMaio Knych *et al*, 2010, Peters *et al*, 2013, Dettwiler *et al*, 2014).

4.1.2: DNA sequencing

Despite the advancement of next generation sequencing (NGS), Sanger sequencing is still a widely used sequencing technology (Lee *et al*, 2013). NGS is ideal for large sequencing studies due to the ability to run thousands to millions of reactions in parallel (Van Dijk *et al*, 2014). For smaller studies (< ~ 1400 bp), Sanger sequencing is often preferred due to having a lower error rate than NGS (Lee *et al*, 2013). Automated Sanger sequencing allows all four fluorescently tagged-dideoxynucleotides (ddNTPs) to be mixed in one reaction tube and run together for subsequent capillary electrophoresis and laser detection at different wavelengths. This results in an output chromatogram with peaks of different colours for each base (Kircher and Kelso, 2010). Automatic Sanger sequencing can produce quality reads up to around 700-900 bp, thereby meaning sequencing in the forward and reverse direction is required for longer sequences (Kircher and Kelso, 2010).

4.1.3: Molecular cloning methods

Most cloning vectors utilise *E. coli* as a host although other organisms can be used, such as yeast and mammalian cells (Joska *et al*, 2014, Okayama, 2012). For protein expression, plasmids must contain a strong promotor before the multiple cloning site (MCS). The *lac* promoter is the most widely used however bacteriophage promoters such as T7 and sp6 often give much stronger expression (Rosano and Ceccarelli, 2014). Promoters for specialist applications are also available, for example cold-induction of the cspA promoter at 15°C (Hayashi and Kojima, 2008). The pCold I vector employs the cspA promotor in order to allow for protein expression at lower

temperatures while it also contains a 6x polyhistadine-tag (his-tag) between the promoter and the MCS for protein purification using antibodies or affinity chromatography (Terpe, 2006). This vector system has been successfully employed for the recombinant expression of many eukaryotic proteins that have been hard to express via conventional bacterial methods (Sugiki *et al*, 2014).

Baculovirus has also been used extensively for expressing eukaryotic proteins that are difficult to express in bacterial systems (Van Oers *et al*, 2014). The Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is a baculovirus used for recombinant protein expression in *Spodoptera frugiperda* (sf9 or sf21) cells (Carinhas *et al*, 2011). The Bac-to-Bac expression system (Life Technologies) allows for an efficient method of cloning a gene of interest and recombining it into a bacmid (baculovirus) shuttle vector for subsequent transfection of insect cells. The pFastBac series of vectors are used as donor plasmids for initial cloning in *E. coli* and contain a baculovirus promotor and a Tn7 transposable element which is used for site-specific recombination into the bacmid. DH10Bac cells containing bacmid DNA are used for recombination, allowing for production of sufficient recombinant baculovirus with an overnight incubation (Sung *et al*, 2014).

4.1.4: CYP P450 protein structure prediction

The way in which a protein folds is crucial to its function, and therefore any means of determining protein folding can be an effective tool for predicting its functional traits (Dorn *et al*, 2014). Xray crystallography and NMR spectroscopy have proved invaluable for analysing how structure affects function however it is also possible to analyse protein structure through *in silico* methods (Pavlopoulou and Michalopoulos, 2011). Homology modelling is based on the observation that proteins with a high

degree of primary structure similarity are likely to have similar tertiary structure (Watson *et al*, 2005). It uses a 'template' of a protein with a known (experimentally-determined) structure and predicts the structure of the query protein based on an alignment of this template with the query sequence (Kelley and Sternberg, 2009). There are many homology modelling tools available (see Dorn *et al*, 2014), one of the most widely used of which is the Phyre2 server (Kelley *et al*, 2015). This software involves comparison of the query with a database of protein sequences in order to find similarity, after which an alignment between the query and a protein with a known structure is carried out such that a homology model can be compiled. Homology modelling is particularly beneficial for the study of enzymes and has been used extensively for P450s (Ito and Lewis, 2009). Analysis of the active site of P450s enables the user to compare active site structure between similar isoforms to predict drug metabolising properties (Zhang *et al*, 2012). Using this approach, it becomes possible to make assumptions about the functional integrity of an enzyme therefore meaning a more targeted approach can be taken to *in vitro* functional screening (Ito and Lews, 2009).

4.1.5: Current study

For the current study, RT-PCR was used to detect P450 expression in equine liver. Amplified P450 sequences were isolated and cloned along with six commercially-produced clones into two expression vectors – a cold-induction vector (pCold) for *E. coli* protein expression and an insect cell vector (pFastBac 1) for sf9 protein expression via integration into a bacmid shuttle vector. All clones were sequence verified using Sanger sequencing and 3D structural predictions carried out using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2).

4.2: Results

4.2.1: Amplification and sequencing of P450 sequences from equine liver

One full-length novel sequence was successfully amplified from equine liver cDNA – 'CYP2C26-like'. Additionally, one previously known P450, CYP3A97, was amplified. (table 4.2.1). After amplification, gel bands containing PCR products were excised (figure 4.2.1) and cloned into expression vectors using the restriction sites in the primers (see table 2.1, chapter 2). Following this, all clones were sequence verified (sections 4.2.2 and 4.2.3). The majority of P450 sequences that primers were designed for did not fully amplify however some gene fragments were successfully amplified and subsequently sequence verified (table 4.2.1). E.cab.P450.4, E.cab.P450.17, P450.Equ.30, Ecab.P450.13 and CYP1B1-like were all partially amplified.

Sequence Name	Liver Expression Detected?	Expected size (bp)	PCR product size (bp)
Ecab.P450.1 (CYP2E1)	No	973	N/A
Ecab.P450.4 (CYP2R1-like)	Yes	681	571
Ecab.P450.5 (CYP1A2-like)	No	1545	N/A
Ecab.P450.13 (CYP1B1-like)	Yes	1311	483
Ecab.P450.17 (CYP2D14-like)	Yes	592	571
Ecab.P450.18 (CYP2R1-like)	No	1170	N/A
P450.Equ.18 (CYP4F22-like)	No	1211	N/A
P450.Equ.23 (CYP46A1-like)	No	1270	N/A
P450.Equ.30 (CYP2D14-like)	Yes	507	475
P450.Equ. 37 (CYP27B1)	No	827	N/A
CYP1A1-like	No	1563	N/A
CYP1B1-like	Yes	1632	1084
CYP2U1-like	No	1167	N/A
CYP3A97	Yes	1535	1535
CYP2C26-like	Yes	1319	1319

Table 4.2.1: Summary of amplification of sequences via RT-PCR. Size in base pairs (bp) is given for the full length sequences as well as for what was obtained after sequencing of PCR products.
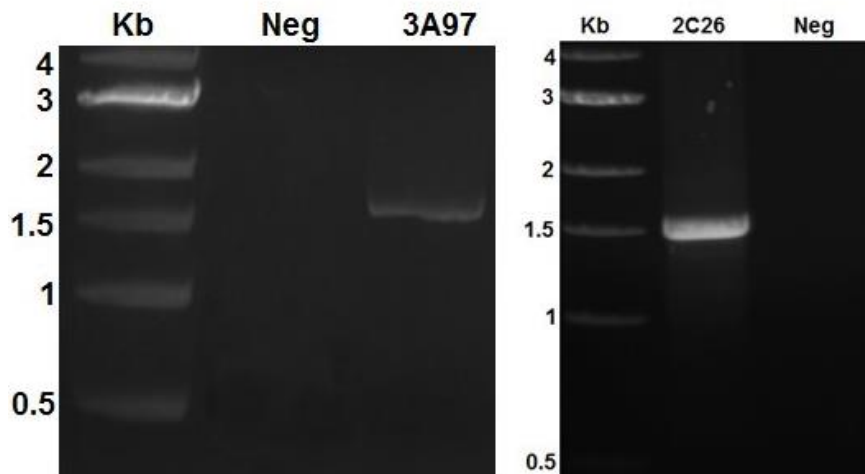
Figure 4.2.1: RT-PCR products using primers for CYP3A97 (left) and CYP2C26 (right). Negative controls (Neg) were run without template DNA. Band sizes of the 1 kilobase (Kb) DNA marker are highlighted.
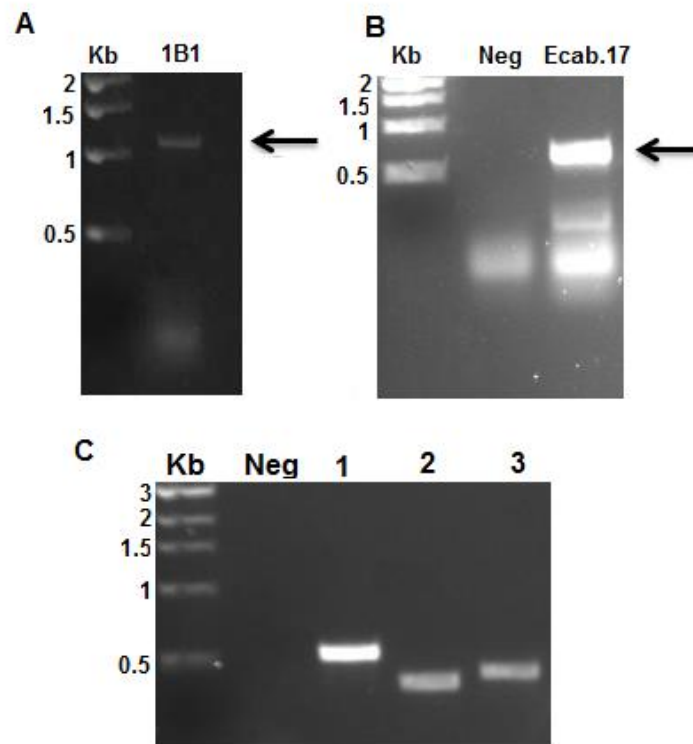
Figure 4.2.2: Gene fragment RT-PCR products. A: 1B1-like, B: Ecab.P450.17, C: Ecab.P450.4 (1), P450.Equ.30 (2) and Ecab.P450.13 (3). Neg = negative control (reaction mixture without template DNA). Arrows highlight sequenced products and Band sizes of the 1 kilobase (Kb) DNA marker are also highlighted.

4.2.2: Cloning into pCold expression vector and sequence verification

P450s cloned into the pCold expression vector were sequence verified to ensure no base changes had occurred. Six sequences were incorporated into the expression vector (table 4.2.3). CYP2D50, CYP1A1 and CYP3A96 were full matches for the reference sequence, with changes having occurred in the sequences of all other constructs. CYP3A97 was not cloned into the correct reading frame due to the deletion of an adenine in the TEE region of the vector (figure 4.2.3), therefore it could not be carried through to the next stage (see table 4.2.2 for a summary of this data).

CYP2D50, CYP3A96 and CYP1A1 were all 100% matches with their reference sequences while mutations occurred in CYP2A13 (3 changes – G 735> A, T929>C, A969 >T) and CYP2C92 (7 changes- G4>C, G1191>A, T1361>A, T1438>C, T1460>C, C1466>T, C1479>A), highlighted on figures 4.2.4 and 4.2.5 respectively. Despite various attempts, CYP2C26 and CYP2E1 were not cloned into the pCold vector.

pCold construct	RefSeq accession no.	% match
CYP1A1	XM_005602921.1	100%
CYP2A13	NM_001111337.1	99.88% (see figure 4.2.3)
CYP2C92	NM_001101652.1	99.53% (see figure 4.2.4)
CYP2D50	NM_001111306.1	100%
CYP3A96	NM_001146163.2	100%
CYP3A97	NM_001146164.2	Not in frame – see figure 4.2.1

Table 4.2.2: Comparison of pCold constructs with NCBI reference (RefSeq) DNA sequences. Acession numbers of reference sequences are highlighted along with the percentage match between cloned and reference sequences.

Once the DNA sequences had been analysed, they were translated into protein sequences using ExPASy Translate (http://web.expasy.org/translate/) to observe any amino acid differences caused by the nucleotide changes. For CYP2A13, there was one amino acid change – L310>P (figure 4.2.2). For CYP2C92, there were two amino acid changes, S478>F and Y486>C), figure 4.2.3.

Figure 4.2.3: Translated protein sequence for CYP3A97 cloned into the pCold vector.

Nucleotide sequences were translated using ExPASy translate (http://web.expasy.org/translate) and open reading frames are highlighted in pink. Figure shows A: alignment of vector sequences, B: The reading frame construct was cloned into and C: The reading frame with the his-tag present.

Figure 4.2.4: Summary of Clustal Omega alignment between NCBI reference sequence (NM_001111337.1) and the cloned CYP2A13 pCold construct. DNA sequence regions with a 100% match are illustrated with a dashed line while base variations are highlighted along with their position in the sequence. The one change in the amino acid sequence, highlighted in red (L310>P). 6-his tag is highlighted in blue and haem-binding region is highlighted in yellow.

Figure 4.2.5: Summary of Clustal Omega alignment between NCBI reference (NM_001101652.1) sequence and the cloned CYP2C92 pCold construct. DNA sequence regions with a 100% match are illustrated with a dashed line while base variations are highlighted along with their position in the sequence. The two changes in the amino acid sequence, highlighted in red (S478>F and Y486>C). Also highlighted are substrate recognition regions (grey, Zawaira *et al*, 2011) and the haem-binding region (yellow).

4.2.3: Cloning into pFastBac 1 expression vector and sequence verification

The seven sequences cloned into the pCold expression vector were also cloned into the pFastBac 1 expression vector for expression in sf9 insect cells. CYP2C26 was the only sequence not cloned into pFastBac 1. Table 4.2.3 displays a summary of sequencing data for the six clones along with percentage matches with reference sequences.

pFastBac 1 construct	RefSeq accession no.	% match
CYP1A1	XM_005602921.1	98.82% (figures 4.2.5 and 4.2.6).
CYP2A13	NM_001111337.1	99.87% (figure 4.2.7)
CYP2C92	NM_001101652.1	99.46% (figure 4.28)
CYP2D50	NM_001111306.1	100%
CYP2E1	NM_001111303.2	100%
CYP3A96	NM_001146163.2	100%
CYP3A97	NM_001146164.2	96.33%

Table 4.2.3: comparison of pFastBac 1 constructs with NCBI reference (RefSeq) DNA sequences. DNA sequences. Acession numbers of reference sequences are highlighted along with the percentage match between cloned and reference sequences.

Figures 4.2.6-4.2.9 display the nucleotide and amino acid changes that occurred during the cloning process. Three constructs were 100% matches for their reference sequence (CYP2D50, CYP2E1 and CYP3A96) while the other constructs ranged from 96.33% (CYP3A97) similarity to 99.87% (CYP2A13) similarity.

The CYP1A1 DNA sequence was truncated at the beginning and end compared to the reference sequence although the translated protein sequence had two extra residues on the end (threonine and arginine), as well as having an entirely different sequence from 513-526 (figure 4.2.6).

```
CYP1A1_pFast    ------------------------------------------------------------   0

CYP1A1_RefSeq   ATGTAACCAGCCCTCAGATCAAGAGACAGAATGTTACCAGCATCCTAGAACCTCCTTGTG  60


CYP1A1_pFast    --------------------------------------   22

CYP1A1_RefSeq   CTCCTTCCCCCAGCCACCTTGAGATCCCTACGCGGATC  120


CYP1A1_pFast    CTGCAGTTTGCAGTAAACACAGAGGATGAAGAAACACGCTG--  1580

CYP1A1_RefSeq   GTGCGCCCTCAGGGGACTGAAAGCCCTGCAGCCTAGACTCTGT  1680


CYP1A1_pFast    -AATTGGATCTAG-------------------------------------   1592

CYP1A1_RefSeq   CTACCTGACCTGCTTGGGCAGCCAGGCCAGGGTGCTGGCCTGGGGGGTCTAG  1732


CYP1A1_RefSeq   ATACAGTGCCTGGGGGGATTGCCTCCCTCTGCC  2193
```

```
CYP1A1_pFast    MFSVFGFSVPISATELLLTSAIFCLVFWLVRAWQPQIPKGLKSPPGPWGWPLLGHVLTLG   60
CYP1A1_RefSeq   MFSVFGFSVPISATELLLTSAIFCLVFWLVRAWQPQIPKGLKSPPGPWGWPLLGHVLTLG   60
                ************************************************************

CYP1A1_pFast    KNPHLALSRLSQRYGDVMQIRIGSTPVLVLSGLDTVRQALVRQGDDFKGRPDLHSFTLIS  120
CYP1A1_RefSeq   KNPHLALSRLSQRYGDVMQIRIGSTPVLVLSGLDTVRQALVRQGDDFKGRPDLHSFTLIS  120
                ************************************************************

CYP1A1_pFast    DGQSMTFSPDSGPVWAARRRLAQNALKSFSIASDPASMSSCYLEEHVSKEAEYLIRKFQE  180
CYP1A1_RefSeq   DGQSMTFSPDSGPVWAARRRLAQNALKSFSIASDPASMSSCYLEEHVSKEAEYLIRKFQE  180
                ************************************************************

CYP1A1_pFast    LMAGVGHFDPYKYVVMSVANVICAMCFGRRYDHDDEELLNLINLNNEFGEVAASGNPADF  240
CYP1A1_RefSeq   LMAGVGHFDPYKYVVMSVANVICAMCFGRRYDHDDEELLNLINLNNEFGEVAASGNPADF  240
                ************************************************************

CYP1A1_pFast    IPILRYLPNSALDTFKDLNKKFYIFMQKMIKEHNKTFEKGHIRDITDSLIEHCQDKRLDE  300
CYP1A1_RefSeq   IPILRYLPNSALDTFKDLNKKFYIFMQKMIKEHNKTFEKGHIRDITDSLIEHCQDKRLDE  300
                ************************************************************

CYP1A1_pFast    NANIQLSDEKIINVVLDLFGAGFDTVTTAISWSLLYLVTRPSMQKKIQEELDTVIGRARQ  360
CYP1A1_RefSeq   NANIQLSDEKIINVVLDLFGAGFDTVTTAISWSLLYLVTRPSMQKKIQEELDTVIGRARQ  360
                ************************************************************

CYP1A1_pFast    PRLSDRPQLPYMEAFILETFRHSSFVPFTIPHCTTRNTSLSGFYIPKGHCVFVNQWQINH  420
CYP1A1_RefSeq   PRLSDRPQLPYMEAFILETFRHSSFVPFTIPHCTTRNTSLSGFYIPKGHCVFVNQWQINH  420
                ************************************************************

CYP1A1_pFast    DQKLWGDPSEFRPERFLNPNGTINKALSEKVVLFGLGKRKCIGETIGRLEVFLFLAILLQ  480
CYP1A1_RefSeq   DQKLWGDPSEFRPERFLNPNGTINKALSEKVVLFGLGKRKCIGETIGRLEVFLFLAILLQ  480
                ************************************************************

CYP1A1_pFast    QVEFSVPPGVKVDMTPIYGLSMKHARCEHFQVQLQFAVNTEDEETR  526
CYP1A1_RefSeq   QVEFSVPPGVKVDMTPIYGLSMKHARCEHFQVQVRPQGTESPAA--  524
                *********************************::      .  .
```

Figure 4.2.6: Sequence alignment between the CYP1A1 pFastBac 1 construct and the CYP1A1 reference sequence (XM_005602921.1). Differences between sequences occurred at both ends of the alignment while the middle portion was a 100% match. For the protein sequence, Residues that vary between the sequences are highlighted in red. Highlighted in grey are the potential substrate recognition regions (Chinta *et al*, 2005) and highlighted in yellow is the haem-binding region.

The CYP2A13 DNA sequence had a 17 AA insert (figure 4.2.7) which caused the

reading frame after this to be disrupted, meaning the sequence had low similarity
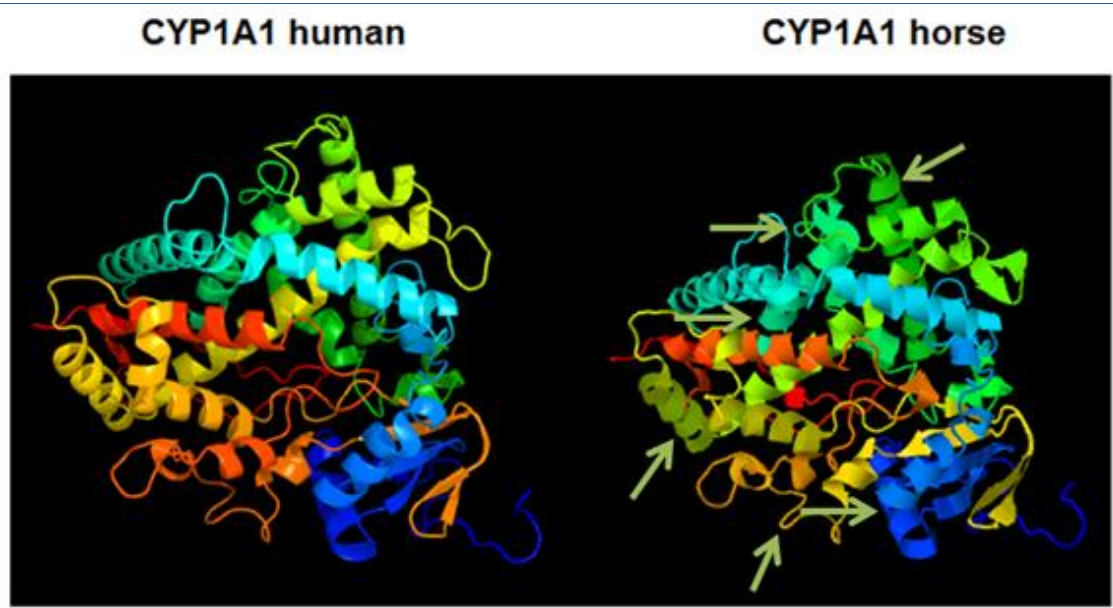
after this insertion (highlighted in red).
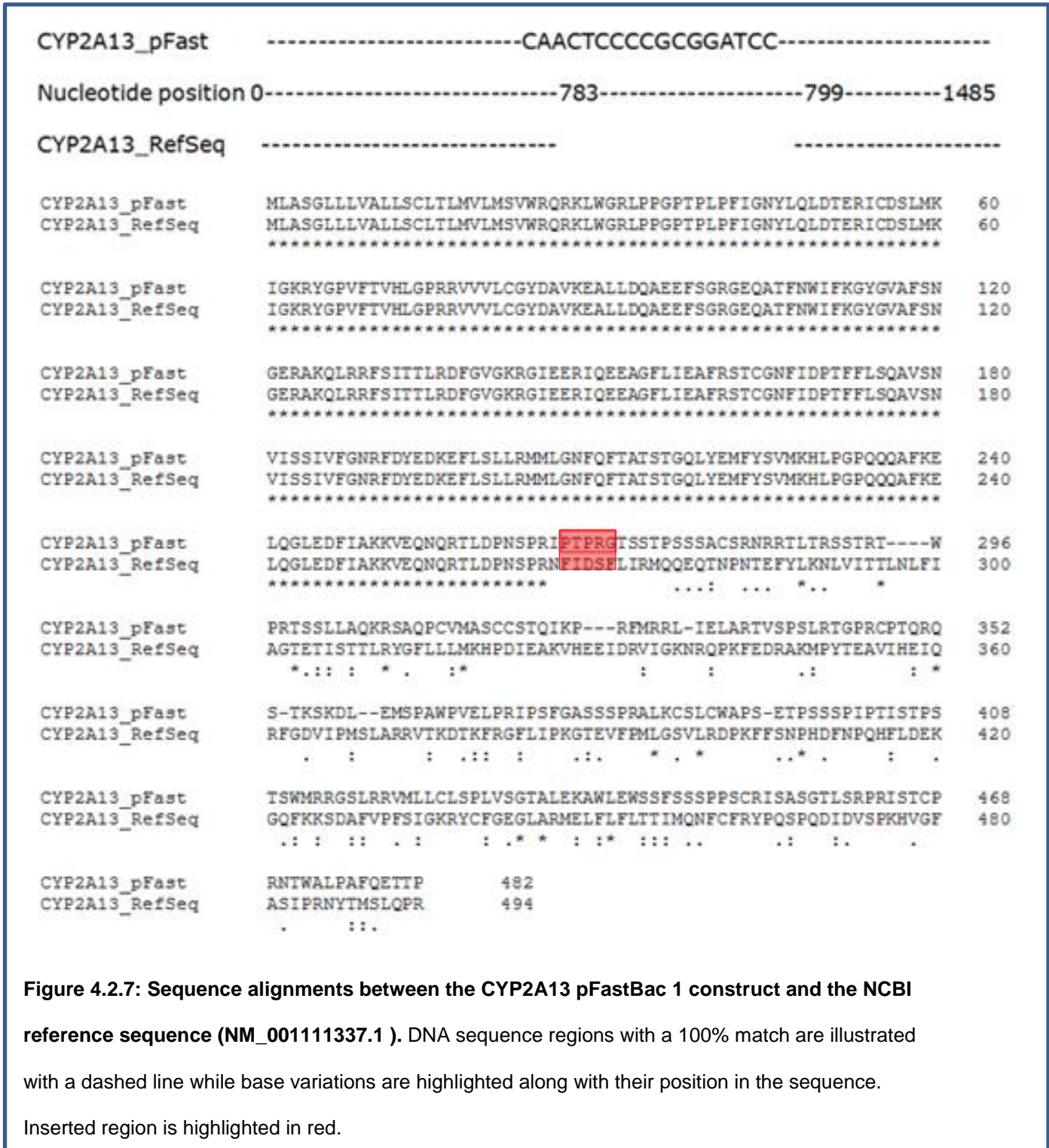
Figure 4.2.7: Sequence alignments between the CYP2A13 pFastBac 1 construct and the NCBI reference sequence (NM_001111337.1). DNA sequence regions with a 100% match are illustrated with a dashed line while base variations are highlighted along with their position in the sequence. Inserted region is highlighted in red.

When sequenced, CYP2C92 pFastBac construct had 7 nucleotide changes (G4>C, G1243>A, T1360>A, T1437>C, T1460>C, A1462>A, C1466>T) and three protein residue changes (F478>S, Y484>C and a deletion of V490) – two of which (F478>S AND Y484>C) were the same as for the pCold construct (figure 4.2.8).
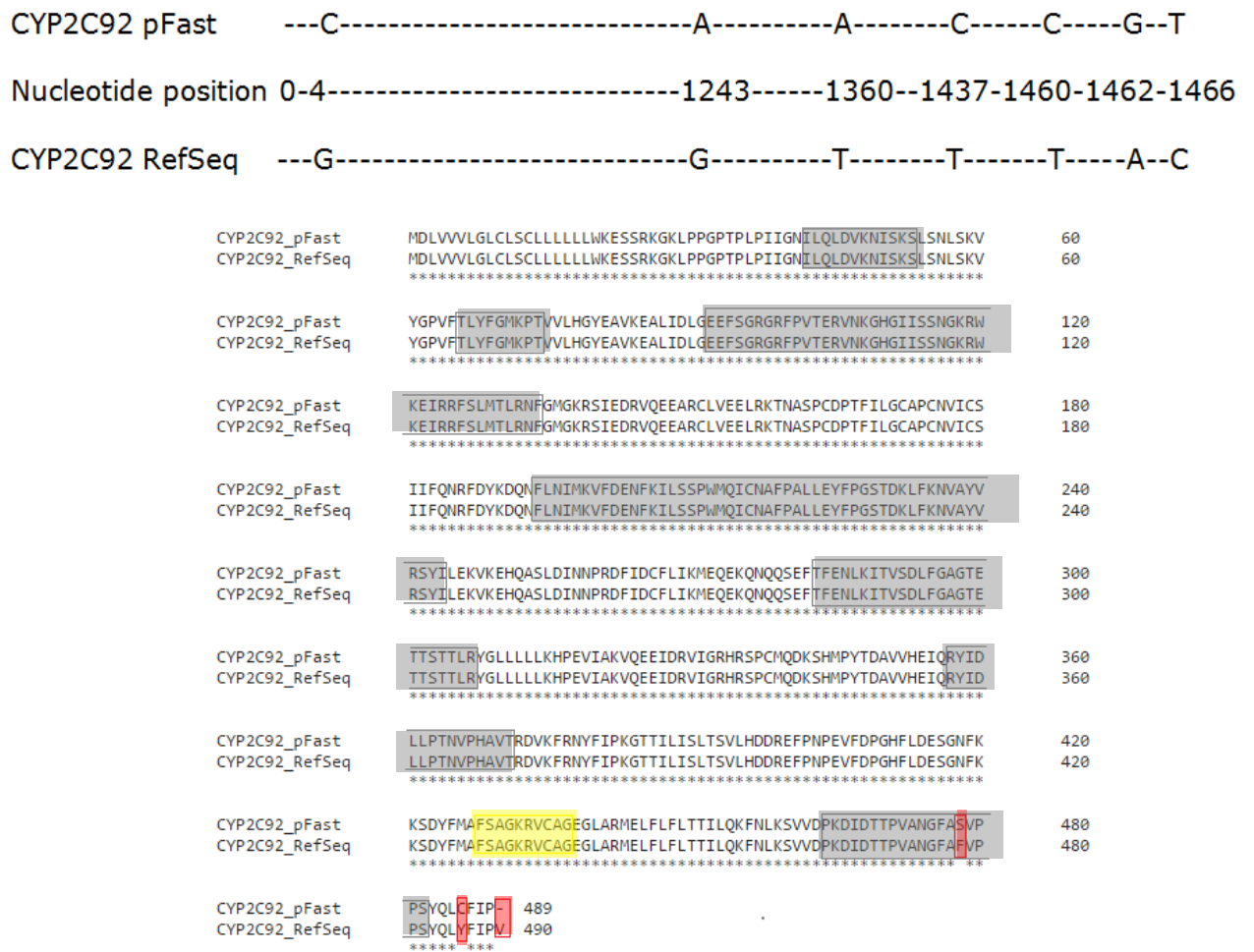
Figure 4.2.8: Sequence alignments between the CYP2C92 pFastBac 1 construct and the NCBI reference sequence (NM_001101652.1). DNA sequence regions with a 100% match are illustrated with a dashed line while base variations are highlighted along with their position in the sequence. The three changes in the amino acid sequence, are highlighted in red (S>F (478), Y>C (486) and no valine at position 490). Also highlighted are substrate recognition regions (grey) and the haem-binding region (yellow).

CYP3A97 had seven mutations in the DNA sequence (figure 4.2.9) which translated to three amino acid differences inside the protein sequence and truncated end (missing the last four residues). One mutation (T119>I) was within a theoretical substrate recognition region.
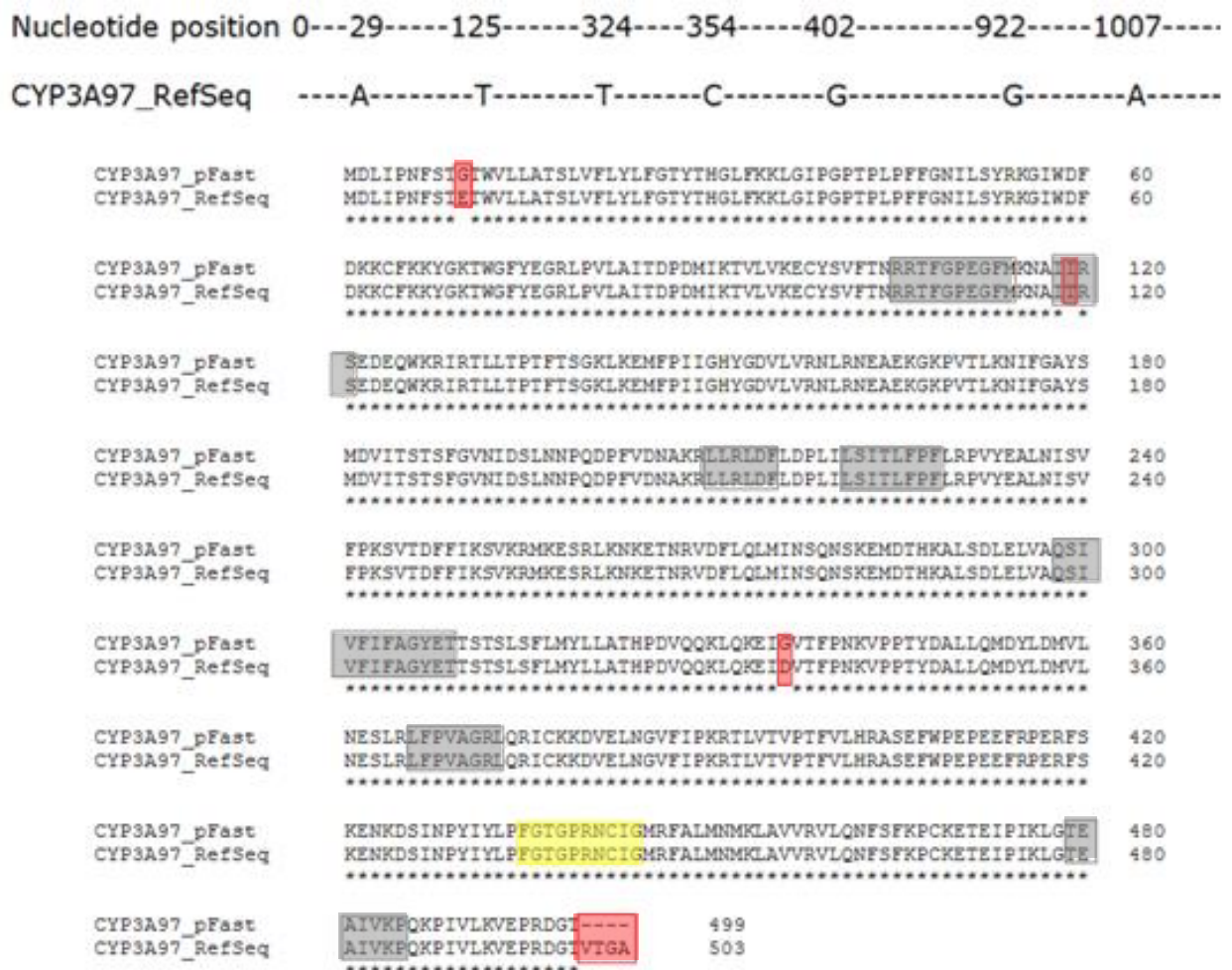
Figure 4.2.9: Sequence alignments between the CYP3A97 pFastBac 1 construct and the NCBI reference sequence (NM_001146164.2). DNA sequence regions with a 100% match are illustrated with a dashed line while base variations are highlighted along with their position in the sequence. The four changes in the amino acid sequence are highlighted in red (E10 > G, T119 > I, D336 > G and an end terminal truncated by four amino acids (VTGA)). Also highlighted are substrate recognition regions (grey) and the haem-binding region (yellow).

4.2.4: Protein structure predictions

There have not been any equine P450 structures published to date. Homology modelling was used on those sequences that had been successfully cloned into the pCold construct during the current study to infer if functional changes may have occurred along with the sequence changes.

CYP2D50 and CYP1A1 were compared to the human isoforms since the pCold constructs were a 100% match for their reference sequence. The 3D structure is highly similar between the two isoforms although there are a number of small, yet potentially significant, differences (figure 4.2.10 and figure 4.2.11). Substrate recognition regions vary between both enzymes, with the residues in the horse isoforms being highly dissimilar from those in the SRSs for the human isoforms. SRS regions are highlighted on both figures.

Figure 4.2.10: Phyre2 prediction for CYP2D50 compared with CYP2D6. Highlighted is the haem-binding region (yellow box and arrow), and potential substrate binding regions (grey boxes and arrows), based on the known SRSs for CYP2D6. Colouring is based on a rainbow from the N to C terminal (blue to red). Alpha helices, beta sheets and coils are shown.

CYP1A1 human   **CYP1A1 horse**

```
CYP1A1_horse   MFSVFGFSVPISATELLLTSAIFCLVFWLVRAWQPQIPKGLKSPPGPWGWPLLGHVLTLG   60
CYP1A1_human   ----MLFPISMSATEFLLASVIFCLVFWVIRASRPQVPKGLKNPPGPWGWPLIGHMLTLG   56
                   : * : :****:**:*.*******::** :**:**** .*********:**.****

CYP1A1_horse   KNPHLALSRLSQRYGDVMQIRIGSTPVLVLSGLDTVRQALVRQGDDFKGRPDLHSFTLIS   120
CYP1A1_human   KNPHLALSRMSQQYGDVLQIRIGSTPVVVLSGLDTIRQALVRQGDDFKGRPDLYTFTLIS   116
               ********.**:***.*:*******.******.:***************:***** .:*****

CYP1A1_horse   DGQSMTFSPDSGPVWAARRRLAQNALKSFSIASDPASMSSCYLEEHVSKEAEYLIRKFQE   180
CYP1A1_human   NGQSMSFSPDSGPVWAARRRLAQNGLKSFSIASDPASSTSCYLEEHVSKEAEVLISTLQE   176
               :****.****************** .*********** .*************.** .:**

CYP1A1_horse   LMAGVGHFDPYKYVVMSVANVICAMCFGRRYDHDDEELLNLINLNNEFGEVAASGNPADF   240
CYP1A1_human   LMAGPGHFNPYRYVVVSVTNVICAICFGRRYDHNHQELLSLVNLNNNFGEVVGSGNPADF   236
               **** ***.**:***.**:.*****.********:.:*** .*:****.****..*******

CYP1A1_horse   IPILRYLPNSALDTFKDLNKKFYIFMQKMIKEHNKTFEKGHIRDITDSLIEHCQDKRLDE   300
CYP1A1_human   IPILRYLPNPSLNAFKDLNEKFYSFMQKMVKEHYKTFEKGHIRDITDSLIEHCQEKQLDE   296
               ********* :*.::*****.*** *****.*** ****************:**.*.:***

CYP1A1_horse   NANIQLSDEKIINVVLDLFGAGFDTVTTAISWSLLYLVTRPSMQKKIQEELDTVIGRARQ   360
CYP1A1_human   NANVQLSDEKIINIVLDLFGAGFDTVTTAISWSLMYLVMNPRVQRKIQEELDTVIGRSRR   356
               ***:*********:********************.** .* .*:***********:**:

CYP1A1_horse   PRLSDRPQLPYMEAFILETFRHSSFVPFTIPHCTTRNTSLSGFYIPKGHCVFVNQWQINH   420
CYP1A1_human   PRLSDRSHLPYMEAFILETFRHSSFVPFTIPHSTTRDTSLKGFYIPKGRCVFVNQWQINH   416
               ****** .:***********************. ***:***.*******:*********

CYP1A1_horse   DQKLWGDPSEFRPERFLNPNGTINKALSEKVVLFGLGKRKCIGETIGRLEVFLFLAILLQ   480
CYP1A1_human   DQKLWVNPSEFLPERFLTPDGAIDKVLSEKVIIFGMGKRKCIGETIARWEVFLFLAILLQ   476
               ***** .**** *****.*.:*.*.**.** *****:.**.*.*.***.*********

CYP1A1_horse   QVEFSVPPGVKVDMTPIYGLSMKHARCEHFQVQLQFAVNTEDEETR   526
CYP1A1_human   RVEFSVPLGVKVDMTPIYGLTMKHACCEHFQMQLRS----------   512
               :****** ************.****.*****:**:**:
```

Figure 4.2.11: Phyre2 prediction for horse CYP1A1 compared with that for human CYP1A1.
Highlighted is the haem-binding region (yellow box and arrow), and potential substrate binding
regions (grey boxes and arrows), based on the known SRSs for CYP1A1. Colouring is based on a
rainbow from the N to C terminal (blue to red). Alpha helices, beta sheets and coils are shown.

The protein sequence for the CYP2C92 pCold construct varied from the reference sequence by two residues (S>F (position 540) and Y>C (position 548)). The S > F mutation occurs within an SRS and there is a small structural difference apparent (figure 4.2.12, horizontal arrow). The second mutation (Y > C) also has resulted in a small structural change.

CYP2A13 had one amino acid change from the reference sequence (residue 310, L>P) however the 3D structure prediction suggests that this has not caused any conformational changes (figure 4.2.13).
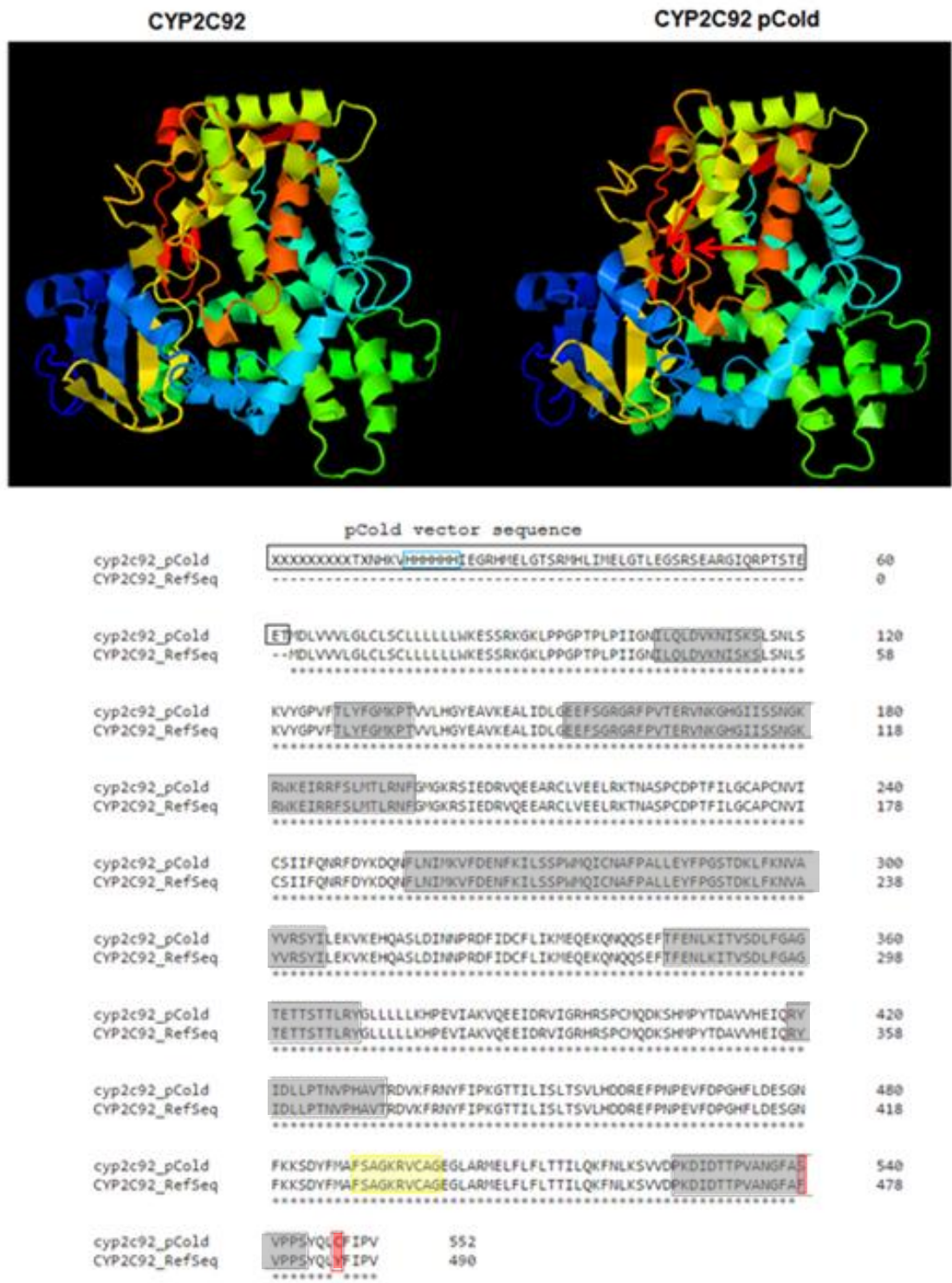
Figure 4.2.12: Phyre2 prediction for CYP2C92. The CYP2C92 pCold construct and the CYP2C92 reference sequence are compared. Residues that are different between the two sequences are highlighted (red boxes and arrows). Also highlighted are the haem-binding region (yellow box and arrow), and potential substrate binding regions (grey). Colouring is based on a rainbow from the N to C terminal (blue to red). Alpha helices, beta sheets and coils are shown.

Figure 4.2.13: Phyre2 prediction for CYP2A13. The one residue variation between the pCold construct and the NCBI reference sequence is highlighted with a red arrow (Phe310). Also highlighted is the haem-binding region (yellow). Colouring is based on a rainbow from the N to C terminal (blue to red). Alpha helices, beta sheets and coils are shown.
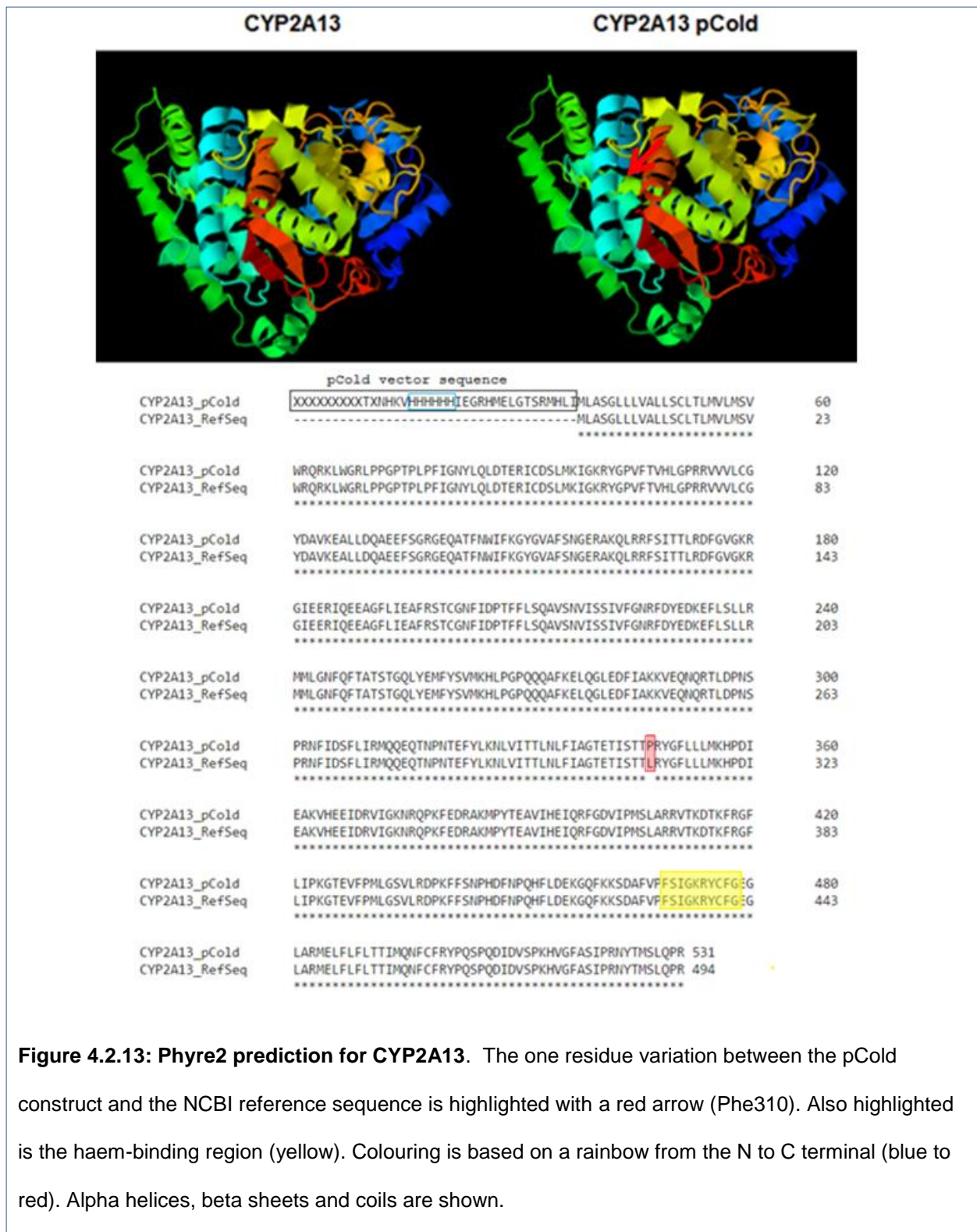
4.3: Discussion

4.3.1: Amplification of cDNA from equine liver

This chapter describes efforts to produce and clone equine P450 cDNAs for recombinant protein expression and functional studies. Two full sequences were obtained using RT-PCR while all other sequences either did not amplify at all or full length sequences were not amplified (tables 4.2.1 and 2.2.2). Quality of cDNA is one possibility that could explain some of the problems in sequence amplification, although great care was taken to ensure that degradation was kept to a minimum (immediately aliquoting samples to avoid frost/defrost cycles, use of RNase free water). cDNA was synthesised using random hexamer primers, which may explain some of the difficulty obtaining full length sequences, as this method can result in only partial sequences being amplified (Harbers, 2008). Of those sequences that were partially amplified, Ecab.P450.13 and CYP1B1-like had the biggest difference between the expected actual PCR product sizes, suggesting the full length cDNA may not have been present within the reaction mixture. For future work, gene specific primers should be used for cDNA synthesis in order to validate whether the full length transcripts are present. Additionally, equine liver samples should be added to a storage reagent such as RNAlater immediately after excision to ensure RNA degradation is kept to a minimum.

The isoforms not detected in equine liver were CYP2U1, CYP1A1, CYP4F22, CYP46A1, CYP27B7, CYP1A2 and CYP2E1. Some of these isoforms are not expressed in the liver of other species therefore this result was as expected. CYP2U1, CYP4F22, CYP27B1 and CYP46A1 are only expressed to any significant extent in extrahepatic tissues in other species (Devos *et al*, 2010, (Ohno *et al*, 2015,

Milagre *et al*, 2010, Adams and Hewison, 2012) while CYP1A1 is only expressed in the liver of other species when induced (Galván *et al*, 2005). CYP2E1 and CYP1A2 are highly expressed in the liver of other species and were not detected, which also conflicts with previous reports of high expression of these enzymes in equine liver (Tyden *et al*, 2014).

Five isoforms were detected in equine liver - CYP2R1, CYP2D14, CYP2C26, CYP1B1 and CYP3A97. CYP2R1 is expressed in human and mouse liver (Zhu *et al*, 2013) while CYP2C26 is also expressed in the liver of other species (Sakuma *et al*, 1994). Despite the fact CYP1B1 was detected in equine liver within the current study, it does not have significant hepatic expression in other species (Palenski *et al*, 2013). For CYP2D14, little research has been carried out on this isoform however it has been reported to be expressed in cow liver (Tsuneoka *et al*, 1992). CYP3A97 was used as a positive control, since it has previously been isolated from equine liver (DiMaio Knych *et al*, 2010), and was detected and fully amplified within the current study.

4.3.2: Cloning and sequence verification

Two separate expression systems were chosen for P450 expression. The sf9 cell system was selected due to its establishment as the 'gold standard' for recombinant P450 production and the bacterial system was selected as an alternative should insect cell protein expression fail or produce too low yields (Qing *et al*, 2004, Van Oers *et al*, 2014). Constructs cloned into each vector were sequence verified on both strands and compared with their reference sequences. Some had mutations in their sequences, however CYP1A1, CYP2D50 and CYP3A96 had no mutations when cloned in the bacterial pCold vector and CYP2D50, CYP3A96 and CYP2E1 had no

mutations in the pFastBac 1 vector system. All other constructs had over 95% sequence similarity; with the pFastBac CYP3A97 having the lowest score (96.33%). For pCold, one construct (pCold CYP3A97) was cloned into the wrong frame due to a nucleotide deletion (figure 4.2.1) For the pFastBac 1 vector CYP2A13 had a frameshift mutation (a 17 AA insert, figure 4.2.5) and CYP1A1 was truncated at the beginning and end of the sequence, with the translated protein sequence having additional residues on the end but otherwise was unchanged (figure 4.2.4). These mutations may have been introduced due to decreased viability of the *E. coli* cultures as a consequence of repeted use of glycerol stocks, or alternatively inadequate viability of the batch of cells used for transformations (Sivashanmugam *et al*, 2009). Other reasons for mutations during *E. coli* cloning include plasmid instability and toxicity of the protein of interest to the host (Rosano and Ceccarelli, 2014).

4.3.3: Protein structure predictions

Protein homology modelling was carried out on four CYP sequences, two of which had changes compared with the reference sequences (section 4.2.3). Models were constructed via the Phyre2 homology modelling server (Kelley and Sternberg (2009), http://www.sbg.bio.ic.ac.uk/phyre2/). Using this software CYP2C92 was predicted as having various conformational changes and since one amino acid change was in a known SRS (figure 4.2.3) it is possible its substrate specificity could be altered as a result. Despite having a difference at the primary structure level, the CYP2A13 pCold construct was predicted to be identical to the reference sequence. CYP2D50 and CYP1A1 were compared with their human orthologs in order to observe conformational differences at the potential substrate recognition sites. Both isoforms had various changes at these sites. Future work comparing substrate specificities

between horse and human will be required to determine if these conformational differences are important for function. Ligand modelling could also be used as an alternative to *in vitro* methods, as it is a technique that has been employed within the pharmaceutical industry in particular to predict P450 metabolism of specific substrates (Raunio *et al*, 2015).

Taken together, these results give an insight into the expression of equine liver cytochrome P450s while also suggesting there may be functional variations between the equine and human isoforms.

Chapter 5: Protein expression and verification

5.1: Introduction

Recombinant protein production has been a crucial step in advancing the fields of

molecular biology and biotechnology, allowing specific proteins to be produced in

large quantities that were not previously possible (Rosano and Ceccarelli, 2014). *E.*

coli systems are by far the most well-characterised and thus most popular choice for

protein expression (Terpe, 2006). Gram positive bacteria such as *Bacillus and*

Lactococcus are also a popular choice, due to their high levels of protein secretion

into the extracellular media (Vavrová *et al*, 2010, Morello *et al*, 2007). The gram

negative bacterium *Caulobacter crescentus* is also used for its secretory ability (due

to exploitation of the RsaA secretion signal), although it is limited with regard to the

size of proteins that can be produced (Amat *et al*, 2010). Other bacteria that have

been used for protein expression include *Streptomyces* (Binda *et al*, 2013) and

Pseudomonas (Retallack *et al*, 2012).

Bacterial expression systems are attractive for their ease of use, fast growth and

high protein production, however eukaryotic expression systems may sometimes be

necessary if certain post-translational modifications are required or if the protein of

interest is insoluble in bacterial systems (Rosano and Ceccarelli, 2014). These

include yeast (Mattanovich *et al*, 2012) plants such as Arabidopsis, rice and tobacco

(Rigano and Walmsley, 2005, Borghi, 2010), baculovirus (Van Oers *et al*, 2014), and

various mammalian cell lines – some of the most frequently used of which are

Chinese hamster ovary (Omasa *et al*, 2010), human embryotic kidney (Lin *et al*,

2015) and baby hamster kidney (Conner *et al*, 2005). Yeast is a popular choice due

to ease of use, affordability and their ability to produce high protein yields, although variations in glycosylation patterns may pose a problem for some proteins (Mattanovich *et al*, 2012). Mammalian cell lines offer the advantage of having the full array of post-translational modifications required for functional expression, however they are often costly, time-consuming and have significantly lower protein expression levels compared with bacterial and yeast systems (Khan, 2013).

Baculovirus-infected Insect cells are a popular alternative to mammalian systems and offer higher protein titres and relative ease of use. For these reasons, they are one of the most popular expression systems for mammalian proteins (Van Oers *et al*, 2014). Baculovirus expression systems work by incorporating the gene of interest into a bacmid virus particle, which is then used to infect the insect cells of choice (generally sf9, sf21 or High Five[TM] cells), Van Oers *et al*, 2014. Baculovirus was first used as an expression vector due to its ability to produce high levels of occlusion bodies (polyhedra), under the control of the polyhedron promoter, in the nucleus of infected cells (Jarvis, 2009). Exploitation of this polyhedron promoter allowed for the polyhedral gene to be replaced with a gene of interest, and therefore production of high quantities of recombinant protein (Jarvis, 2009).

5.1.1: Recombinant P450 production

Recombinant proteins are highly useful for studying P450 activity and have been used extensively in the area of human P450 research, as well as for research into P450s in many other species (Stringer *et al*, 2009, Gonzalez and Korzekwa,1995). Recombinant CYPs for human and some other organisms are commercially available and recombinant protein expression has become an important part of the drug development process (Zhang *et al*, 2012). Microsomes and hepatocytes are

useful for giving a more holistic approach to *in vitro* methods of drug development than recombinant enzymes since they provide a pool of drug-metabolising enzymes (Fasinu *et al*, 2012), although there are some questions as to the applicability of microsome data to *in vivo* systems due to underestimation of clearance rates (Chiba *et al*, 2009). The advantage of recombinant P450s is that they offer a specific insight into the metabolism of a drug – allowing one isoform to be studied in detail without the presence of other P450s.

For recombinant P450 expression, bacterial systems are able to generate the highest protein yield although the ways in which they vary from eukaryotic cells can be problematic, especially with regards to post-translational modifications such as glycosylation and phosphorylation (Khow and Suntrarachun, 2012). Successful production of proteins can be a significant problem in bacterial expression of eukaryotic DNAs (Rosano and Ceccarelli, 2014). Insect cell systems have been particularly popular in P450 research as they are relatively easy to maintain and generate higher protein yields than mammalian cells (Stringer *et al*, 2009). Insect cells provide one of the most popular and well-established systems for P450 expression and have been used for P450s in numerous species such as mosquitos (Duangkaew *et al*, 2011) pigs (Yao *et al*, 2011), dogs (Zhou *et al*, 2010, zebrafish (Wang-Buhler *et al*, 2005) and many more (Ohnishi *et al*, 2012, Sakamoto *et al*, 2012, Niu *et al*, 2011). Insect cells have the advantage over many other eukaryotic expression systems in that they do not have endogenous P450 expression (Stringer *et al*, 2009).

5.1.2: P450 carbon monoxide binding assay

Since the discovery of P450s, confirmation and quantification of an enzyme's status as a P450 has often relied on use of the carbon monoxide binding assay (Guengerich *et al*, 2009). This method involves binding of carbon monoxide to the ferrous (reduced) form of the enzyme in order to observe its absorbance – if a reading of 450 nm is made then the enzyme can be classed as a functional P450 enzyme (Guengerich *et al*, 2009). It can also be used to quantify the level of P450s within a sample by determining the difference in absorbance between 450 nm and 490 nm. Mass spectrometry and immunoblot analysis have also been used to quantify P450s however these do not allow for differentiation between inactive and active forms of the enzyme, therefore the carbon mononxide binding is ideal when this differentiation is required (Gröer *et al*, 2014).

5.1.3: Recombinant equine P450s

To date, equine P450s have been produced via recombination in insect cells (DiMaio Knych and Stanley, 2008) and hamster lung fibroblasts (Schmitz *et al*, 2014). Hamster lung fibroblasts, like insect cells, have no endogenous P450 expression (Schmitz *et al*, 2014).

During the current study, two different expression techniques were used – one using an insect cell system and the other using a cold-induction *E. coli* system. The cold-induction system (using the pCold I expression vector) was designed to express high protein levels at $15^{o}C$ due to induction of the cspA (cold shock) promoter with addition of IPTG after temperature reduction. Cell growth and production of endogenous bacterial proteins are halted upon this temperature change (Qing *et al*, 2004). Cold-induction expression is useful for when higher temperatures may result

in incorrect folding or the formation of inclusion bodies; a common problem in *E. coli* expression of eukaryotic proteins (Rosano and Ceccarelli, 2014). Cold-induction bacteria have been successfully used to produce many proteins which were difficult to express at higher temperatures (Qing *et al*, 2004, Hayashi and Kojima, 2008).

The aim of this chapter was to produce functional P450 proteins for subsequent functional analysis. Sf9 insect cells were used due to being the most highly established technique for recombinant P450 production while *E. coli* was used for its higher protein production capacity. Expression was confirmed via Western blotting, mass spectrometry and the carbon-monoxide binding assay.

5.2: Results

5.2.1: Sf9 insect cell expression

Cells infected with baculovirus constructs were harvested at time points ranging from 0 to 72hrs in order to ascertain time for optimal protein production by observing band size. Figure 5.2.1 displays expression attempts which show clear bands at approximately the desired size for the P450 constructs. From this, 24 hours post-infection seems to be the optimal time for maximal protein production. Western blots using anti-P450 antibody were carried out to validate protein production however these generated negative results (no bands were present). Immunoprecipitation (IP) for CYP2D50 baculovirus cells appeared to be successful, with a final elute band appearing at ~52KDa. Despite apparent successful IP, Western blot analysis was negative.
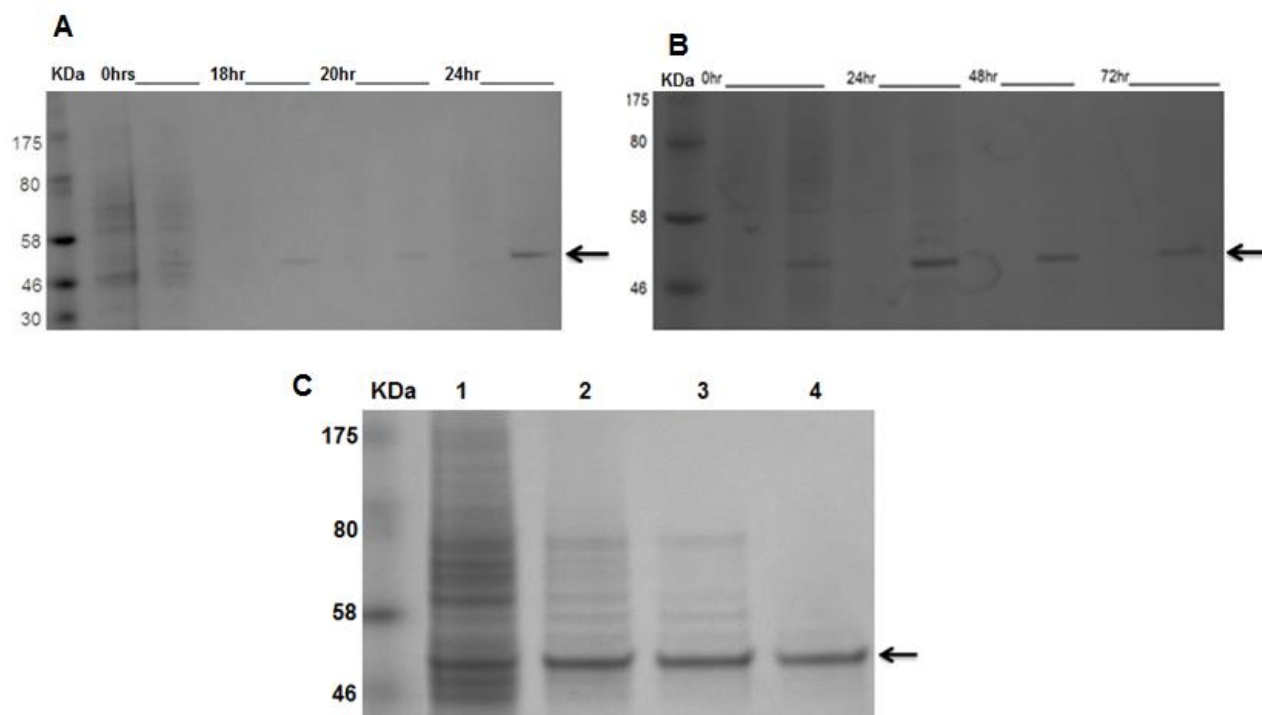
Figure 5.2.1: Sf9 coomassie-stained gels of baculovirus-infected cells. A: CYP2E1, B: CYP2D50

- samples at each time point were run along with a negative control (cells without baculovirus, on the

left of each sample set). C: immunoprecipication using anti-P450 antibody with CYP2D50 cells -

eluate from wash steps of immunoprecipication (1-3) was run along with the final elute (4).

5.2.2: Bacterial pCold expression

In order to validate that expression of the desired P450 proteins had occurred,

Western blots were carried out on the lysate of each construct. All clones underwent

multiple expression attempts, and four constructs were validated to have produced

P450 proteins – CYP2A13, CYP1A1, CYP2D50 and CYP2C92. IPTG titrations

ranging from 0mM-1mM were carried out in order to determine optimal IPTG

concentration to induce protein expression for each construct – Western blots using

an anti-P450 antibody are displayed in figure 5.2.3. Table 5.2.1 summarises

concentrations used for subsequent expression studies.

Figure 5.2.3: Western blots of IPTG titrations for all expressed P450s. A: CYP2C92, B:

CYP2D50, C: CYP1A1, D:CYP2A13. A cytochrome P450 polyclonal antibody was used and 30µl

protein was loaded per well.
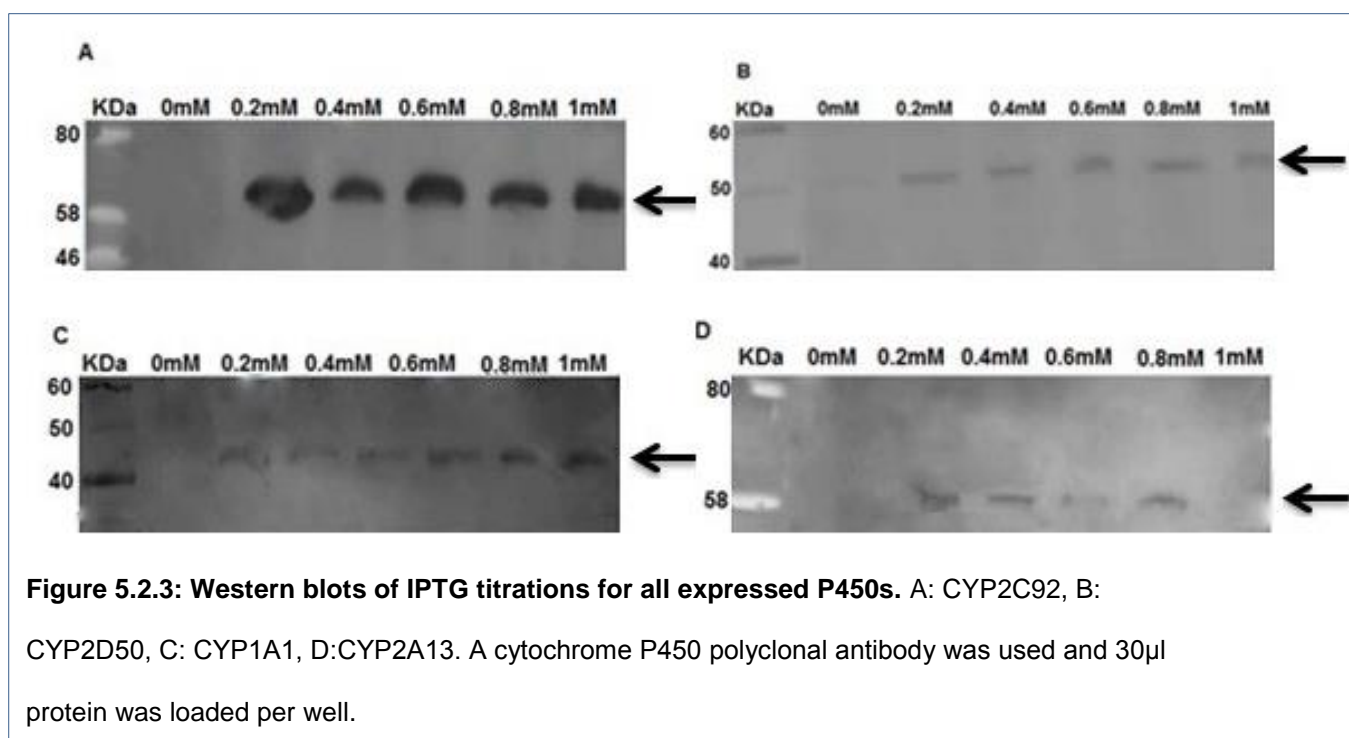
P450 construct	Optimum IPTG concentration
CYP2C92	0.2mM
CYP2D50	0.6mM
CYP1A1	1mM
CYP2A13	0.8mM

Table 5.2.1: Optimum ITPG concentrations for each P450 pCold construct as determined by

Western blot analysis. IPTG concentrations ranged from 0mM to 1mM.

Figures 5.2.4-5.2.7 show Western blots of samples taken at five different stages

post-induction using optimal IPTG concentrations and clearly show the

corresponding protein band (~55-58KDa) increasing. Two different antibodies (anti-

P450 and anti-his tag) were used for each construct – one against cytochrome P450

and one against the his-tag, which was present in the pCold vector (section 4.1.3).

Once expression was confirmed, large-scale (1 litre culture) batches of protein were

produced for functional assays (to ensure uniformity across all assays) and protein

content was measured – the result of which is displayed in table 5.2.2. Purified

protein was stored at -80°C in 1ml aliquots.

	CYP1A1	CYP2A13	CYP2C92	CYP2D50
Batch 1	9mg/ml	5mg/ml	6mg/ml	7mg/ml
Batch 2	2.4mg/ml	7.5mg/ml	5.5mg/ml	8mg/ml
Batch 3	6mg/ml	---------------	5mg/ml	6.5mg/ml
Batch 4	---------------	---------------	---------------	8.5mg/ml

Table 5.2.2: Protein content (mg/ml) for large batches produced for each pCold construct.

Protein concentration was measured via Bradford assay (Sigma-Aldrich) according to manufacturer's

protocol and a spectrophotometer with detection at 595nm.
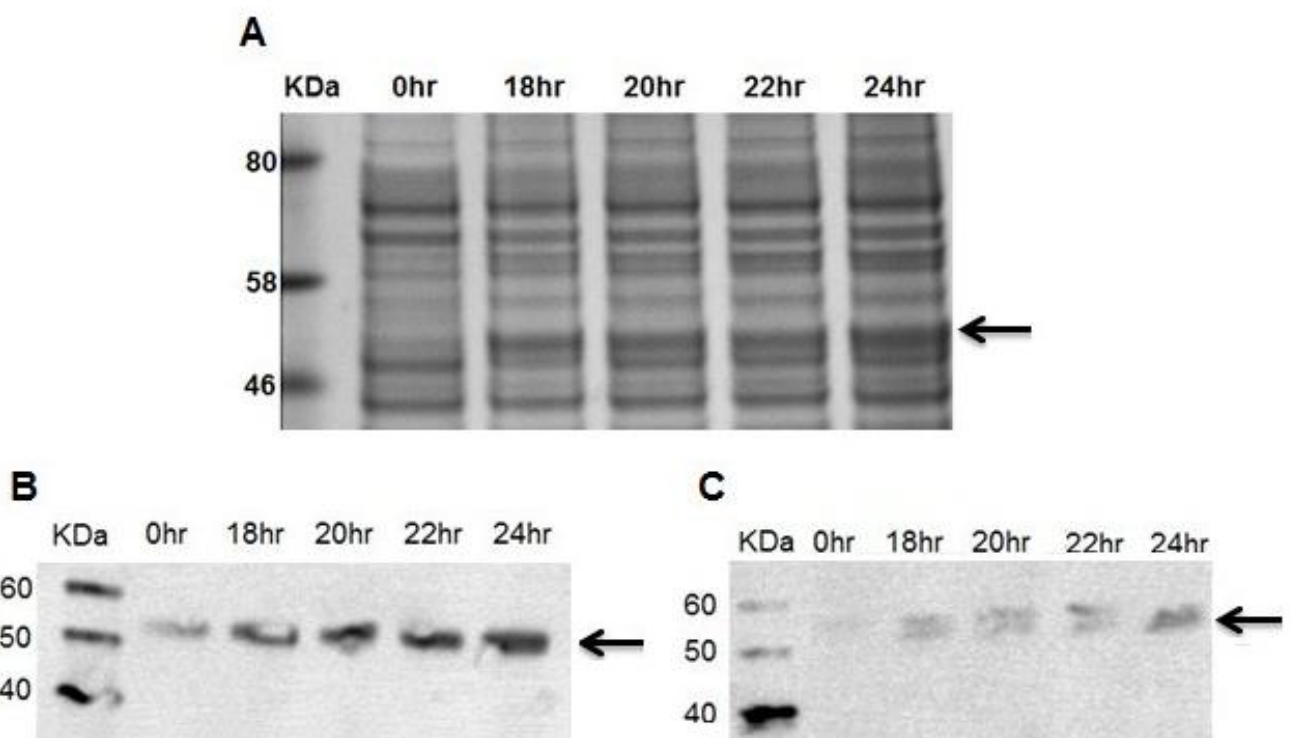
Figure 5.2.4: Coomassie-stained gel (A) and Western blots of CYP2D50 in the pCold vector.

Samples were taken at different time points during expression ranging from 0 hours to 24 hours post-

induction and were probed with B: an anti-his tag antibody and C: an anti-P450 antibody. Band size

for both is around 55-58KDa.
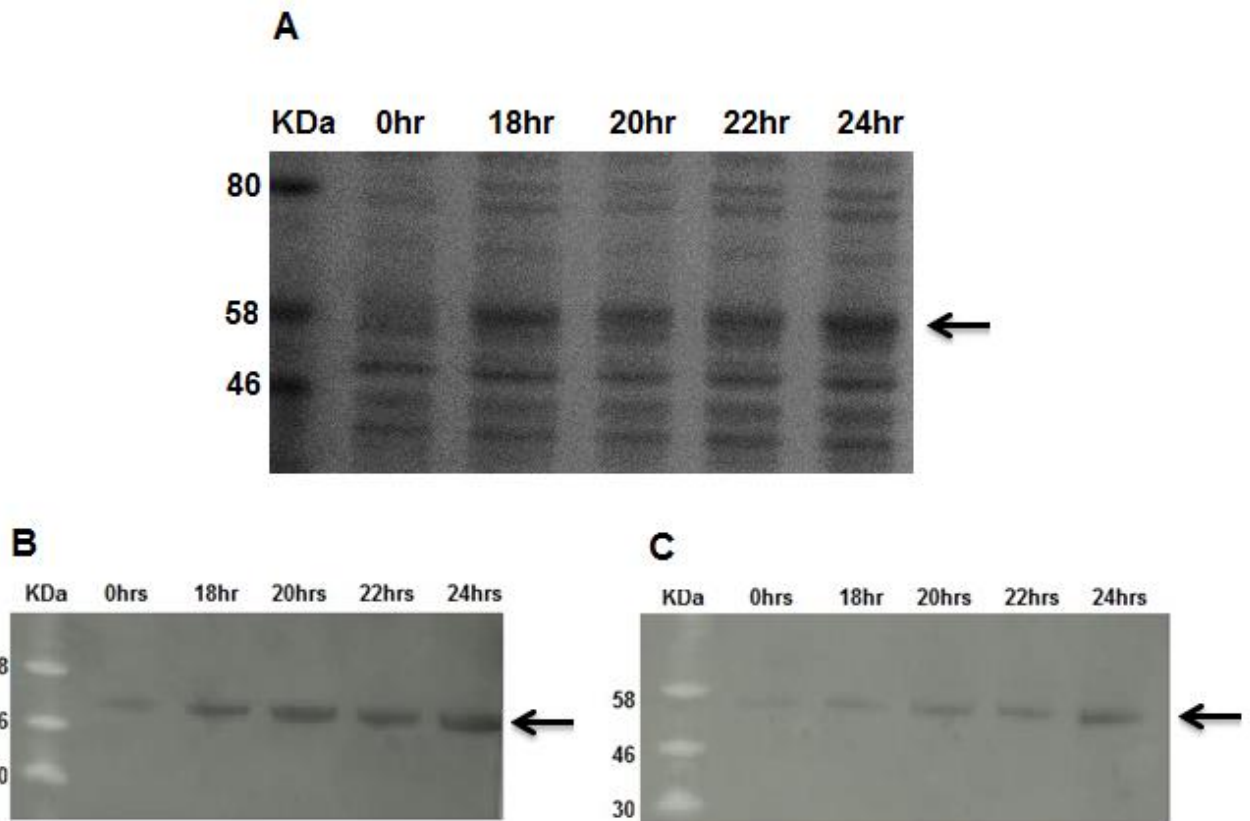
Figure 5.2.5: Coomassie-stained gel (A) and Western blots of CYP2C92 expressed in the pCold vector. Samples were taken at different time points during expression ranging from 0 hours to 24 hours post-induction and were probed with A: an anti-his tag antibody and B: an anti-P450 antibody. Band size for both is around 55-58KDa.
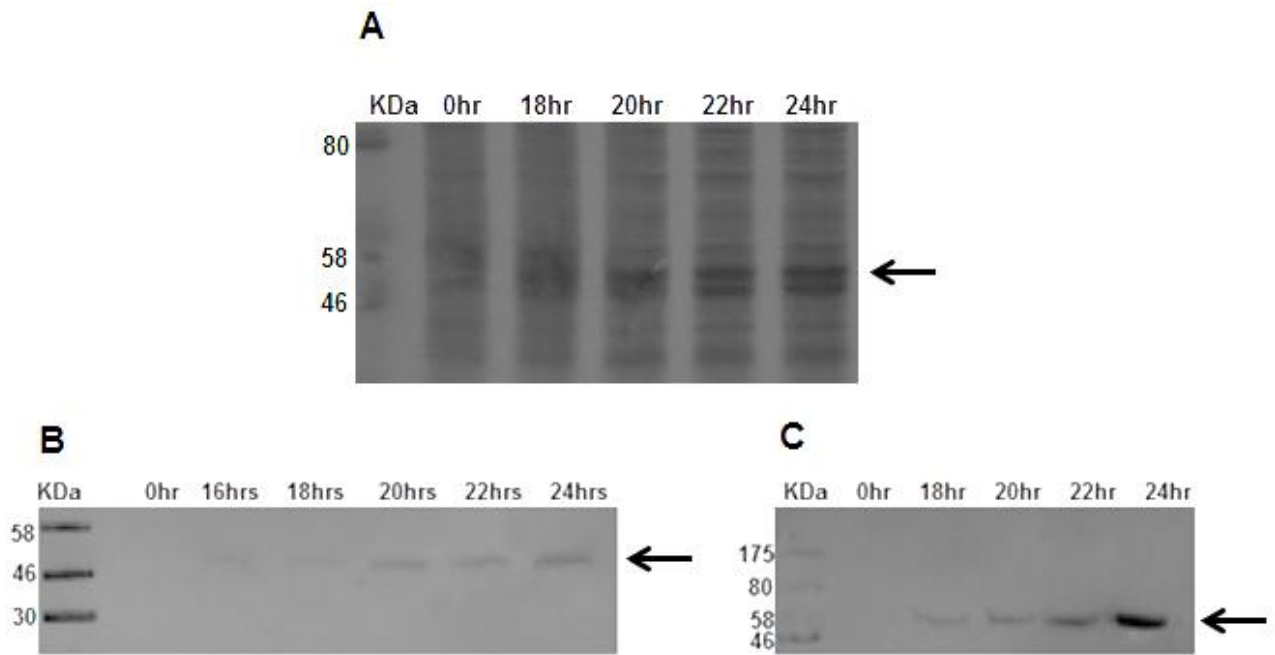
Figure 5.2.6: Coomassie-stained gel (A) and Western blots of CYP2A13 expressed in the pCold vector. Samples were taken at different time points during expression ranging from 0 hours to 24 hours post-induction and were probed with A: an anti-his tag antibody and B: an anti-P450 antibody. Band size for both is around 55-58KDa.
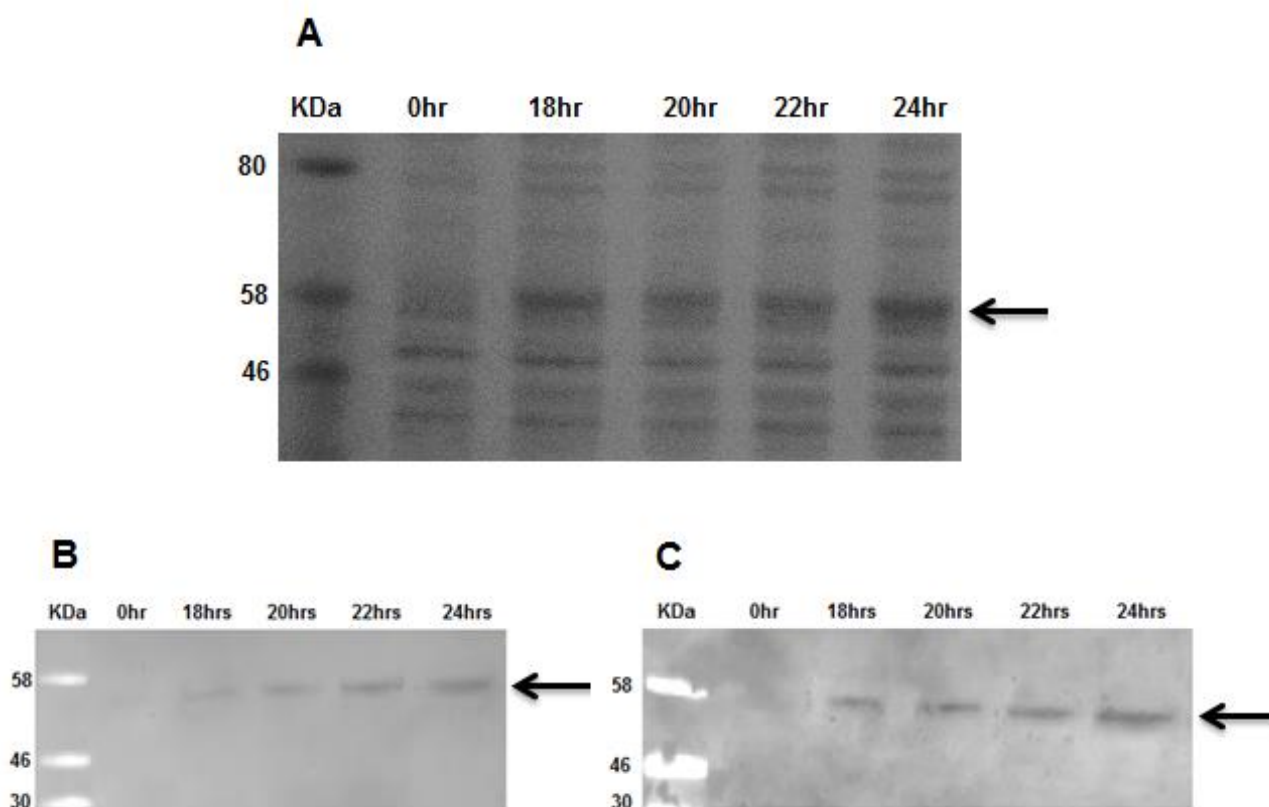
Figure 5.2.7: Coomassie-stained gel (A) and Western blots of CYP1A1 expressed in the pCold vector. Samples were taken at different time points during expression ranging from 0 hours to 24 hours post-induction and were probed with A: an anti-his tag antibody and B: an anti-P450 antibody. Band size for both is around 55-58KDa.

5.2.2.1: His-Tag Purification

To purify the P450 proteins, the his-tag integrated into the pCold cloning vector was used by adding the samples to his columns. Figure 5.2.8 displays a stained protein gel of this, with a band of expected size in the final eluate. The products from the purification were stored at -80°C for use in functional assays (see chapters 6-10). Due to the low yield of this method (protein concentrations of eluted protein were around 0.06ml/ml-0.125mg/ml with only 100μl being produced at a time), purified protein was not used for most of the functional assays.

133
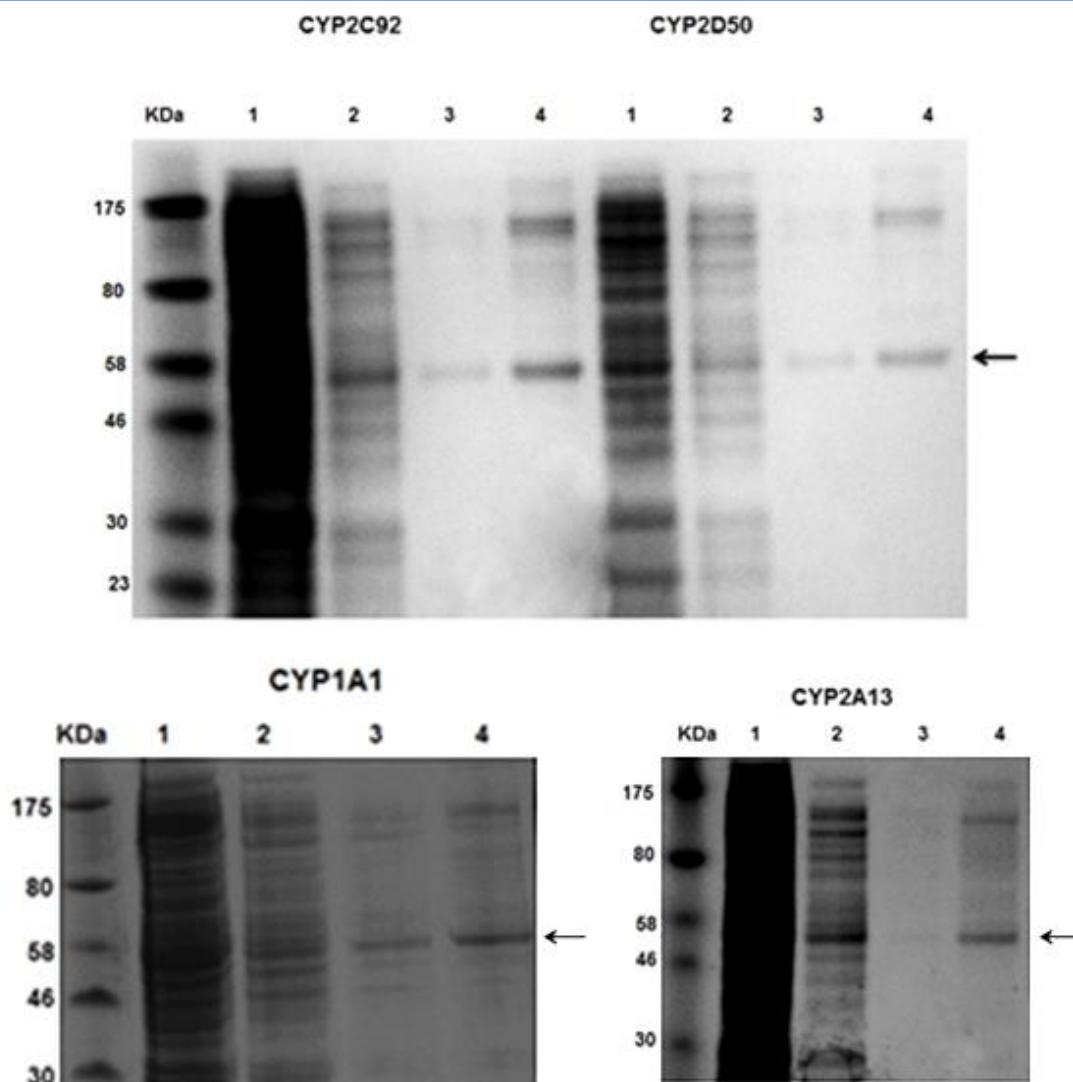
Figure 5.2.8: His-Select Purification of all four expressed pCold constructs. Supernantants (1-3) from wash steps were run alongside the elution product (4) where a band of the expected ~55KDa size was present.

5.2.3: Carbon monoxide binding spectrum

The P450 carbon monoxide binding assay is used for determining if an enzyme is a P450, via measuring the absorbance pre- and post-addition of carbon monoxide at 420nm and 450nm (Guengerich *et al*, 2009). All four pCold P450 constructs

(CYP2D50, CYP2C92, CYP2A13 and CYP1A1) were tested in this way, and lysate using three different lysis techniques (lysis in Cellytic B buffer, lysis with lysozyme and lysis with sonication) was used in case denaturation occurred due to one particular method (section 2.31) Lysate in the lysozyme and the sonicated buffer did not show peaks (at 420nm, 450nm or 490nm) and so only those in Cellytic B were able to be measured (table 5.2.3).

Microsome samples were used as positive controls – one suspended in PBS and the other in Cellytic B buffer. In PBS, the microsomal system displayed a classic peak at 450nm however in Cellytic B a large peak at 420nm was generated. The recombinant P450s had smaller 420nm readings however all had much larger 420nm than 450nm aborbance values. Figure 5.2.9 displays the readings generated for microsomes in Cellytic B and CYP2C92 in Cellytic B, both of which have peaks at 420nm.

	Microsomes in PBS	Micorosmes in Cellytic B	CYP2C92 in Cellytic B	CYP2A13 in Cellytic B	CYP2D50 in Cellytic B	CYP1A1 in Cellytic B
420 nm (abs)	0.028	0.103	0.015	0.045	0.046	0.012
450 nm (abs)	0.087	0.008	0.005	0.071	0.022	0.003

Table 5.2.3: Summary of peaks detected in samples bound to carbon monoxide. Absorbance readings were taken at 420nm and 450nm for all samples after being zeroed using samples without carbon monoxide.
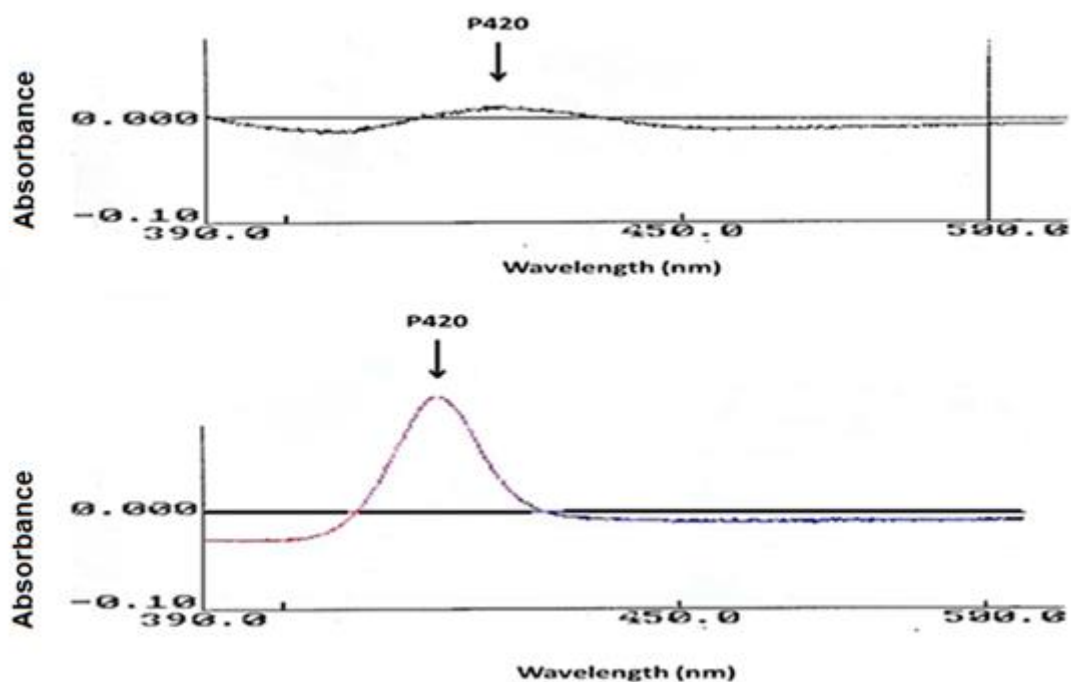
Figure 5.2.9: Carbon monoxide binding spectra for CYP2C92 in Cellytic B (top) and positive control microsomes in Cellytic B (bottom). Both samples show a peak at 420nm with no peak at 450nm.

5.2.4: Mass spectrometry analysis of recombinant proteins

To provide a final validation step, purified bacterial lysate was analysed via liquid chromatography mass spectroscopy in collaboration with Dr Susan Liddell in order to validate that the specific P450 was present. Data were searched using the MASCOT database with default settings and one P450 generated a match - CYP2C92. CYP2A13 and CYP1A1 were also analysed however only matches for bacterial proteins were obtained – implying protein content may have been too low for detection. For CYP2C92, four peptide sequences were matched with the published CYP2C92 sequence (table 5.2.4). In addition to CYP2C92 peptides, matches with

the *E. coli* proteins elongation factor Tu, 30S ribosomal protein S10 and fructose-

bisphosphate aldolase were made (table 5.2.5).

Peptide	Position in Sequence	MASCOT Score
RFSLMTLRN	125-133	29
RGRFPVTERV	97-106	32
RYIDLLPTNVPHAVTRD	356-373	61
KSHMPYTDAVVHEIQRY	341-357	80

Table 5.2.4: P450 peptide Mass spectrometry matches for CYP2C92. Peptides analysed are

highlighted along with their position within the CYP2C92 sequence and their MASCOT scores, with a

total score for all four peptides of 202 (scores above 61 are classed as highly significant, i.e. unlikely

to be occurring due to chance).

Protein	Peptide	MASCOT Score
Elongation factor Tu (708)	RAGENVGVLLRG	68
	KVGEEVEIVGIKE	61
	KALEGDAEWEAKI	43
	KTTLTAAITTVLAKT	58
	KFESEVYILSKDEGGRH	12
	KMVVTLIHPIAMDDGLRF	74
	RTKPHVNVGTIGHVDHGKT	59
	RGITINTSHVEYDTPTRH	82
	KILELAGFLDSYIPEPERA	86
	RELLSQYDFPGDDTPIVRG	84
	RAIDKPFLLPIEDVFSISGRG	41
	RQVGVPYIIVFLNKC	40
30S ribosomal protein S10 (104)	RLVDIVEPTEKT	57
	RLIDQATAEIVETAKRT	47
fructose-bisphosphate aldolase (81)	SKIFDFVKPGVITGDDVQKV	51
	RFTIAASFGNVHGVYKPGNVVLTPTILRD	21
	KVKAPVIVQFSNGGASFIAGKG	9

Table 5.2.3: Summary of non-P450 MASCOT matches for CYP2C92. Overall MASCOT scores are

given in brackets.

5.3: Discussion

The purpose of this chapter was to express recombinant P450 enzymes in *E. coli* and sf9 insect cells. P450 expression was unable to be confirmed for the insect cell system, with Western blotting analysis generating negative results despite bands appearing around the correct size on coomassie-stained gels (figure 5.2.1). Immunoprecipitation appeared to be successful for the CYP2D50 construct however subsequent Western blotting of this product using an anti-CYP2D6 antibody produced negative results. This may have been due to a lack of reactivity, since this antibody was only confirmed to be active against the human, rat and mouse forms of the enzyme however no other antibodies were available as alternatives.

Four constructs were expressed in *E. coli* – CYP2D50, CYP2C92, CYP1A1 and CYP2A13. Western blotting analysis of lysates confirmed P450 expression. Mass spectrometry was used as an additional means for conforming expression of the desired proteins and for CYP2C92 matches were made with equine CYP2C92 peptides. With this analysis, various matches were also made with bacterial proteins, although his-column purification was carried out before mass spectrometry (section 5.2.2.1) to minimise bacterial contaminants. Native *E. coli* proteins are known to often co-elute with the target protein using this method, due to possessing high affinity for the nickel ions used for his-tag purification (Robichon *et al*, 2011).

A third method was used to validate P450 expression – the carbon monoxide binding assay, which is often seen as the 'gold standard' for P450 verification and quantification (Guengerich *et al*, 2009). A 450 nm peak was not detected for any lysate when bound with carbon monoxide therefore suggesting expressed enzymes may not be functional P450s. Instead, a P420 peak was detected in multiple enzyme

batches. P420 enzymes are generally regarded as denatured forms of P450s (Panicco *et al*, 2008). P420s vary from P450s in the haem-binding region via alteration of the haem-thiolate bond which generally becomes weaker when a P420 species is produced (Omura and Sato, 1964a). Evidence suggests this weakening happens due to the thiolate becoming protonated to form a thiol (Sun *et al*, 2013). There is no literature describing catalytically active P420 enzymes, although it has been reported that converting the inactive P420s to P450s is possible (Dunford *et al*, 2007). Three different lysis techniques were used with this assay in case the 'main' Cellytic B buffer was responsible for the absorbance change however the two other lysis techniques resulted in no change in absorbance upon binding with CO, suggesting the enzyme was denatured such that no carbon monoxide binding was able to occur.

Due to the lack of eukaryotic post-translational modifications and potential incorrect folding in bacterial expression systems, it is possible that the proteins produced were not functional P450s. The cold-induction system was used to minimize this issue, since lowering the temperature can reduce incorrect folding and inclusion body formation (Hayashi and Kojima, 2008). Often, removal of the amino-terminal section of the P450 enzyme that anchors it to the endoplasmic reticulum has been required to produce a functional enzyme (Yun *et al*, 2006). However, *E. coli* systems have been used successfully during some studies without modification of the P450 sequences (Park *et al,* 2014, Locuson *et al*, 2009), although variables such as rotations per minute of cultures, culture conditions and additives to cultures may play a significant role in the production of functional protein (Faiq *et al*, 2014). Further work will be required on the clones produced within the current study in order to determine whether varying these factors alters the spectral properties of the

recombinant proteins. Additionally, removal of the N-terminal of the P450 sequences should be attempted to ascertain whether this is required to produce enzymes that can be functionally classed as P450s.

Chapter 6: Recombinant CYPP450 enzyme kinetic analysis

6.1: Introduction

In humans, the majority of pharmacokinetic data is derived from the pharmaceutical industry, however in the horse this data largely comes from the horseracing industry and relates to doping control (Scarth *et al*, 2010). As with humans, adverse drug interactions can occur in the horse when multi-drug treatments are prescribed (Brumbaugh *et al*, 2001). Various classes of drug are frequently used in the horse, including (but not limited to) non-steroidal anti-inflammatory drugs (Lees and Higgins, 1985), corticosteroids (Harkins *et al*, 1993), anticholinergics (Rumpler *et al*, 2013) and antihistamines (Benoit *et al*, 2008, Petersen and Schott, 2009).

As covered in chapter 1 (section 1.15), little is known about CYP-specific metabolism in the horse. NSAIDs are frequently prescribed during equine veterinary treatment and are one of the most commonly used veterinary medications (National Office for Animal Health, 2014), particularly phenylbutazone (Tobin *et al*, 1986). Other commonly used NSAIDs include ketoprofen and flunixin (Goorich and Nixon, 2006).

6.1.1: Analysis of drug metabolism

Quantitative analysis of equine drug metabolism studies is generally carried out via gas chromatography mass spectrometry (GC-MS) or liquid chromatography mass spectrometry (LC-MS), which is the technique of choice in most analytical laboratories today (McKinney, 2009). GC-MS is still used for some compounds (such as testosterone and other steroids) which are poorly ionized by LC-MS (Teale and Houghton, 2010). Although the vast majority of equine drug metabolism research relies on *in vivo* data, *in vitro* studies are becoming more commonplace (e.g. Scarth

et al, 2010a, Ho *et al*, 2007, Ho *et al*, 2007a). Data relating to recombinant enzyme systems in the horse is much more limited, with only two laboratories thus far having published studies using recombinant equine enzymes (DiMaio Knych and Stanley, 2008, DiMaio Knych *et al*, 2009, DiMaio Knych *et al*, 2010, Peters *et al*, 2013, Dettwiler *et al*, 2014). *In vitro* studies are routinely carried out in human drug research and the data generated is often extrapolated in order to predict the *in vivo* effects of drugs (Venkatakrishnan *et al*, 2001, Mcginnity and Riley, 2001).

6.1.2: Enzyme kinetic analysis

The aim of enzyme kinetic analyses is to work out the velocity of metabolism of a substrate by an enzyme (i.e. how fast the reaction occurs), and there are various methods a user may choose to do this, the most popular of which is by using Michaelis-Menten kinetics (Johnson, 2013). The velocity of metabolism is measured by either monitoring disappearance of a substrate or appearance of a product over time (Johnson, 2013). When it was discovered that enzymes could be saturated, a new way of looking at enzyme kinetics was formed (Brown, 1902). The following equation can be used to illustrate an enzymatic reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{K_2}{\longrightarrow} E + P$$
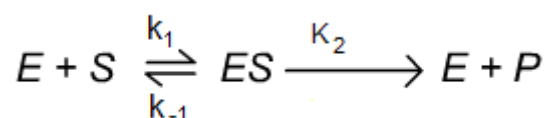
Equation 1

Here, an enzyme-substrate complex is formed (ES) before product formation (P). K_1 and K_{-1} are the forward and reverse reaction rate constants for the ES complex and K_2 is the rate constant for product formation. The preferred means of measuring the

velocity rate is from measuring product formation. The initial rate (v_0) for product formation is obtained from the initial linear slope of the product concentration versus time graph.

6.1.3: Michaelis-Menten kinetics

After Brown's discovery that enzyme activity could be saturated, Michaelis-Menten kinetics was developed (Michaelis and Menten, 1913). This involves looking at the initial rate of a reaction across various substrate concentrations. It is one of the most widely used kinetic analysis tools and allows for the relationship between the initial rate, maximal velocity (V_{max}) and Michaelis-Menten constant (K_m – defined as the drug concentration at half the V_{max}) to be defined (Chen *et al*, 2010).
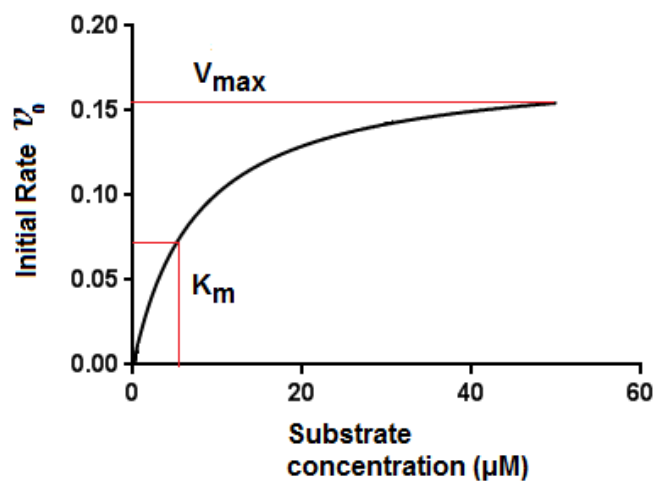
Figure 6.1: Graph displaying the rate of an enzymatic reaction. The initial rate (v_0) is plotted against substrate (drug) concentration. V_{max} (maximal velocity) and K_m (substrate concentration at half of V_{max}) are highlighted.

Michaelis-Menten kinetics can be defined via the following equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 2

Where the initial rate (v_0) is equal to the product of V_{max} and the substrate concentration divided by the sum of K_m and the substrate concentration. Micheaelis-Menten kinetics can be used on its own to study enzyme saturation or the data can be extrapolated in order to estimate the intrinsic clearance of a drug (Houston, 1994).

Although it is ideal to use product formation to determine reaction rate, this is not always possible as the product might not be known, or the commercially produced product may be unavailable/too expensive. In this case, it is possible to apply Michaelis-Menten kinetics to substrate (drug) depletion, and similar values can be obtained as for product formation (with the assumption that only one product is formed). The following equation represents the disappearance of a substrate over time:

$$\frac{d[S]t}{dt} = K_{dep} [S]t$$

Equation 3

Where [S] represents the substrate concentration and t = time. K_{dep} is the depletion rate constant, which is calculated by the slope of the line resulting from the natural logarithm (Ln) transformed substrate response data versus time. An advantage of the substrate depletion approach is that it is not necessary to know the concentration of the substrate and only substrate response data is required (e.g. mass spectrometry response).The initial rate (v_0) for product formation approximates to

$$v_0 = K_{dep}\,[S]_0$$

Equation 4

Where the initial rate of the reaction is equal to K_{dep} multiplied by the initial substrate concentration (t = 0).

6.1.4: Intrinsic Clearance

Data gathered *in vitro* can be used to estimate the intrinsic clearance (CL_{int}) of a drug, which is the ability of the enzymes to metabolise a drug, not taking into account factors such as blood flow and protein binding (Houston, 1994). Intrinsic clearance is estimated using Michaelis-Menten kinetics via the following equation assuming the substrate concentration is less than the K_m:

$$CL_{int} = \frac{V_{max}}{K_m}$$

Equation 5

6.1.5: Substrate Inhibition

There are various types of inhibition that can occur with an enzyme, one of the most common of which is substrate auto-inhibition (for a comprehensive review of all types of inhibition see Lu and Li, 2010). Substrate auto-inhibition can happen at high substrate concentrations due to the substrate blocking the enzyme's activity, or alternatively the product (metabolite) produced by the enzyme inhibiting the enzyme's activity. For either mechanism of substrate inhibition, a typical Michaelis-Menten analysis may not be possible for the data. The inhibition constant (K_i) can be used to determine the reaction velocity rate via the equation:

$$v_0 = \frac{V_{max}[S]}{K_m + [S](1 + \frac{[S]}{K_i})}$$

Equation 6

At very low concentrations ($[S] << K_m$ and K_i), the equation can be simplified, and the reaction velocity rate can be expressed as:

$$v_0 = \frac{V_{max}}{K_m}[S]$$

Equation 7

Therefore, the intrinsic clearance (Cl_{int}) can be obtained when the initial velocity is plotted against the substrate concentration for the initial linear slope of the graph, which is equal to V_{max}/K_m thus CL_{int}.

6.1.5: Rational for current study

For the current study, drugs of multiple therapeutic classes were screened against two recombinant equine P450 enzymes – CYP2D50 and CYP2C92 (isolated in this research, see chapter 5). Non-steroidal anti-inflammatory drugs (NSAIDs) were the focus of the research due to their importance in equine medicine however other drugs studied included fluphenazine, a typical antipsychotic banned by the Association of Racing Commissioners International (Association of Racing Commissioners International, 2015), the synthetic corticosteroid triamcinolone, used frequently to treat joint pain, a common problem in performance horses (Frisbie *et al*, 1996) and dextromethorphan, not frequently used in the horse but tested experimentally to treat cribbing (Rendon *et al*, 2001) and used as a positive control for CYP2D activity for the purpose of the current study. Likewise, diclofenac, only occasionally used as a topical treatment in the horse was used as a positive control for CYP2C activity, since it has been used previously for the equine CYP2C92 isoform (DiMaio Knych *et al*, 2009). See table 6.21.1 for a list of those drugs used along with their human P450 specificities. Once these screens of activity had been carried out, those drugs that appeared to be metabolised by the recombinant P450s were further analysed (sections 6.3-6.10).

6.2: Results

6.2.1: LCMS method development

Ten different drugs were screened against recombinant equine CYP2C92 and CYP2D50, most of which are frequently used in the horse (table 6.2.1). Methods to detect the ten different drugs were developed using high performance liquid chromatography mass spectrometry (table 6.2.2). In addition, for two drugs (phenylbutazone and diclofenac) methods were also developed to monitor for the hydroxylated metabolites, 4-hydroxydiclofenac and oxyphenlbutazone, with the detected ions being of mass 312.06 and 325.24. Metabolite detection was run alongside parent drug detection for subsequent detailed analysis (chapters 7-10) – only parent drugs were analysed for initial screens. Figures 6.2.1 -6.2.10 show the full scan spectra obtained for each drug.

Drug class	Drug	P450 metabolism in human
Antitussive	Dextromethorphan	CYP2D6/CYP3A4 (Yu and Haining, 2001).
NSAID	Diclofenac	CYP2C9 (Miners and Birkett, 1998).
NSAID	Flunixin	Unknown
NSAID	Ketoprofen	CYP2C (Glówka *et al*, 2011).
NSAID	Phenylbutazone	CYP2C9 (Takanohashi *et al*, 2007).
First generation antihistamine	Pyralamine	Unknown (predicted substrate of CYP2B6, CYP2D6 and CYP3A4 by SuperCYP – Preissner *et al*, 2009.
First generation antihistamine	Hydroxyzine	CYP2D6 (Hamelin *et al*, 1998).
Synthetic corticosteroid	Triamcinolone	CYP3A4 (Hagan *et al,* 2010).
Typical antipsychotic	Fluphenazine	CYP2D6 (Shin *et al*, 1999).
Muscarinic anticholinergic	Glycopyrrolate	Unknown

Table 6.2.1: Drugs used for screening assays. Drug therapeutic classes are highlighted along with

their known P450 specificity in human.

Drug	Mass	Mass of ion	Cone voltage	Column	Gradient or isocratic?	Details	Retention time (mins)
Dextromethorphan	271	272.89	50	3x150mm, 5µm	Isocratic	50:50 (A:B), 1ml/min	0.99
Diclofenac	296	296.19	35	3x150mm, 5µm	Gradient	See table 2.42 (chapter 2)	4.55
Flunixin	296	297.26	40	3x150mm, 5µm	Gradient	See table 2.42 (chapter 2)	4.60
Fluphenazine	437	438.26	35	3x150mm, 5µm	Gradient	0-100%B (5 mins), 0.5ml/min	0.81
Glycopyrrolate	318	319.67	40	3x150mm, 5µm	Isocratic	50:50 (A:B), 1ml/min	1.00
Hydroxyzine	374	407.68	35	3x150mm, 5µm	Gradient	0-100% B (10 mins), 0.5ml/min	2.46
Ketoprofen	254	332.63 (MW + formic acid + methanol)	15	3x150mm, 5µm	Gradient	See table 2.44 (chapter 2)	4.56
Phenylbutazone	308	309.19	50	3x150mm, 5µm	Gradient	See table 2.43 (chapter 2)	5.59
Pyrilamine	285	286.12	35	3x150mm, 5µm	Gradient	0-100% B (4 min), 0.5ml/min	1.13
Triamcinolone	394	395.63	40	250x3.2mm, 5µm	Isocratic	50:50 (A:), 0.5ml/min	1.80

Table 6.2.2: Methods used for LCMS analysis of each drug. All methods involved ESI+ mode and were run on C18 columns. Solvent A
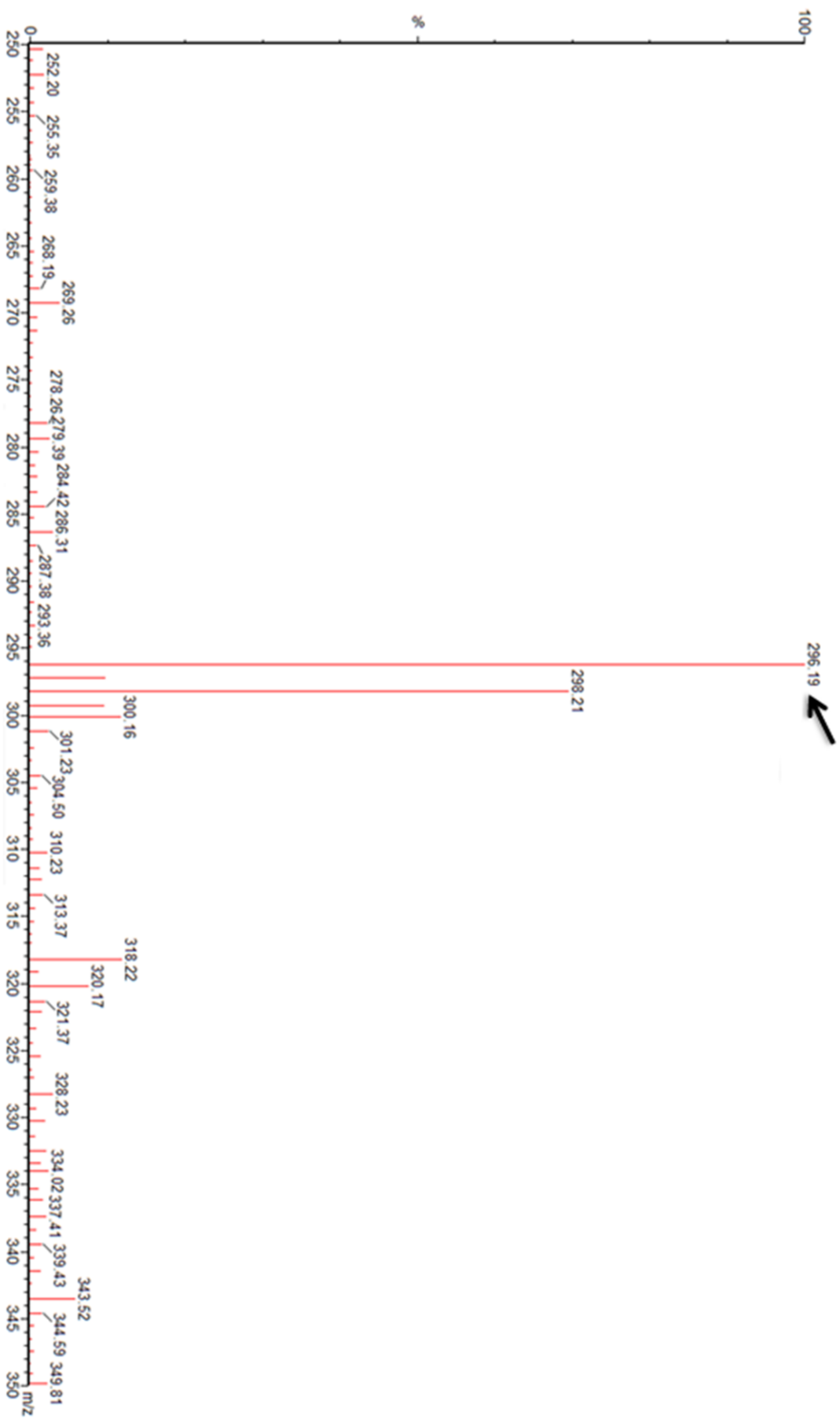
Figure 6.2.1: Full scan spectra for diclofenac. Arrow indicates ion selected for analysis.
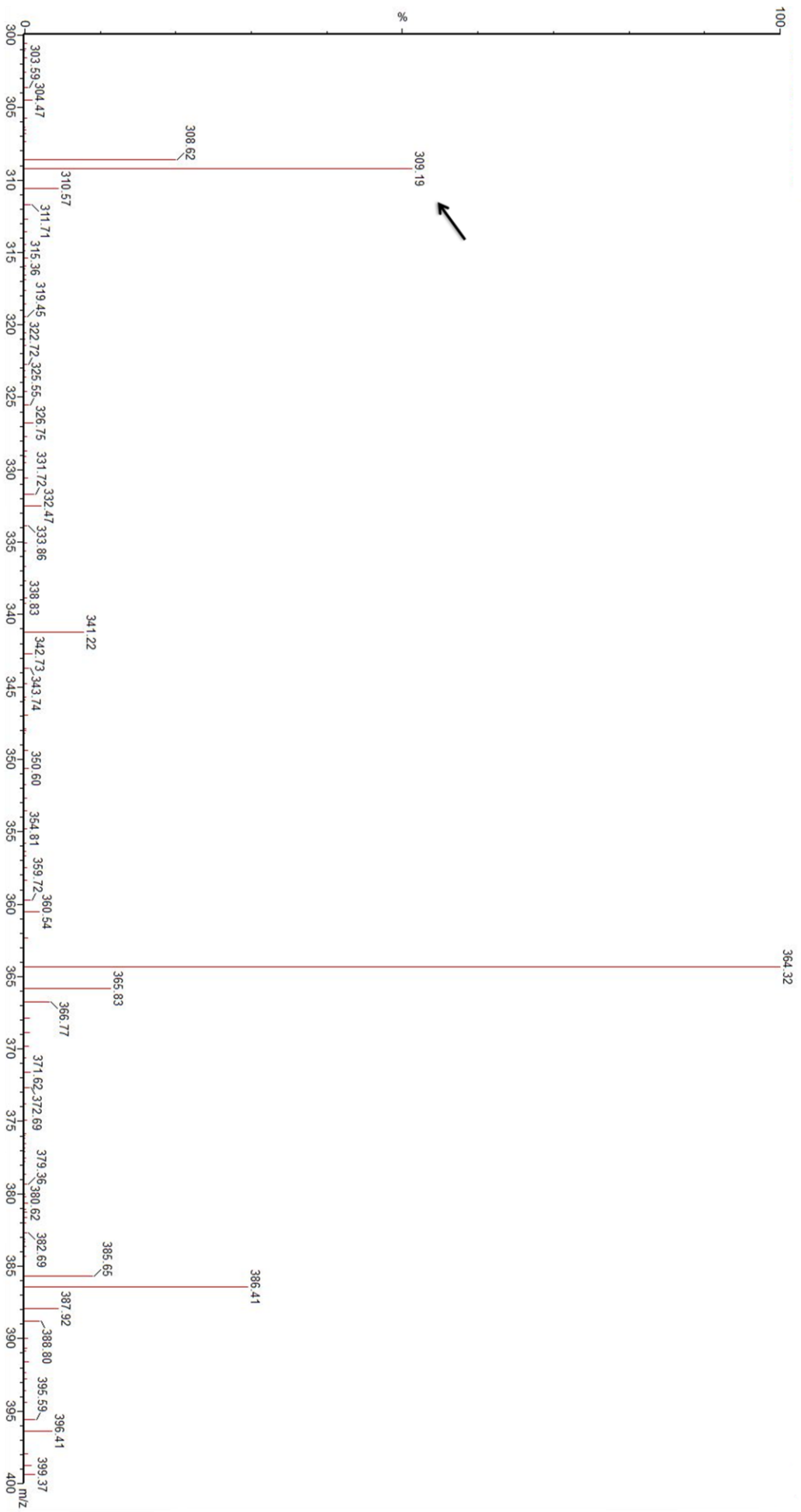
Figure 6.2.2: Full scan spectra for phenylbutazone. Arrow indicates ion selected for analysis.
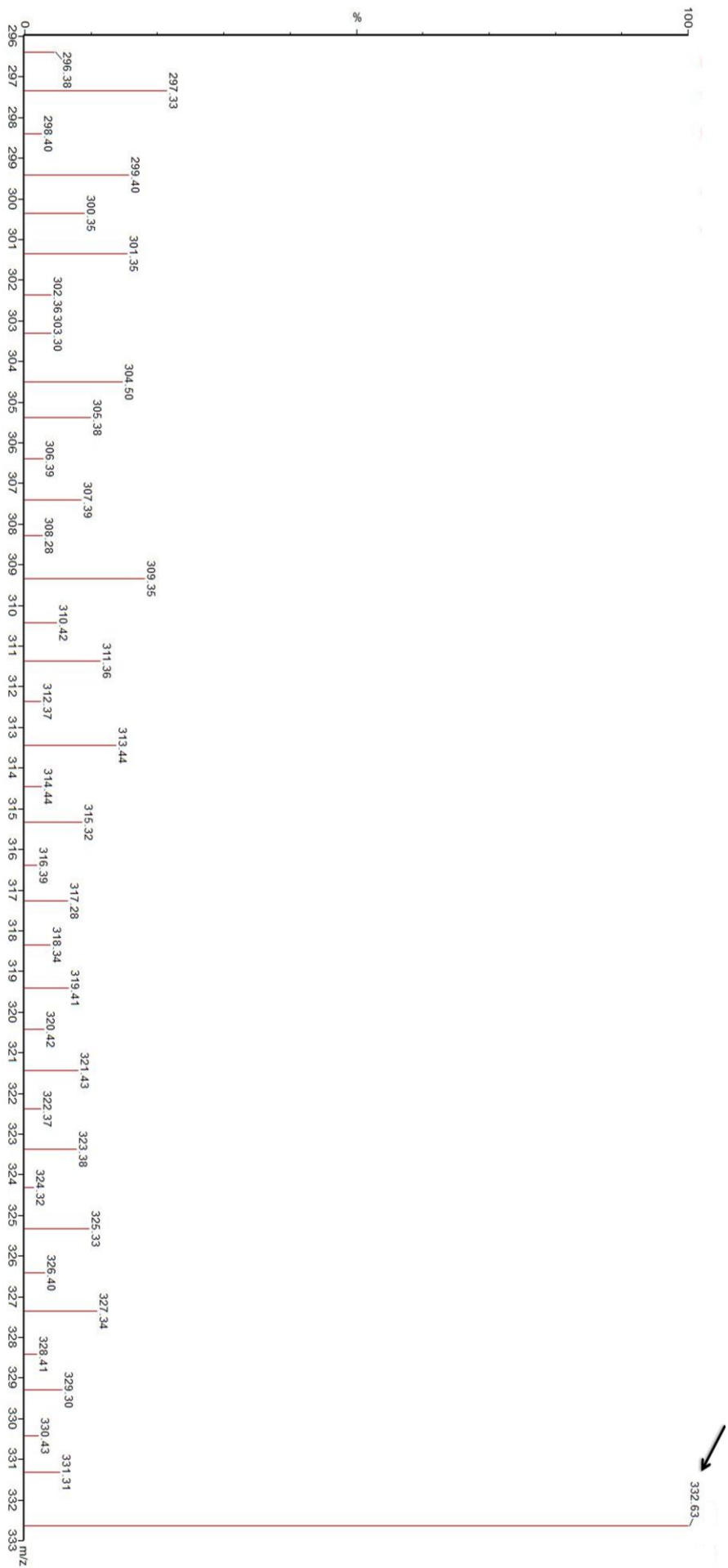
Figure 6.2.3: Full scan spectra for ketoprofen. Arrow indicates ion selected for analysis.
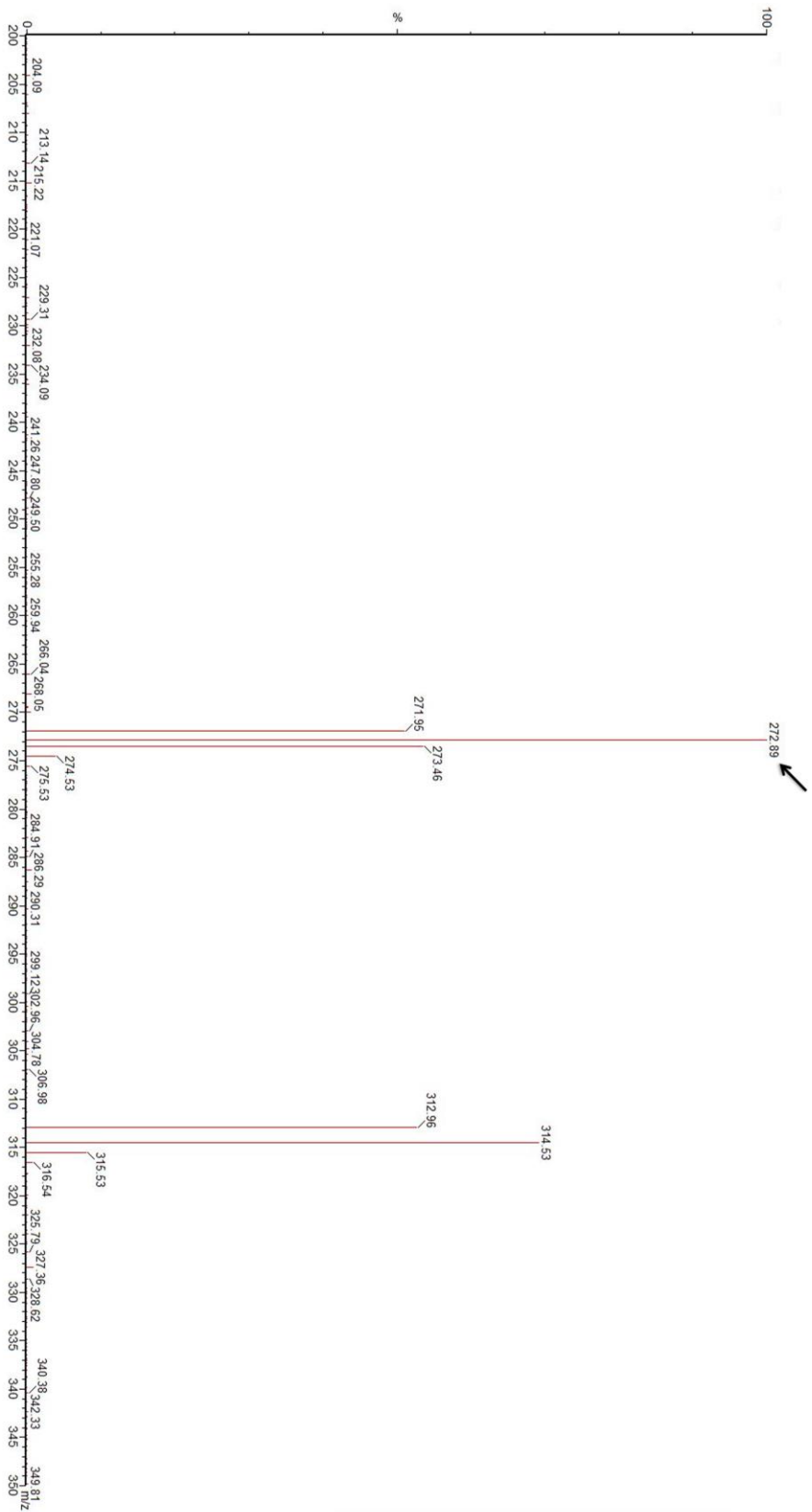
Figure 6.2.4: Full scan spectra for dextromethorphan. Arrow indicates ion selected for analysis.

154
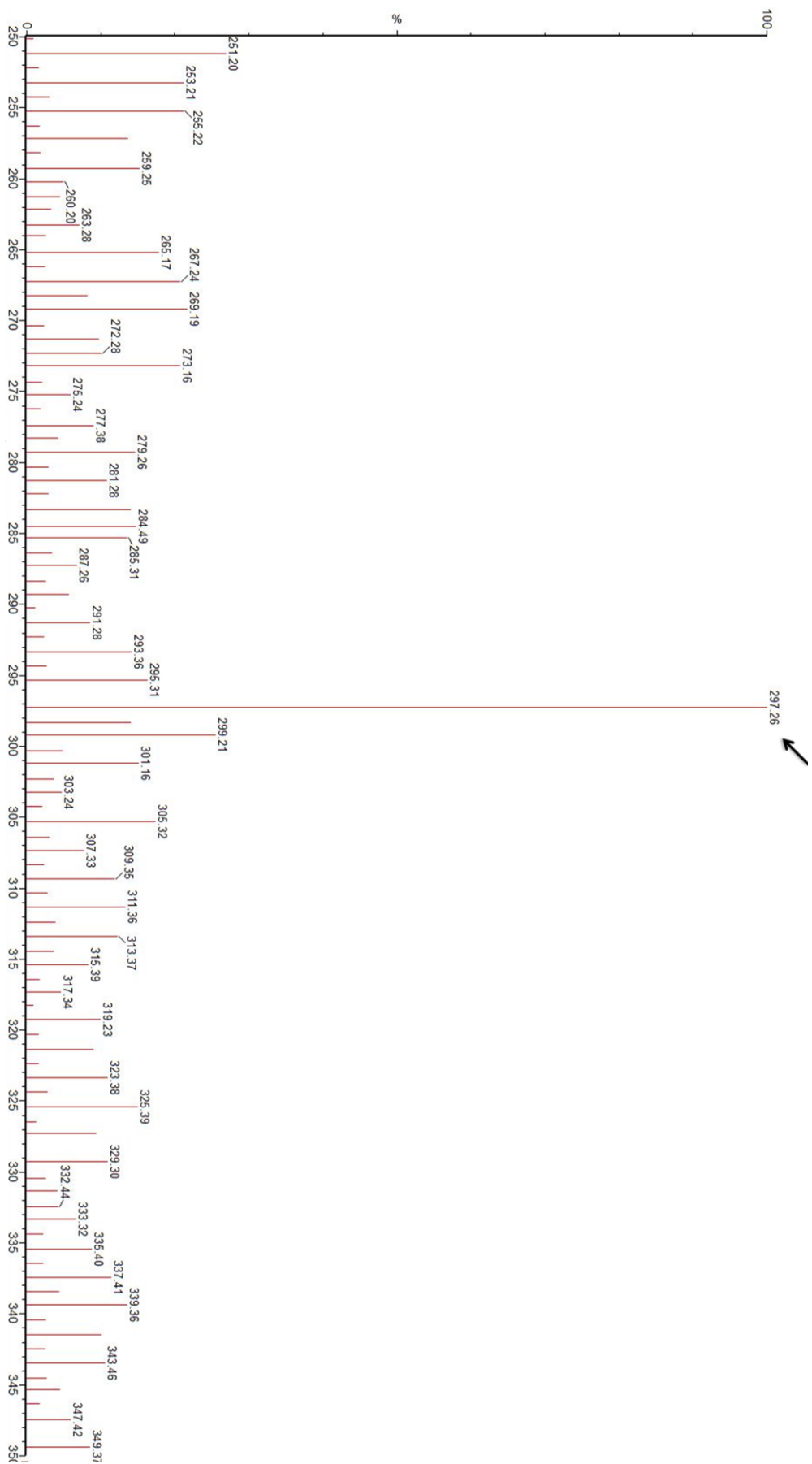
Figure 6.2.5: Full scan spectra for flunixin. Arrow indicates ion selected for analysis.

155
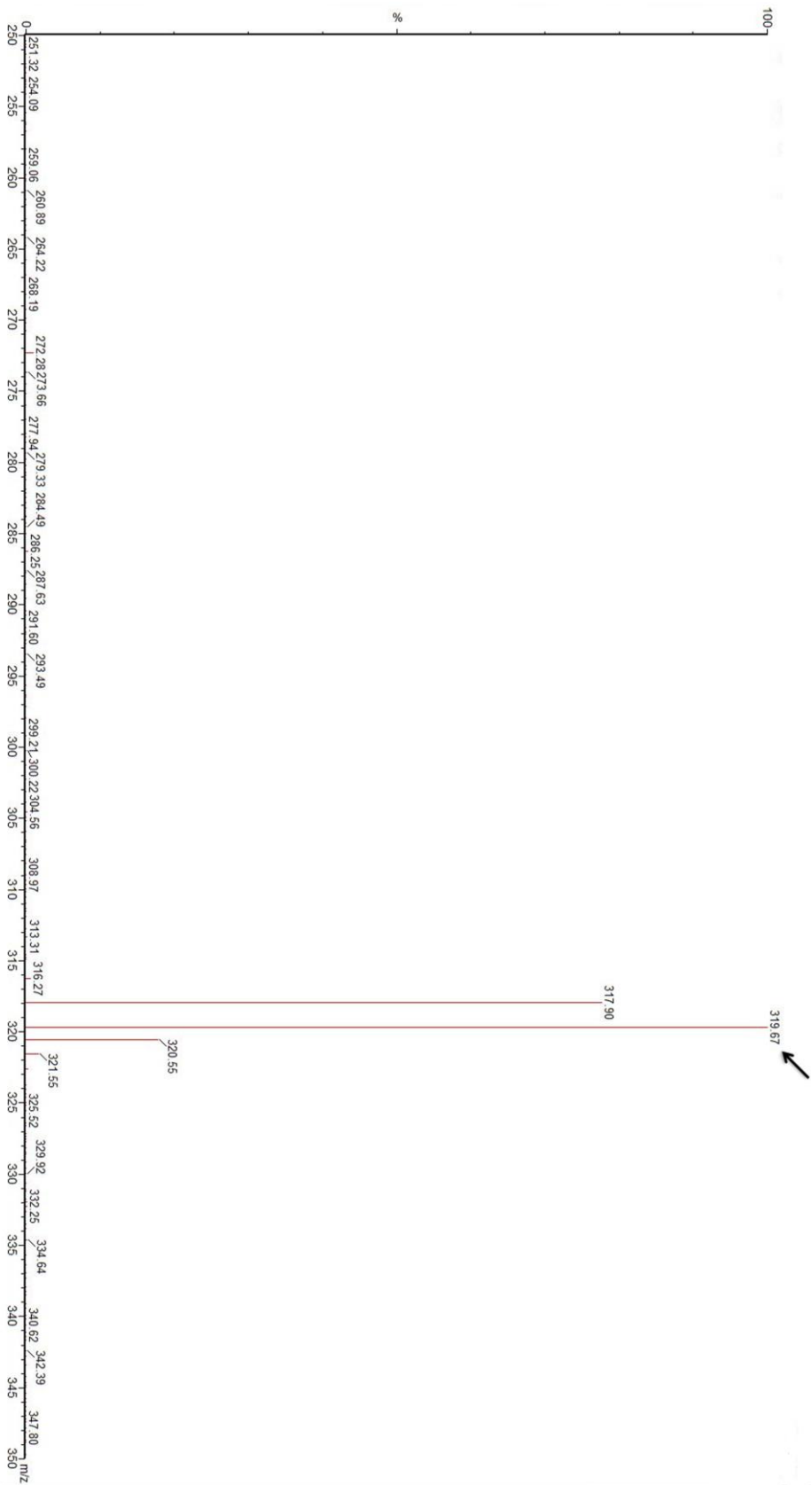
Figure 6.2.6: Full scan spectra for glycopyrrolate. Arrow indicates ion selected for analysis.
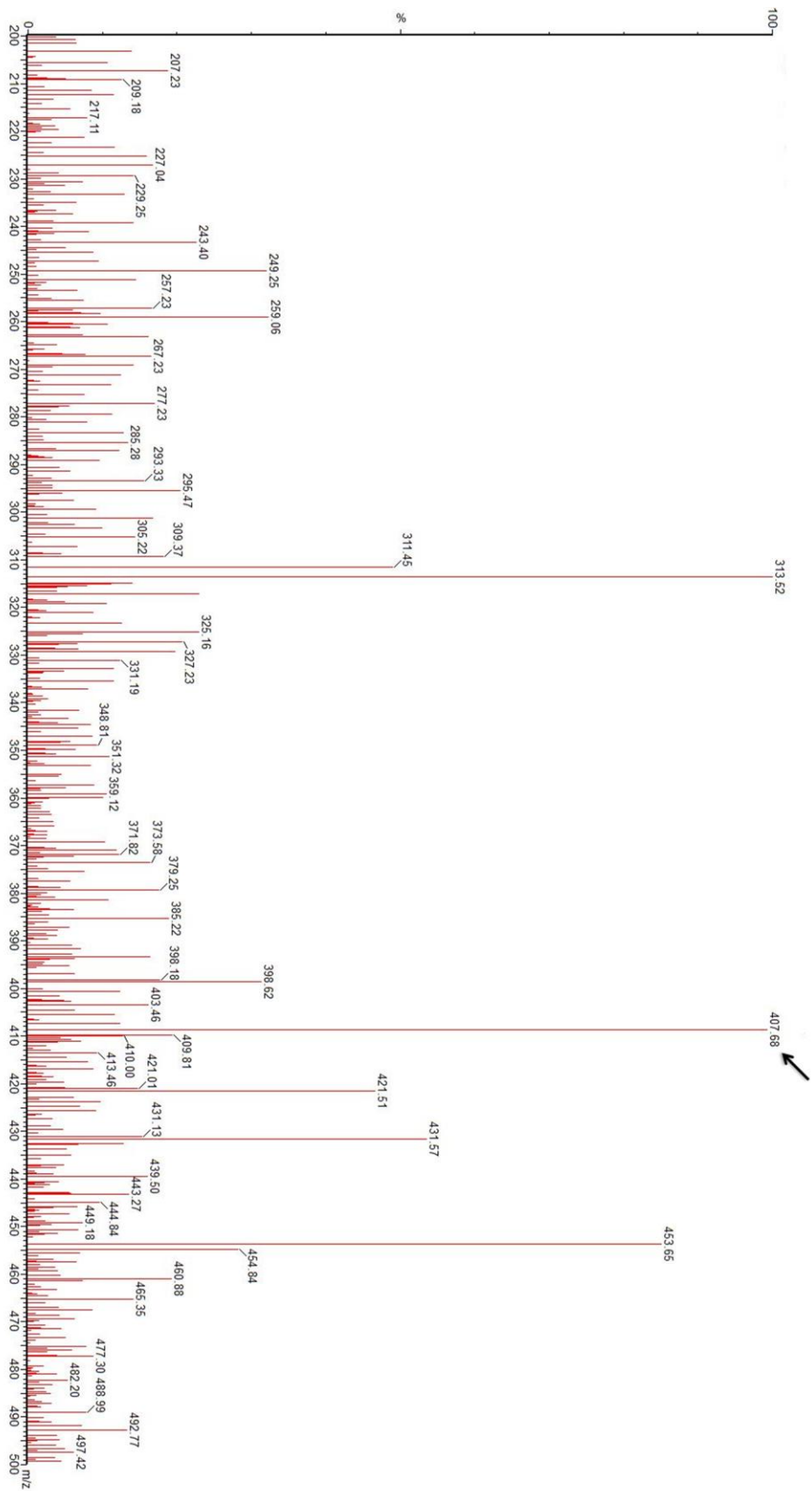
Figure 6.2.7: Full scan spectra for hydroxyzine. Arrow indicates ion selected for analysis.
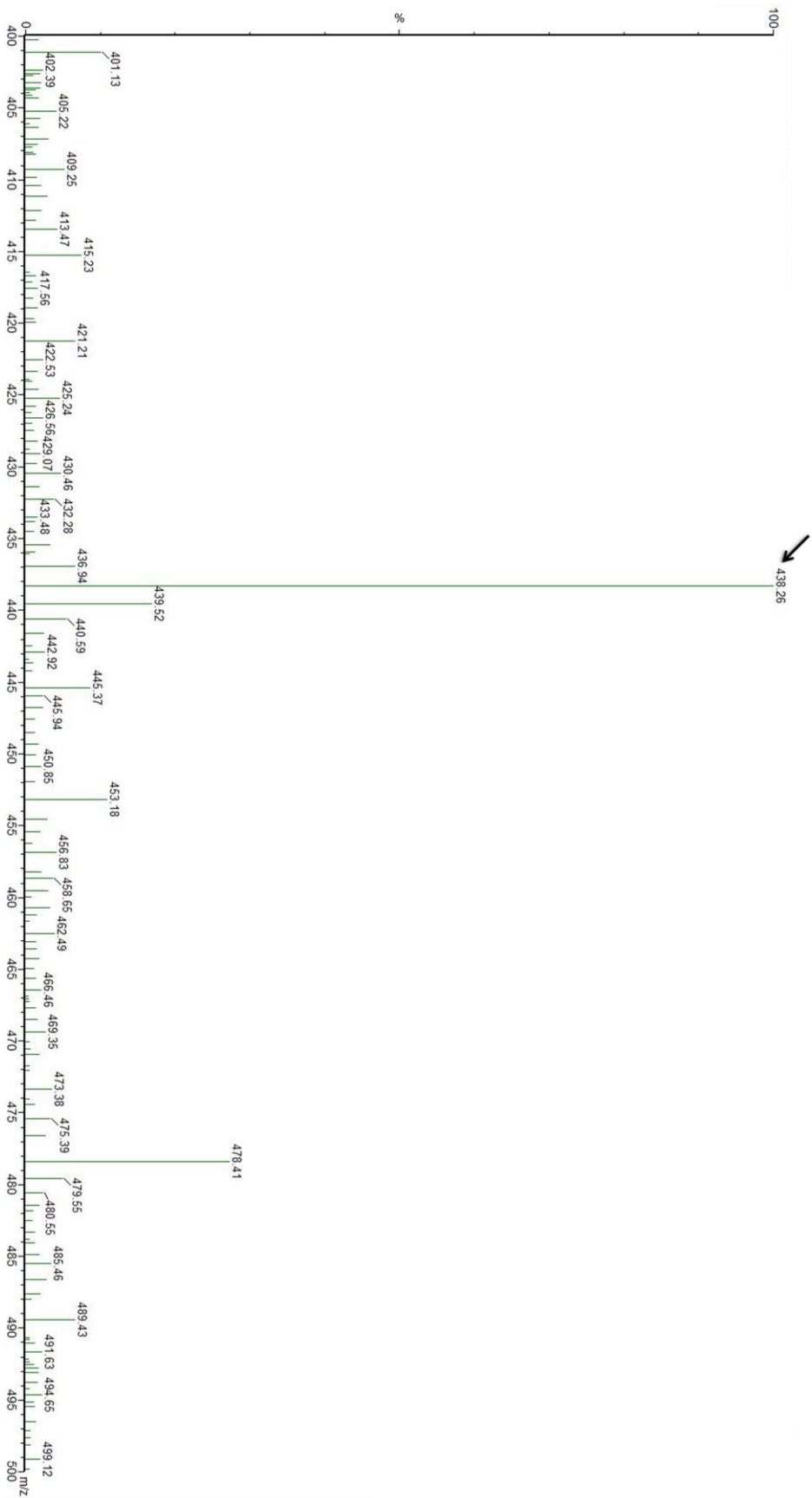
Figure 6.2.8: Full scan spectra for fluphenazine. Arrow indicates ion selected for analysis.
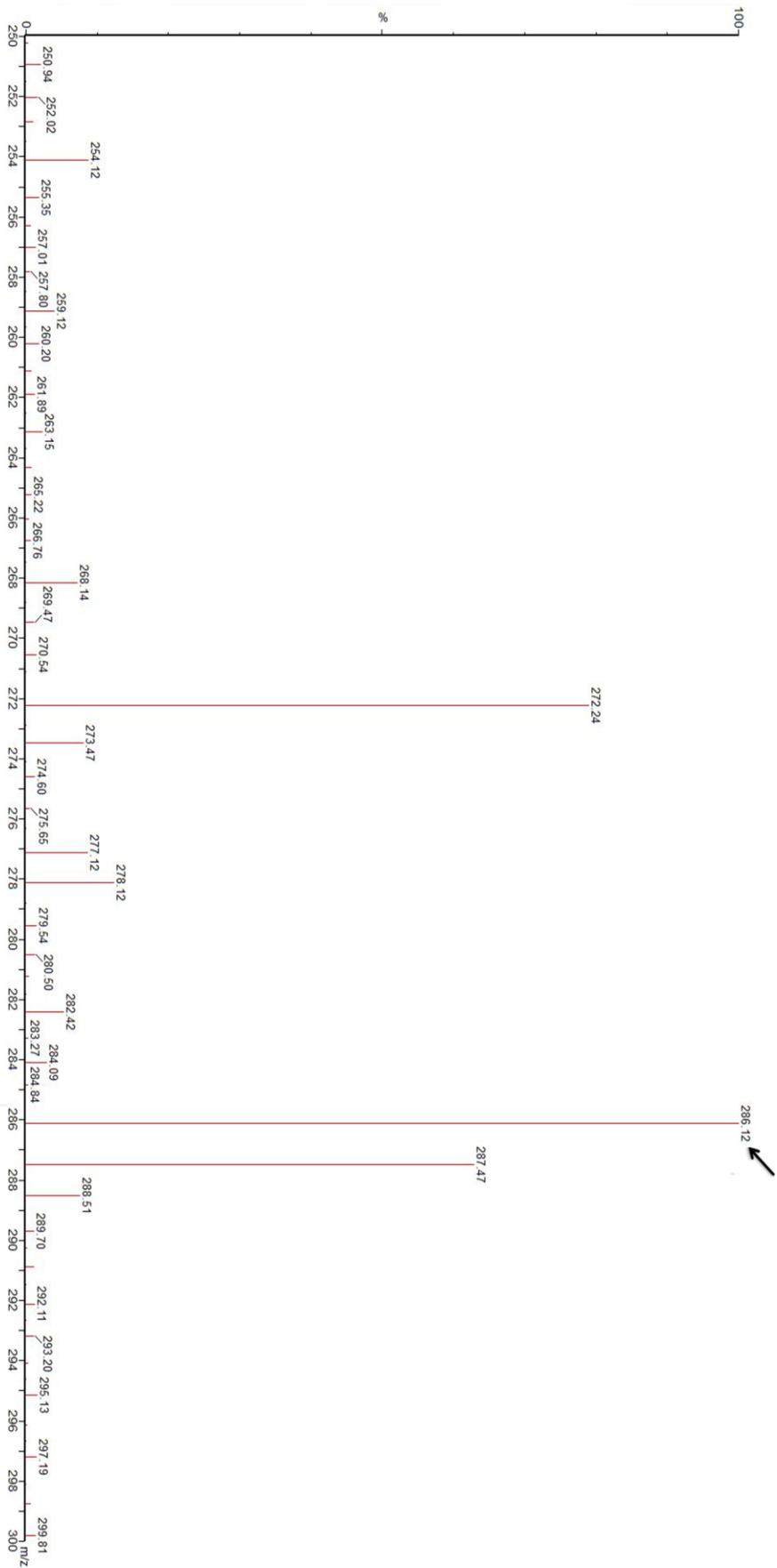
Figure 6.2.9: Full scan spectra for pyralamine. Arrow indicates ion selected for analysis.

159
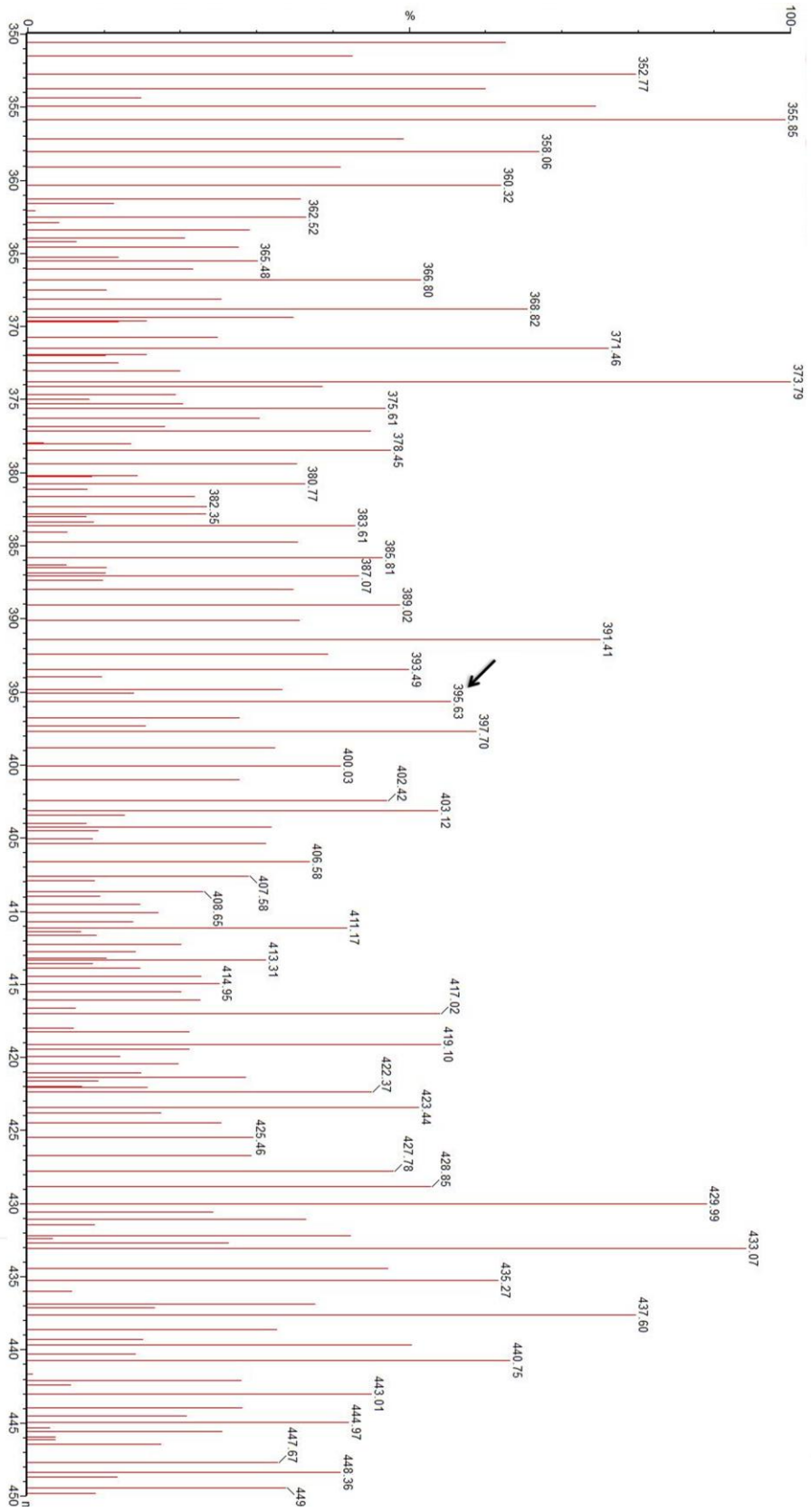
Figure 6.2.10: Full scan spectra for triamcinolone. Arrow indicates ion selected for analysis.

6.22: Preliminary assays

In order to determine whether the recombinant enzymes metabolised any of the selected drugs, preliminary screening assays were carried out using initial drug concentrations of 1µM and samples taken at 0 minutes, 30 minutes and 60 minutes after the addition of NADPH. Tables 6.2.3 and 6.2.3 display this data as a percentage of the mass spectrometry response at 0 minutes. For CYP2C92, diclofenac, flunixin, phenylbutazone and ketoprofen all showed decay at 30 and 60 minutes. For CYP2D50, the only drug that displayed this trend was dextromethorphan, which is a CYP2D6 substrate in human.

Drug	Drug % remaining after 30 mins	Drug % remaining after 60 mins
Dextromethorphan	N/A	N/A
Diclofenac	72	88
Flunixin	91	90
Ketoprofen	61	43
Phenylbutazone	27	27
Pyralamine	110	120
Hydroxyzine	47	69
Triamcinolone	N/A	N/A
Fluphenazine	80	98
Glycopyrrolate	78	85

Table 6.2.3: Results obtained from preliminary screening of all ten drugs with rCYP2C92. Samples were analysed at 0, 30 and 60 mins after the start of the reaction. Data was obtained in arbitrary units and converted to the percentage remaining after 30 and 60 minutes. N/A signifies that data was not obtained.

Drug	Drug % remaining after 30 mins	Drug % remaining after 60 mins
Dextromethorphan	91	85.3
Diclofenac	120	180
Flunixin	99	98
Ketoprofen	61	110
Phenylbutazone	55	120
Pyralamine	120	110
Hydroxyzine	160	73
Triamcinolone	59	95
Fluphenazine	100	100
Glycopyrrolate	110	130

Table 6.2.3: Results obtained from preliminary screening of all ten drugs with rCYP2D50.

Samples were analysed at 0, 30 and 60mins after the start of the reaction. Data was obtained in arbitrary units and converted to the percentage remaining after 30 and 60 minutes.

6.3: Diclofenac metabolism

6.3.1: Introduction

Diclofenac is one of the most frequently used NSAIDs in human and is effectively used for pain relief for various inflammatory disorders. It was introduced in 1979 and, like most NSAIDs, works via inhibition of the cyclooxygenase 1 and 2 enzymes (Gan, 2010). In horse, diclofenac has generally only received research attention in topical formulations (Ferrante *et al*, 2010, Schleining *et al,* 2008, Andreeta *et al,* 2011) and has only been approved for use in practice as a topical treatment (Reeder *et al*, 2009). Because of this, data relating to oral dosing is scarce although not absent. In a 2013 study, Azevedo *et al* compared oral doses of diclofenac with topical, finding good bioavailability with both routes of administration. Additionally, recombinant enzymes have been used to study diclofenac metabolism in the horse. DiMaio *et al*

(2009) looked at diclofenac activity with recombinant CYP2C92 by monitoring the concentration of 4-hydroxydiclofenac, which is a known metabolite of diclofenac in human (Gan, 2010), see figure 6.3. In this study, 4-hydroxydiclofenac was detected using both the recombinant system and when using equine liver microsomes, although metabolite formation was significantly slower (~20 fold) for both these systems compared to the human recombinant CYP2C9 system.
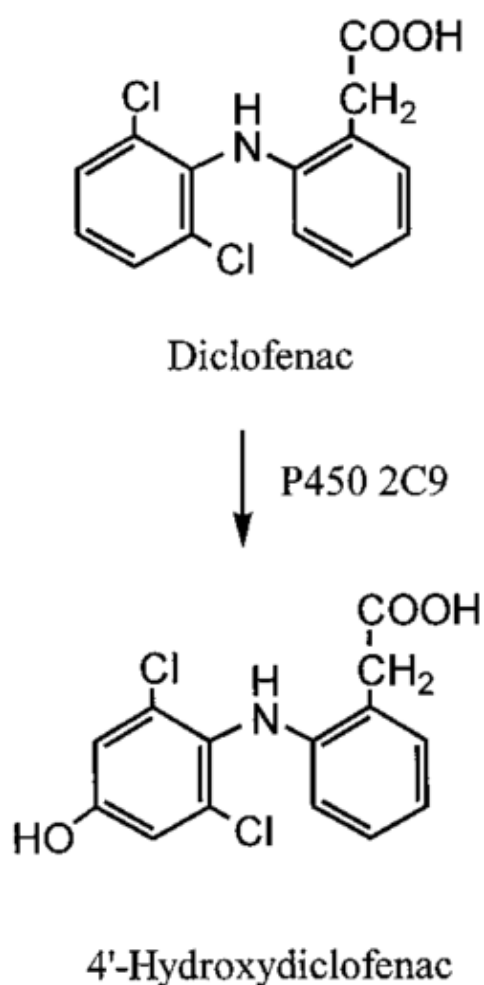
Figure 6.3: Metabolism of diclofenac by CYP2C9. Diclofenac is metabolised by CYP2C9 (in human) to form 4-hydroxydiclofenac via the addition of an hydroxyl group. Adapted from "Interaction of diclofenac and quinidine in monkeys: stimulation of diclofenac metabolism" by Tang, W, Stearns, R.A, Kwei, G.Y, Iliff, S.A, Miller, R.R, Egan, M.A,Yu, N.X, Dean, D.C, Kumar, S, Shou, M, Lin, J.H, Baillie, T.A, 2009, *The Journal of Pharmacology and Experimental Therapeutics*: 291(3), 1068–1074.

6.3.2: Inhibition of diclofenac metabolism by fluconazole

Fluconazole is an antifungal medication frequently used in both veterinary and human medicine (Latimer *et al*, 2001, Kunze *et al*, 1996). In human medicine, it is known to be a potent inhibitor of CYP2C9 (as well as CYP2C19 and CYP3A4), therefore care is needed if drugs metabolised by these enzyme are prescribed at the same time (Miners and Birkett, 1998). Significant drug interactions have been reported between fluconazole and a wide variety of CYP2C9 substrates including warfarin, some selective serotonin reuptake inhibitors (SSRIs), naproxen and diclofenac (Ogu and Maxa, 2000). Fluconazole has been shown to be a potent inhibitor of diclofenac metabolism in human, with a Ki (the concentration of inhibitor needed to decrease the maximal rate of reaction (V_{max}) by 50%) of 17 µM (Hargreaves *et al*, 1994). In the horse, interactions between the general anaesthetics ketamine and midazolam and fluconazole have been reported, with co-administration causing increased recovery time from sedation (Krein *et al*, 2014). In this study, only clinical signs were analysed thus the mechanism of inhibition was not investigated.

For the current study, diclofenac was used as a probe substrate to investigate its metabolism by recombinant equine CYP2C92 and compared to equine liver microsomes and recombinant human CYP2C9. Additionally, fluconazole was incubated with diclofenac in order to determine whether it acts as an inhibitor of this isoform in the horse.

6.4: Results

6.4.1: LCMS analysis of diclofenac and 4-hydroxydiclofenac

Diclofenac and 4-hydroxydiclofenac were monitored in order to detect enzyme activity. The ion of mass 296.19 at a retention time of 4.55 mins was used for diclofenac and the ion of mass 312.05 at a retention time of 4.12 mins was used to monitor apparent 4-hydroxydiclofenac activity (figure 6.4.1). Because of a lack of a authentic 4-hydroxydiclofenac stock, this ion was detected by monitoring incubation samples for an ion 16 daltons (oxygen insertion) larger than that for diclofenac.

6.4.2: Diclofenac metabolism by equine rCYP2C92

Figure 6.4.2 displays an example of the mass spectrometry response for diclofenac with rCYP2C92 at three time points (0, 30 and 60 minutes). Metabolism of diclofenac by CYP2C92 was evident across all concentrations (figure 6.4.3). Substrate enzyme inhibition appeared to be taking place from 100µM onwards and so two higher concentration assays were carried out at 200 µM and 250 µM.
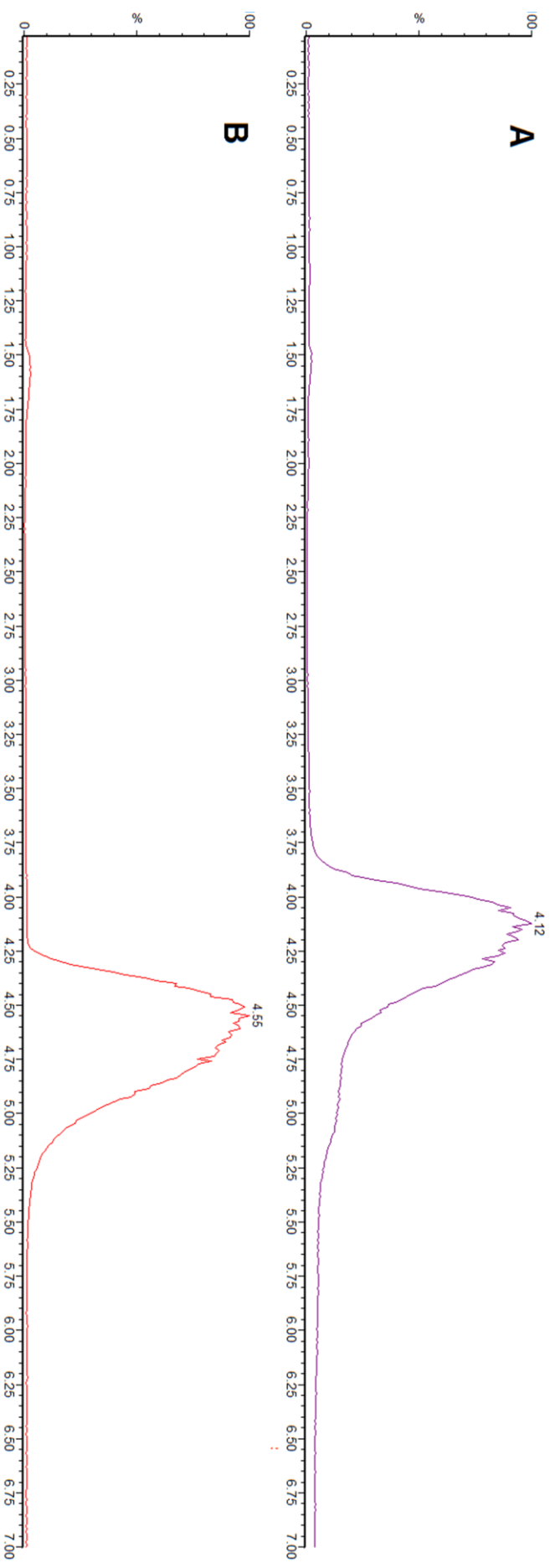
Figure 6.4.1: Chromatograms for diclofenac and 4-hydroxydiclofenac. Diclofenac had a retention time of 4.55 mins and 4-hydroxydiclofenac at a retention time of 4.12 minutes. Displayed in the figure are A: 4-hydroxydiclofenac and B: diclofenac, Due to lack of an authentic 4-hydroxydiclofenac stock, this ion was detected by monitoring for an ion ~16da higher than diclofenac in incubation samples.
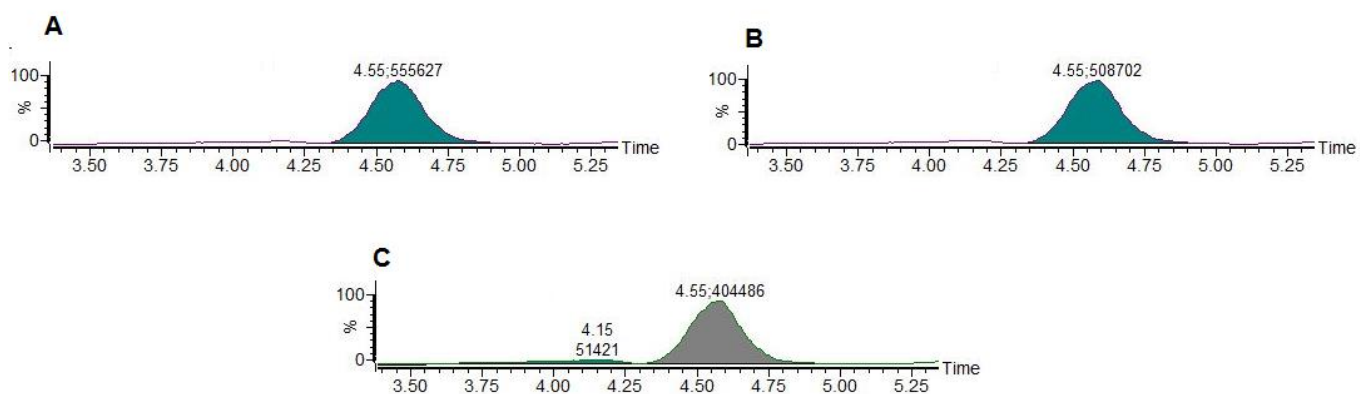
Figure 6.4.2: Chromatograms showing depletion of diclofenac by rCYP2C92 with time (A: 0 mins, B: 30 mins, C: 60 mins). Renention time is displayed on the Y axis of each chromatogram and the mass spectrometry response was calculated as the area under the curve. The retention time was 4.55 (mins).
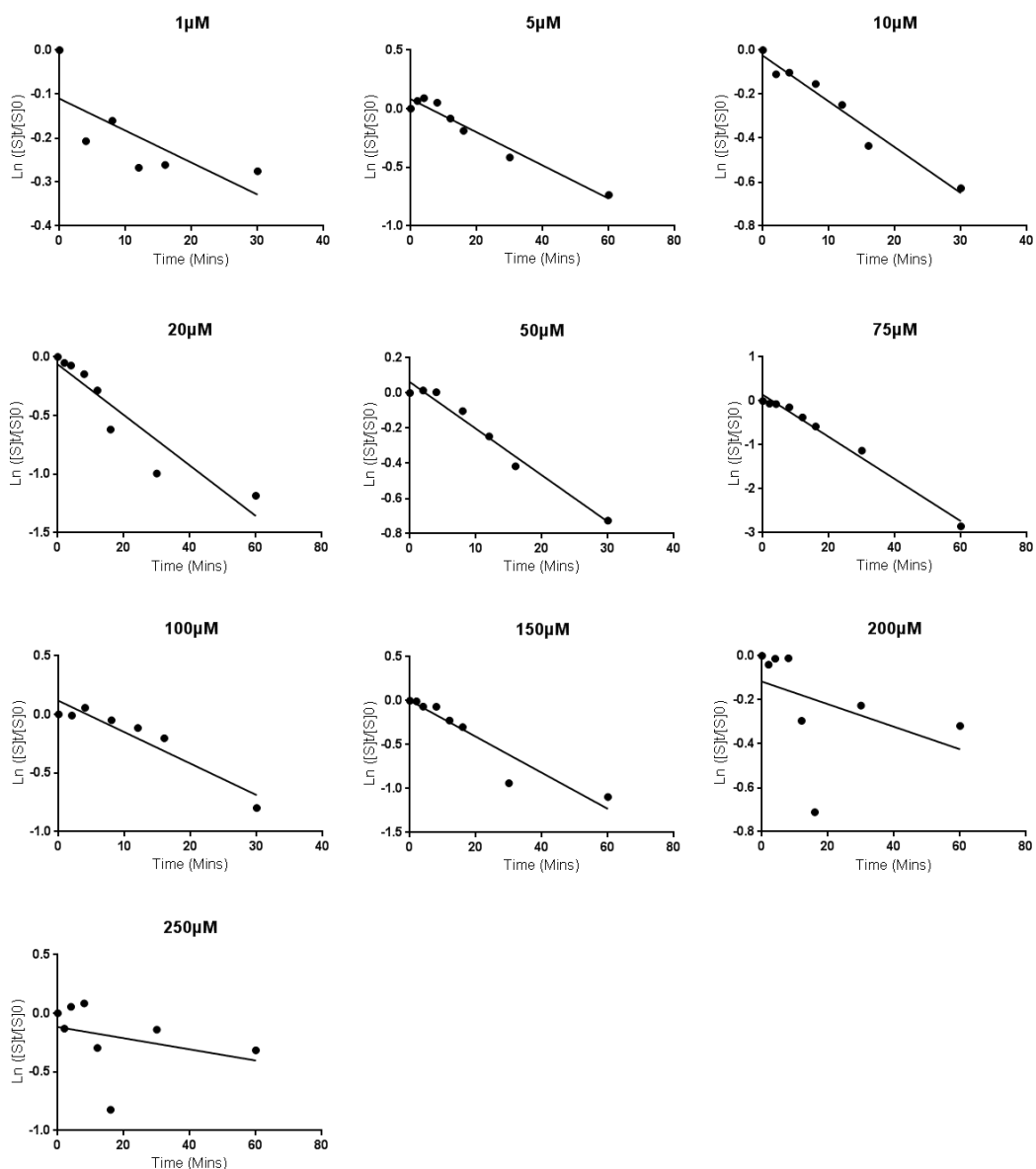
Figure 6.4.3: Data gathered using various diclofenac concentrations with rCYP2C92. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.4.1). Data was generated from one experiment.

A Michaelis-Menten substrate inhibition curve was fitted to the data according to equation 2 in section 6.1.3 (figure 6.4.4), however V_{max} and K_m could not be deconvoluted with any certainty and therefore the initial slope of the Michaelis-Menten curve was used to estimate a CL_{int} value of 3 µl/min/mg of protein (table 6.4.4).

Substrate concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	0.0073	0.0073	79
5	0.014	0.071	47
10	0.021	0.21	47
20	0.022	0.43	30
50	0.027	1.33	47
75	0.048	3.6	5.8
100	0.027	2.7	37
150	0.021	3.0	33
200	0.0051	1.0	72
250	0.0048	1.2	72

Table 6.4.1: K_{dep} and initial rate values for rCYP2C92 with diclofenac. Substrate concentrations ranged from 1-250 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).
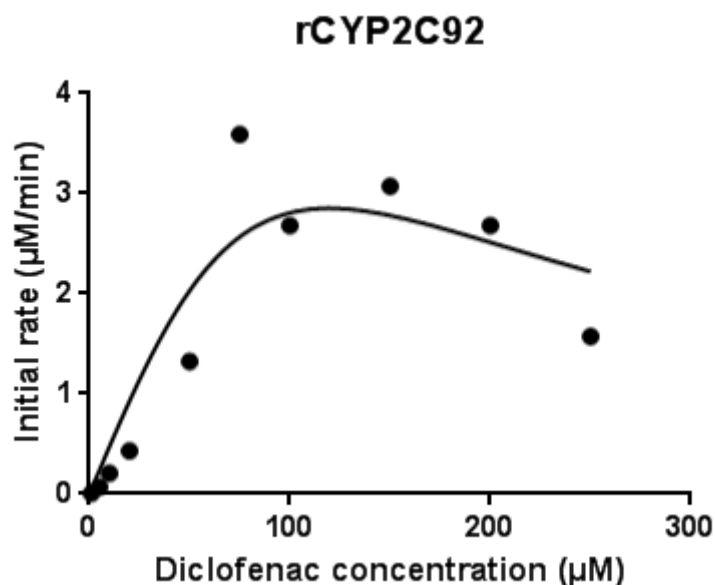
Figure 6.4.4: Michaelis-Menten Substrate inhibition curve for rCYP2C92 with diclofenac. Initial rate is plotted against substrate concentration.

For metabolite appearance, 4-hydroxydiclofenac activity was measured by monitoring for an ion of mass 312.05, however no significant production of this metabolite could be detected at any substrate concentration.

6.4.3: Diclofenac metabolism by equine liver microsomes

Metabolism was evident for equine liver microsomes with diclofenac across all concentrations, except at 1µM (figure 6.4.5).
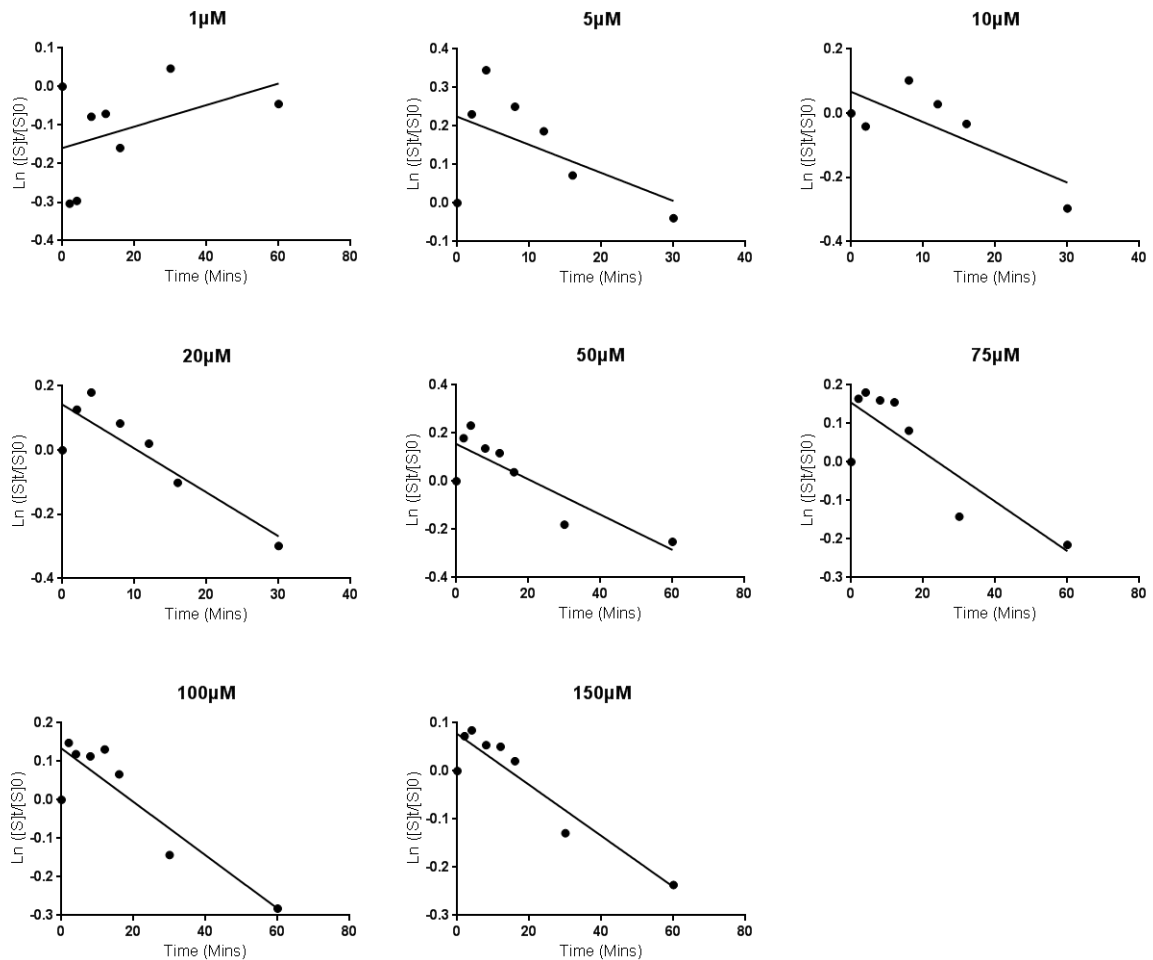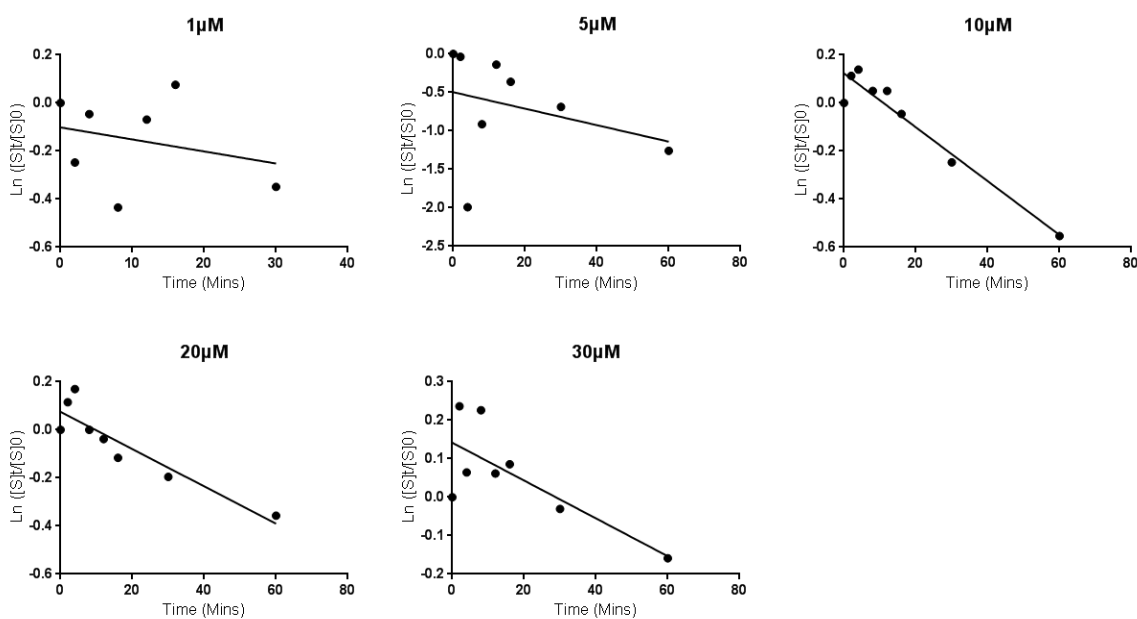

Figure 6.4.5: Data gathered using various diclofenac concentrations with equine microsomes. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.4.2). Data was generated from one experiment.

Substrate concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	-0.0028	0.0028	95
5	0.0073	0.037	97
10	0.0094	0.094	68
20	0.014	0.27	65
50	0.0073	0.37	77
75	0.0064	0.48	80
100	0.0069	0.69	75
150	0.0053	0.80	78

Table 6.4.2: K_{dep} and initial rate values for equine liver microsomes with diclofenac. Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

A partial Michealis-Menten curve was successfully fitted to the depletion data (figure 6.4.6). K_m was estimated at 145 µM and V_{max} at 1.57 µM/min. CL_{int} was calculated as 17.75 µl/min/pmol p450 (table 6.4.4).
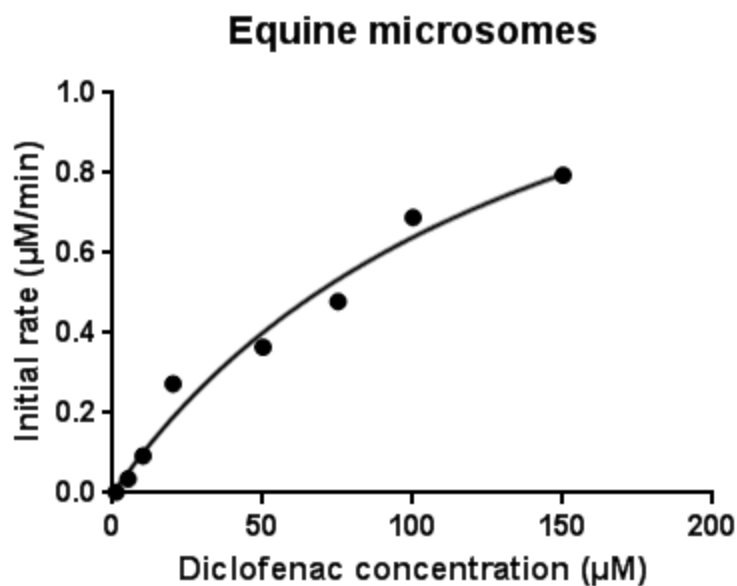
Figure 6.4.6: Michealis-Menten curve for equine liver microsomes with diclofenac. Initial rate (µM/min) is plotted against substrate concentration.

In addition to monitoring diclofenac depletion, the apparent production of 4-hydroxydiclofenac was monitored (figure 6.4.7). Due to absence of an authentic stock solution for 4-hydroxydiclofenac, the concentration of the metabolite could not be determined. The mass spectrometry response for the metabolite (obtained in arbitrary units, and calculated based on the area under the chromatographic peak) was plotted against time (figure 6.4.7) to generate the initial rate in arbitrary units and successfully fitted to a Michaelis-Menten curve (figure 6.4.8). K_m was estimated at 53 µM with a standard error (SE) of 28.
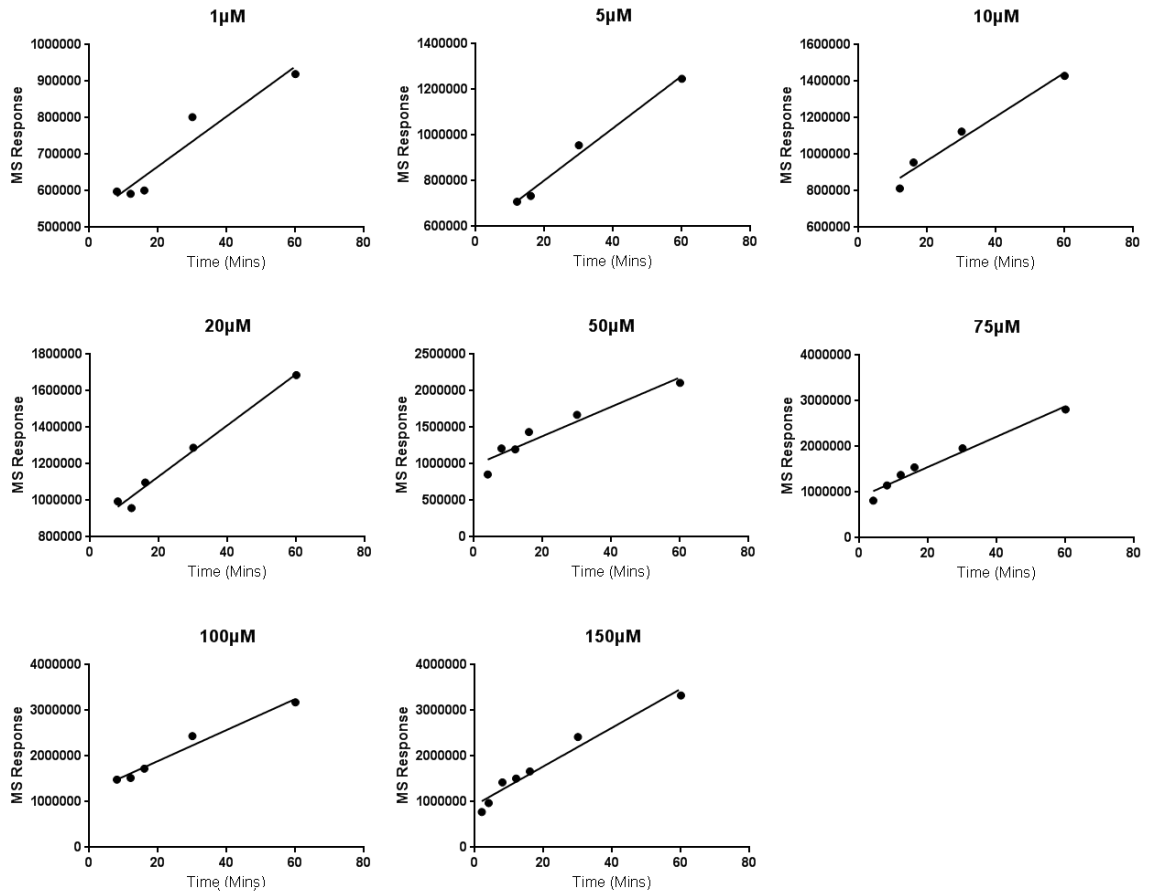
Figure 6.4.7: Production of 4-hydroxydiclofenac with equine liver microsomes at various diclofenac concentrations. MS response (in arbitrary units) is plotted against time.
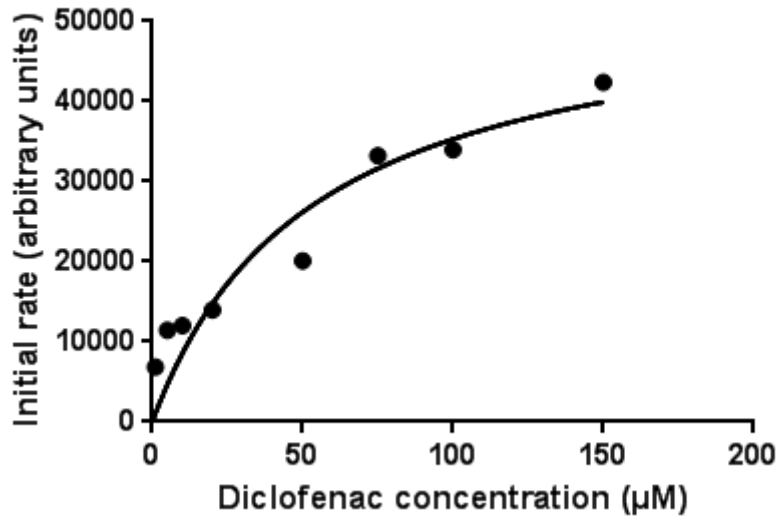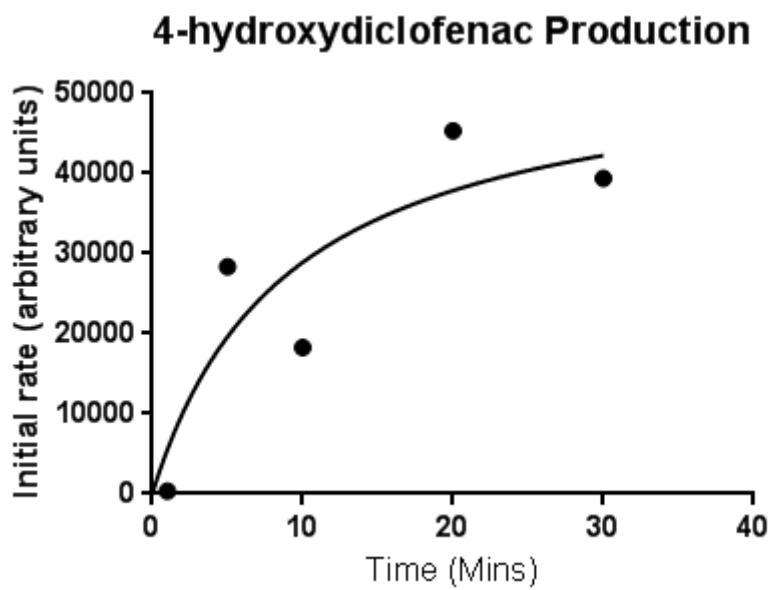
Figure 6.4.8: Michaelis-Menten graph using 4-hydroxydiclofenac production with equine liver microsomes. Substrate concentration is plotted against the mass spectrometry response (in arbitrary units).

6.4.4: Diclofenac metabolism by human rCYP2C9

Since kinetic parameters for diclofenac with human CYP2C9 are already known (Bort *et al*, 1999), fewer concentrations were used (figure 6.4.9) and data was successfully fitted using a Michaelis-Menten curve (figure 6.4.10). K_m and V_{max} estimations were 12.24 µM and 0.23 µM/min respectively, with a CL_{int} of 1.88 µl/min/pmol P450.

Figure 6.4.9: Data gathered using various diclofenac concentrations with rCYP2C9. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.4.3). Data was generated from one experiment.

Substrate concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	0.0050	0.0050	70
5	0.011	0.054	28
10	0.011	0.11	57
20	0.0078	0.16	69
30	0.0049	0.15	85

Table 6.4.3: K_{dep} and initial rate values for rCYP2C9 with diclofenac. Substrate concentrations ranged from 1-30 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).
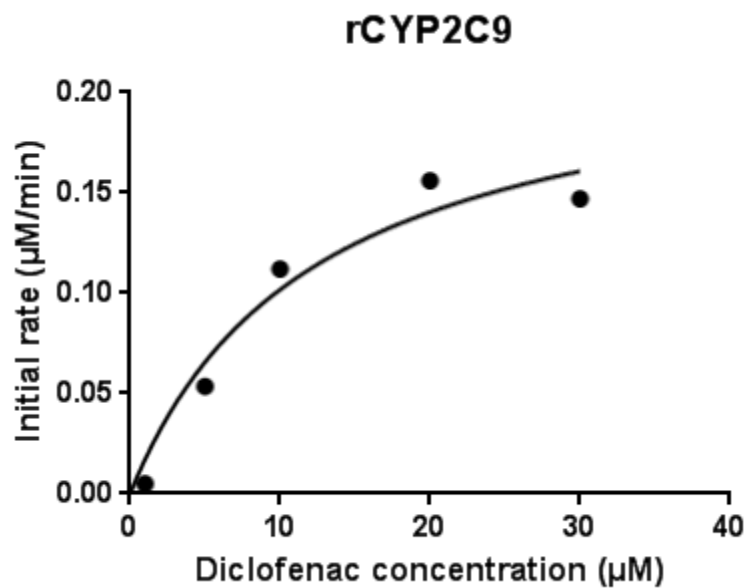
Figure 6.4.10: Michaelis-Menten curve for human recombinant CYP2C9 with diclofenac. Initial rate (in μM/min) is plotted against substrate concentration.

For metabolite production, apparent 4-hydroxydiclofenac production was monitored and mass spectrometry (MS) response vs time was plotted in order to determine the initial rate (figure 6.4.11). A Michaelis-Menten curve was successfully fitted to this data (figure 6.4.12) with K_m estimated at 9.023 μM with a standard error of 9.623.
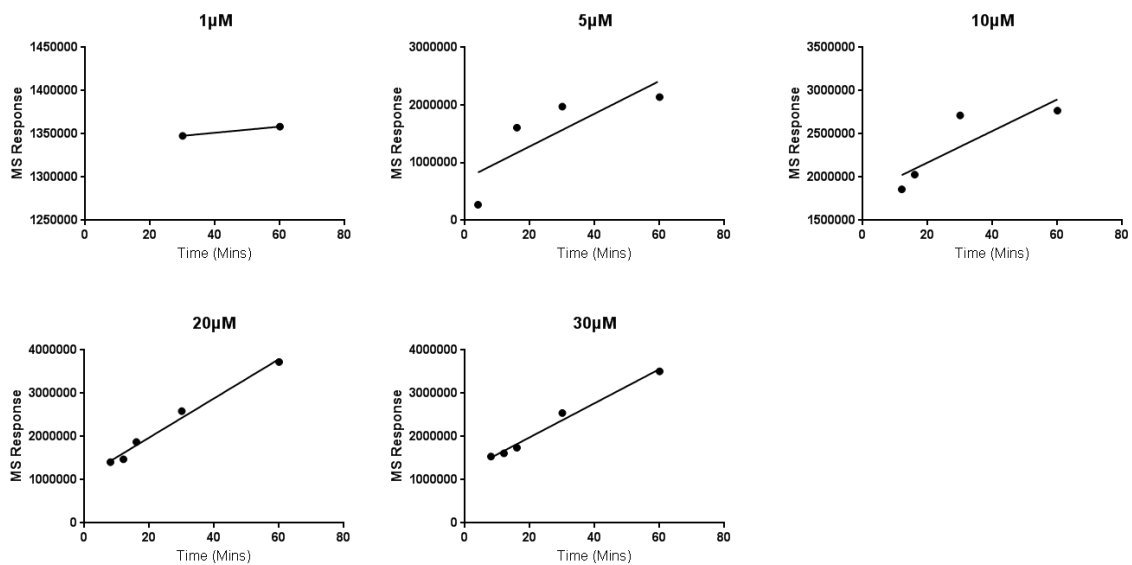
Figure 6.4.11: Production of 4-hydroxydiclofenac with human rCYP2C9 at various diclofenac concentrations. MS response is plotted against time.

Figure 6.4.12: Michaelis-Menten graph of 4-hydroxydiclofenac production with human rCYP2C9 supersomes. Substrate concentration is plotted against the mass spectrometry response (in arbitrary units).

6.4.5: Summary

Table 6.4.4 summarises the depletion data gathered from all three enzyme systems with diclofenac. For apparent 4-hydroxydiclofenac appearance, a K_m of 53.45 µM was obtained for equine liver microsomes and a K_m of 9.023 µM for human rCYP2C9. rCYP2C92 showed substrate auto-inhibition therefore only CL_{int} was obtained.

Enzyme system	K_m [1]	K_m SE[1]	V_{max} [2]	V_{max} SE [2]	CL_{int} [3]
rCYP2C92	N/A	N/A	N/A	N/A	3.00
rCYP2C9	12.24	6.28	0.23	0.02	1.88
Equine Microsomes	145.5	56.17	1.57	0.36	17.75

Table 6.4.4: Summary K_m, V_{max} and Intrinsic clearance (CL_{int}) values for all three enzyme systems with diclofenac. Units = [1] µM, [2] µM/min, [3] µl/min/pmol P450 (µl/min/mg protein for CYP2C92). N/A signifies the value could not be obtained. SE = standard error.

6.4.6: Diclofenac inhibition

Equine rCYP2C92 and equine microsomes were tested against fluconazole, a potent inhibitor of human CYP2C, with diclofenac as the substrate. Fluconazone concentrations ranged from 0-75 µM. Figures 6.4.13 and 6.4.14 display the natural logarithm transformed graphs of this data, with clear substrate depletion evident across all fluconazole concentrations.
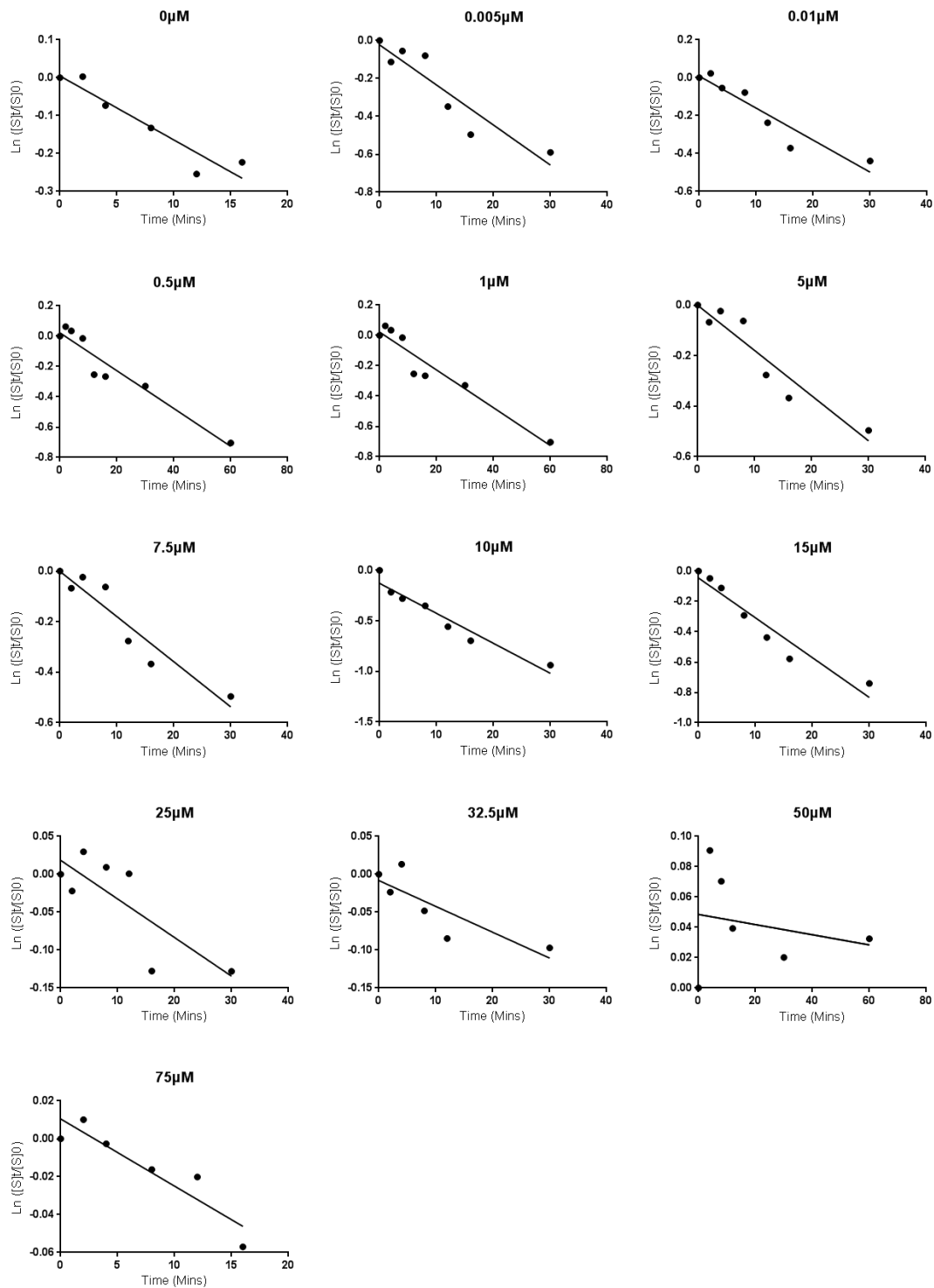
Figure 6.4.13: Data gathered using various fluconazole concentrations with rCYP2C92 and diclofenac as the substrate. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line. Data was generated from one experiment.
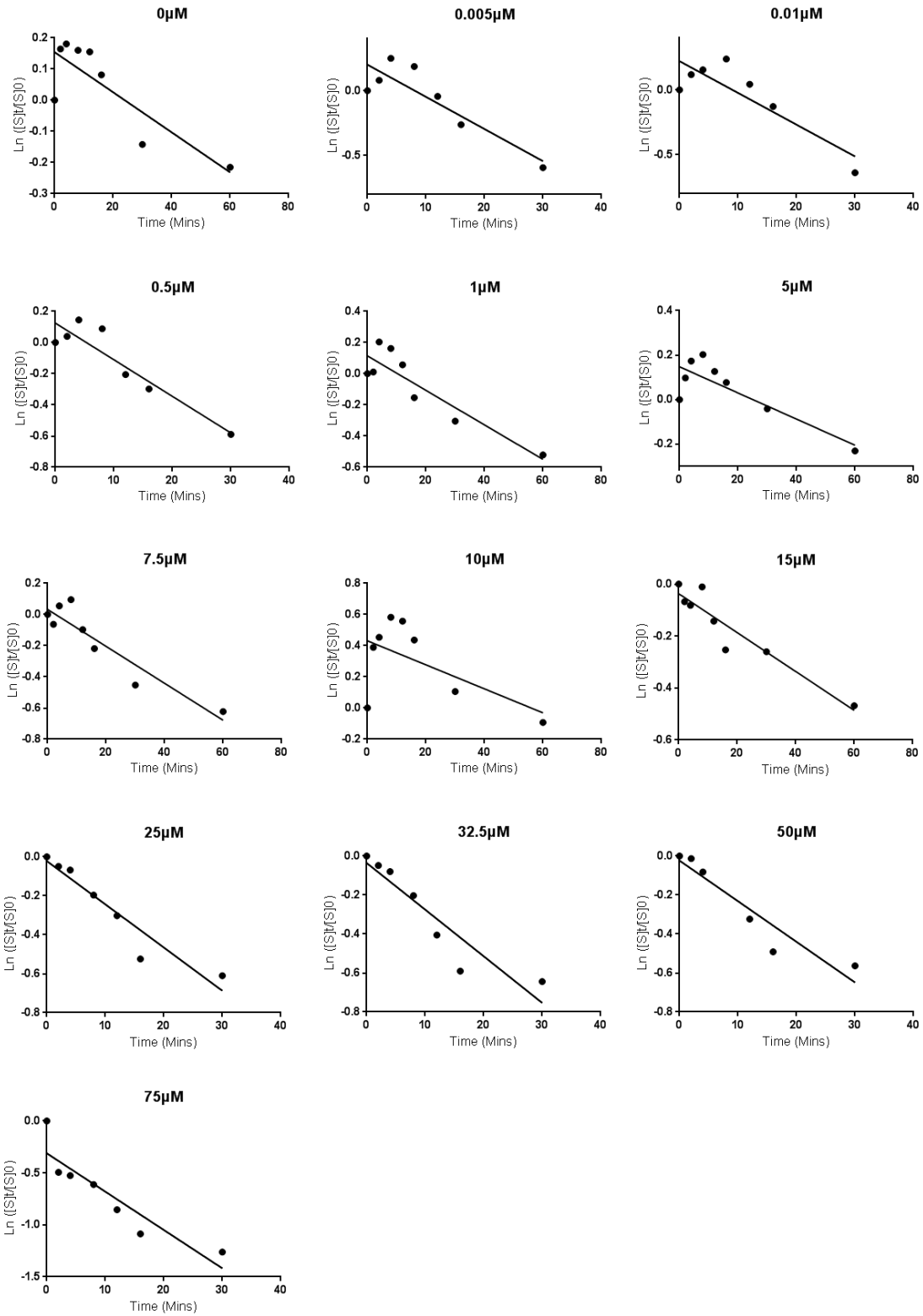
Figure 6.4.14: Data gathered using various fluconazole concentrations with equine microsomes and diclofenac as the substrate. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line. Data was generated from one experiment.

Percentage inhibition was calculated and graphs were plotted (figure 6.4.15). No

inhibition trend was evident therefore Ki could be calculated.
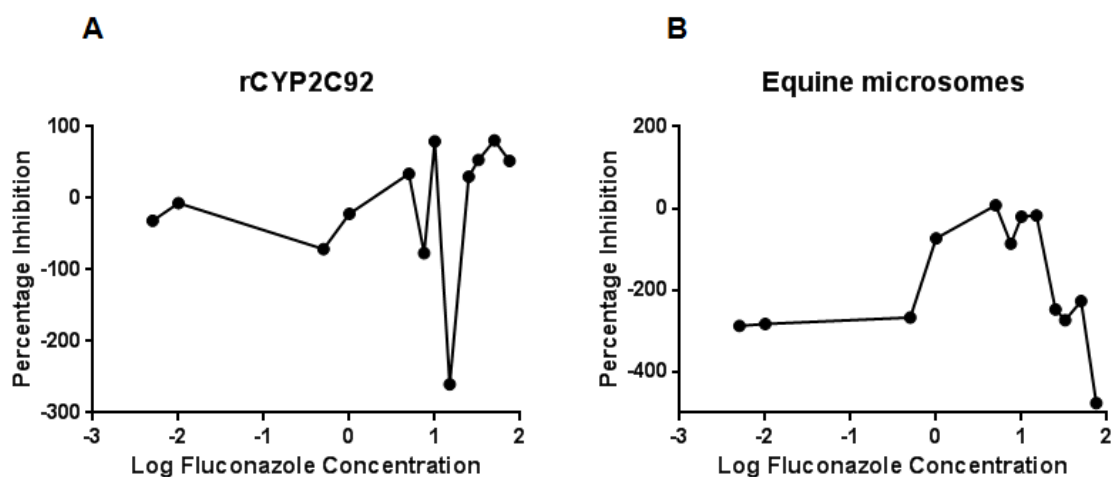
Figure 6.4.15: Percentage inhibition for CYP2C92 (A) and equine microsomes (B) using fluconazole and diclofenac. Fluconazole concentrations were logged (base 10) and plotted against percentage inhibition (normalised using the 0 µM concentration).

6.5: Ketoprofen metabolism

6.5.1: Introduction

Ketoprofen, like other NSAIDs, works via the inhibition of COX-1 and COX-2 (Grubb

et al, 1999). It is widely used in human and veterinary medicine for a variety of

musculoskeletal disorders and is generally used in the racemate form (Scarth *et al*,

2011). Unlike most NSAIDs however, ketoprofen is not majorly metabolised by

P450s in human, although it does have a P450 pathway, which converts it into

hydroxyketoprofen and hydroxybenzolketoprofen (Alkatheeri *et al*, 1999, Skordi *et al*,

2004). Its major pathway in human (accounting for 80% of metabolism) is phase II

metabolism (Grubb et al, 1999). In mice and rats, ketoprofen is metabolised to a much greater extent by P450s (Yamasaki *et al*, 2010). When metabolised by P450s, CYP2C9 is the P450 isoform involved (Zhou *et al*, 2009a).

6.5.2: Ketoprofen in equine medicine

Compared with other NSAIDs, ketoprofen has a higher safety margin and lower toxicity in the horse (Rehman *et al*, 2012). Differences in the rate of elimination of the S(+) and R(-) isomers have been found in horse (which are combined to create the racemate formulation), with the R(-) form being eliminated substantially faster (Verde *et al*, 2001).

As far as phase I metabolism is concerned, hydroxybenzolketoprofen has been detected in equine urine (Benoit *et al*, 1992) as has hydroxyketoprofen (Brink *et al*, 1998). No attempts to characterise the enzyme isoforms involved in phase I metabolism of ketoprofen in the horse have been made to date. Analysis of ketoprofen metabolism generally involves looking for the parent drug itself rather than for the hydroxylated metabolite (Baeyens *et al*, 1999). During the current study, phase I metabolism of ketoprofen was investigated by using recombinant equine CYP2C92 and compared to equine liver microsomes and recombinant human CYP2C9.

6.6: Results

6.6.1: LCMS analysis of ketoprofen

To monitor for ketoprofen metabolism, the ion of mass 336.63 was used, coming out at a retention time of 4.56 minutes (figure 6.6.1). No authentic metabolite of ketoprofen was available so only the parent drug was monitored.
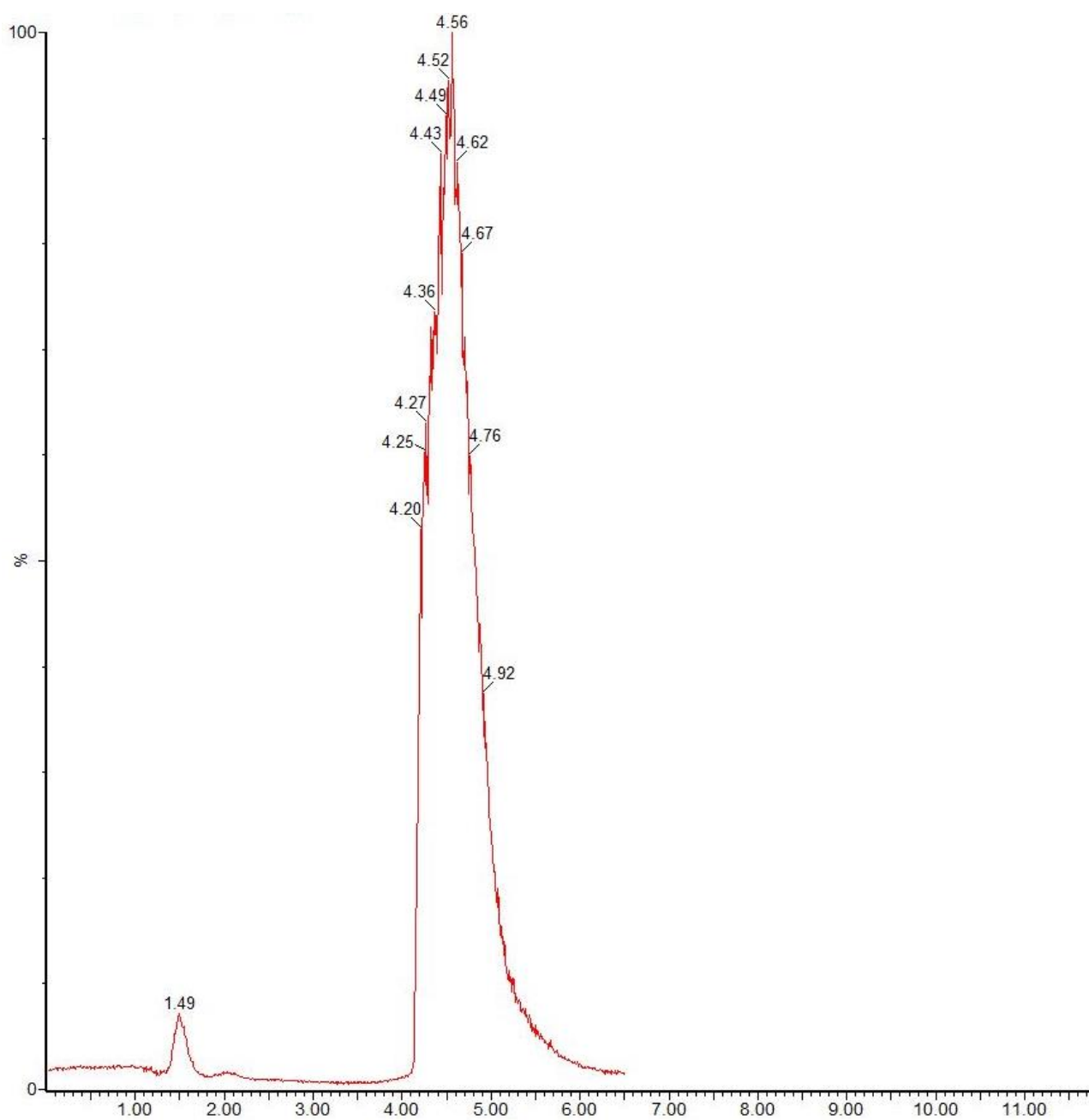
Figure 6.6.1: Single ion chromatogram for ketoprofen. The ion of mass 332.63 (Ketoprofen +

formic acid + methanol) was used to monitor ketoprofen depletion. Retention time was 4.56 minutes.

6.6.2: Ketoprofen Metabolism by Equine CYP2C92

There appeared to be metabolism of ketoprofen at the lower substrate concentrations, with substrate levels being noticeably depleted at 1 µM and 5 µM. By 10 µM metabolism becomes negligible (figure 6.6.2) with no evident depletion trend thereafter.
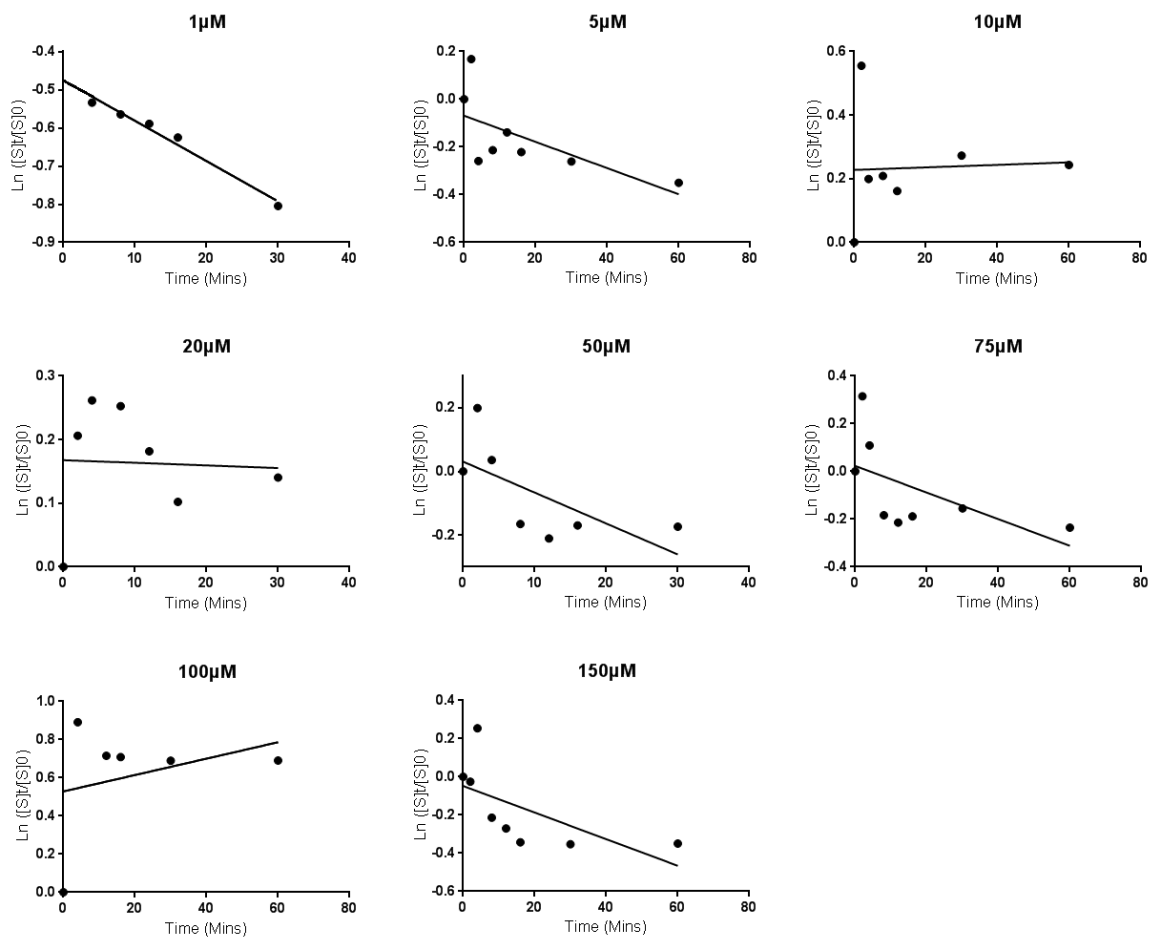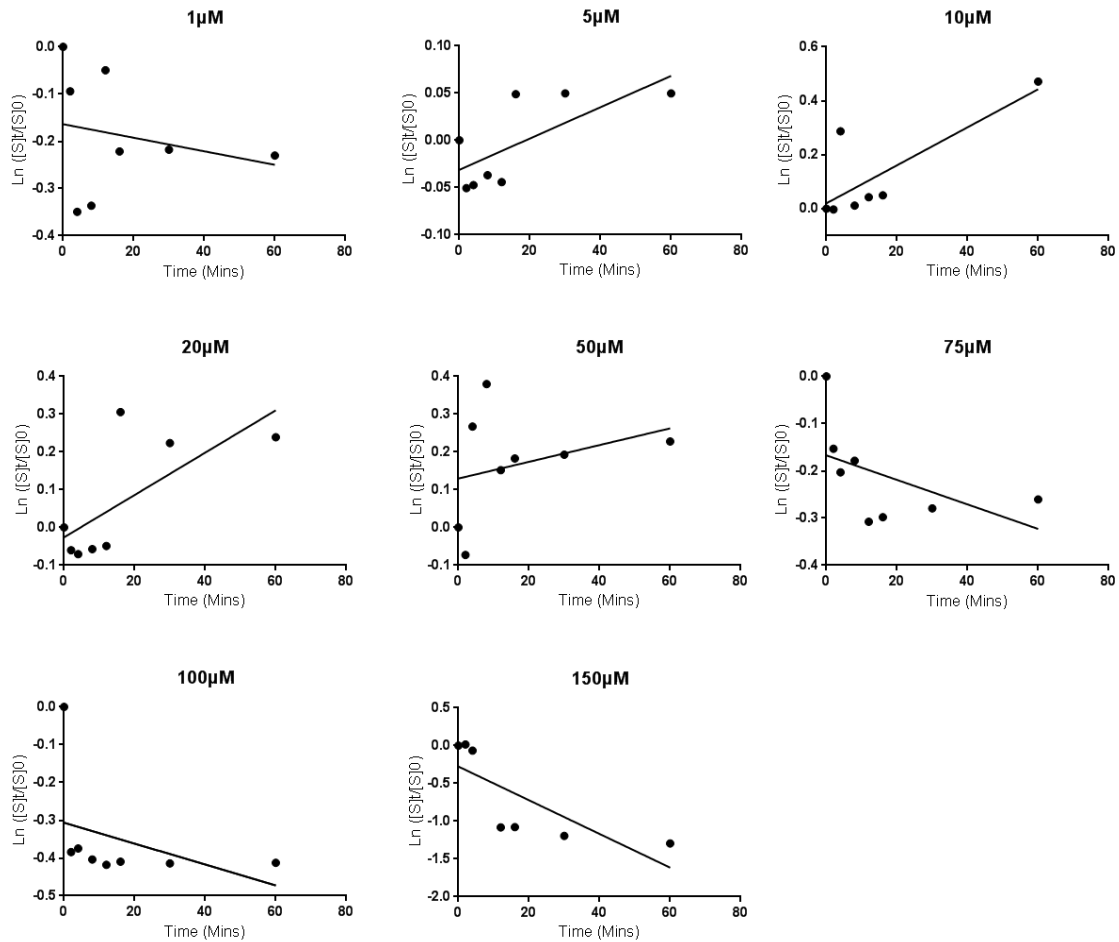

Figure 6.6.2: Metabolism of ketoprofen by rCYP2C92. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.6.1). Data was generated from one experiment.

A Michealis-Menten curve could not be fitted to the data therefore K_m, V_{max} and CL_{int} could not be obtained.

Ketoprofen Concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	0.011	0.011	60
5	0.0055	0.028	70
10	0.00040	0.0040	130
20	0.00040	0.0080	120
50	0.0097	0.49	82
75	0.0056	0.42	79
100	-0.0043	0.43	200
150	0.0050	0.75	70

Table 6.6.1: Initial rate and depletion constant values for equine rCYP2C92 with ketoprofen.

Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

6.6.3: Ketoprofen metabolism by equine liver microsomes

At 1µm there appeared to be metabolism of ketoprofen by equine microsomes (figure 6.6.4) however with increasing concentration no detectable metabolism was noted.
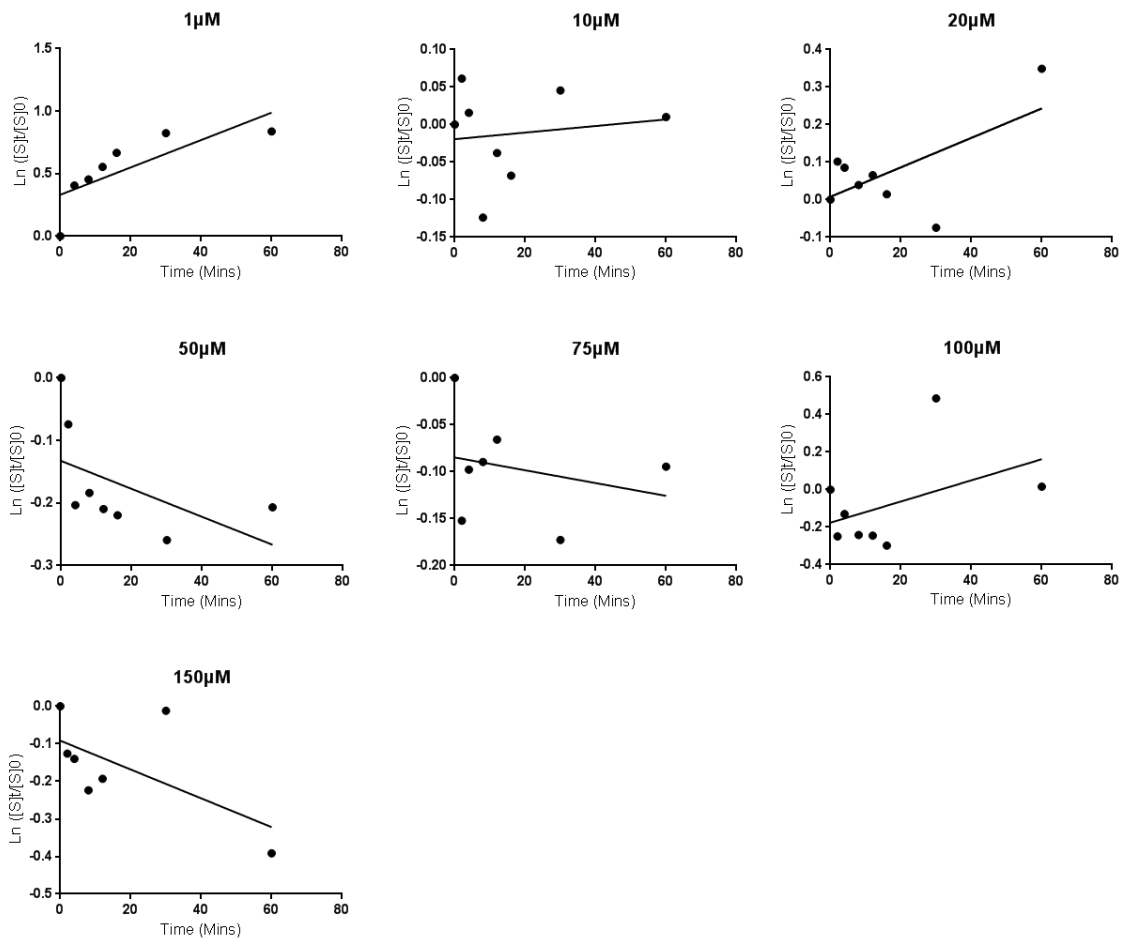
Figure 6.6.4: Metabolism of ketoprofen by equine liver microsomes. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.6.2). Data was generated from one experiment.

Due to a lack of apparent metabolism of ketoprofen across most concentrations by equine liver microsomes, a Michaelis-Menten analysis was not carried out.

Ketoprofen Concentration (µM)	K_{dep} (/min)	v_0 (uM/min)	% drug remaining after 60 mins
1	0.0014	0.0014	79
5	-0.0017	0.0085	110
10	-0.0070	0.070	160
20	-0.0056	0.11	130
50	-0.0022	0.11	130
75	0.0026	0.19	77
100	0.0028	0.28	66
150	0.022	3.35	27

Table 6.6.2: Initial rate and depletion constant values for equine liver microsomes with ketoprofen. Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

6.6.4: Metabolism of ketoprofen by human rCYP2C9

No substrate depletion was evident for the human CYP2C9 isoform with ketoprofen (figure 6.6.6) therefore K_m, V_{max} and CL_{int} could not be calculated.

Figure 6.6.6: Metabolism of ketoprofen by human CYP2C9. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. Kdep is represented by the slope of the line (refer to table 6.6.3). Data was generated from one experiment.

Ketoprofen Concentration (μM)	K_{dep} (/min)	v_0 (uM/min)	% drug remaining after 60 mins
1	-0.0064	0.0064	230
5	N/A	N/A	73
10	0.00030	0.0030	100
20	-0.00020	0.0040	140
50	0.00080	0.040	81
75	0.037	2.8	90
100	-0.0038	0.38	100
150	0.0021	0.32	67

Table 6.6.3: Initial rate values for human rCYP2C9 with ketoprofen. Substrate concentrations ranged from 1-150 μM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

6.7: Flunixin metabolism

6.7.1: Introduction

Flunixin is one of the most frequently prescribed veterinary NSAIDs and is one of the most potent used in the horse (Beretta *et al*, 2005). It has been well-characterised in the horse and is used for, in addition to muscoskeletal disorders, prevention of endotoxic shock and to alleviate symptoms of colic (Carrick *et al*, 1989). It can be administered orally, intramuscularly or intravenously (Pellegrini-Masini *et al*, 2004). Flunixin is hydroxylated by P450s (figure 6.7), with the resulting metabolite having been detected in horse plasma and urine (Jaussaud *et al*, 1987), however the majority of pharmacokinetic studies on flunixin involve looking for flunixin itself rather than 5-hydroxyflunixin (Luo *et al*, 2004). It is not used in human; however it is permitted for use in cattle and pigs in addition to horses (Pairis-Garcia *et al*, 2013). Due to its limited use, no data is available about P450-specific metabolism of flunixin.

In the current study, P450-specifc metabolism was assessed by using recombinant equine and human P450s as well as equine liver microsomes in order to determine whether CYP2C isozymes are responsible for flunixin metabolism.
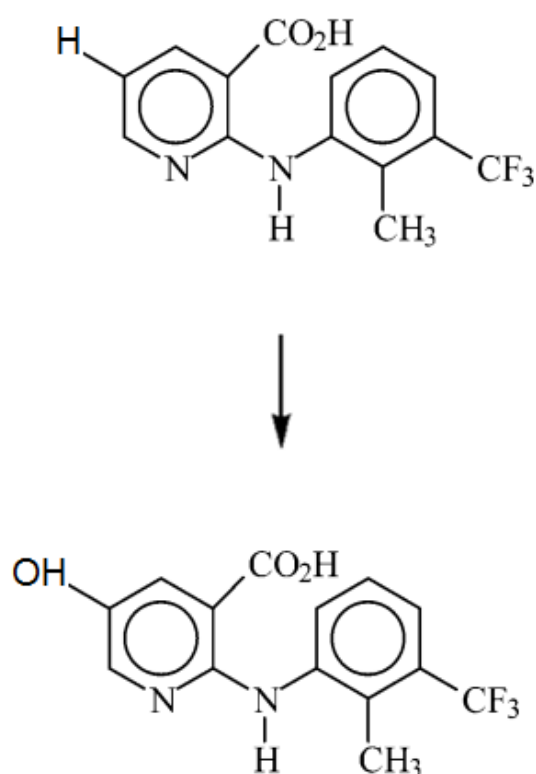

Figure 6.7: Metabolism of flunixin to 5'hydroxyflunixin. Adapted from "Determination and confirmation of 5-hydroxyflunixin in raw bovine milk using liquid chromatography tandem mass spectrometry" by Boner, P.L, Liu, D.D.W, Feely, W.F, Wisocky, M.J, Wu, J, 2003, *Journal of Agricultural and Food Chemistry*: 51, 3753–3759.

6.8: Results

6.8.1: LCMS analysis of flunixin

The ion of mass 297.26 was used to monitor for flunixin depletion (figure 6.8.1). The retention time was 4.6 mins (refer to chapter 2 for full details of HPLC conditions).
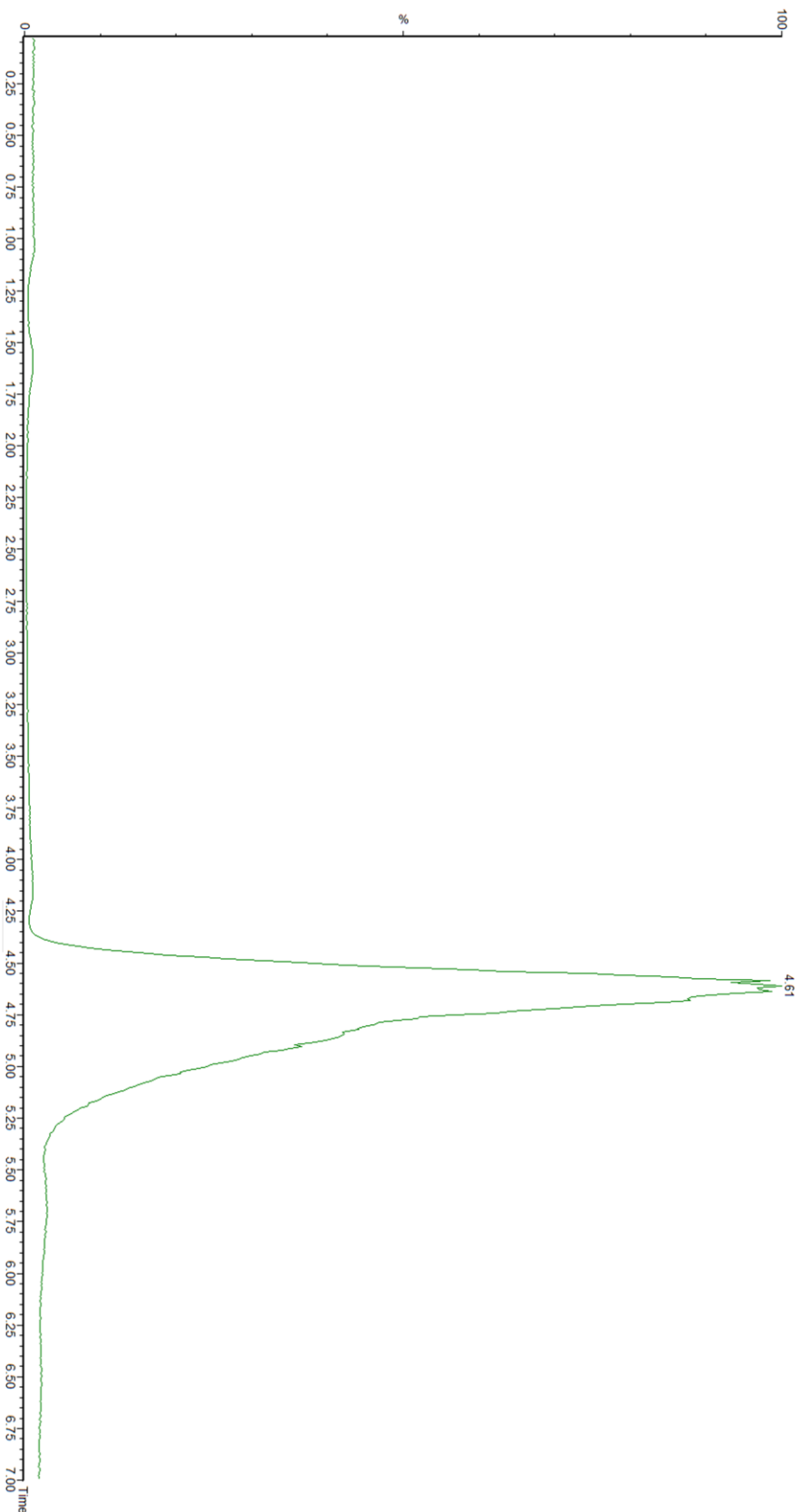
Figure 6.8.1: chromatogram for monitoring flunixin disappearance. The ion of mass 297.26 was monitored at a retention time of 4.61 mins.

6.8.2: Flunixin metabolism by rCYP2C92

For the recombinant CYP2C92, there appeared to be significant turnover of flunixin (figure 6.8.2); metabolism was evident at all concentrations. A Michealis-Menten curve was successfully fitted to the data (figure 6.8.3), with the data closely fitting the curve. K_m was estimated at 43.89 µM and V_{max} at 1.96 µM/min (table 9.2.4). The subsquent CL_{int} value was 297.7 µL/min/mg of protein.
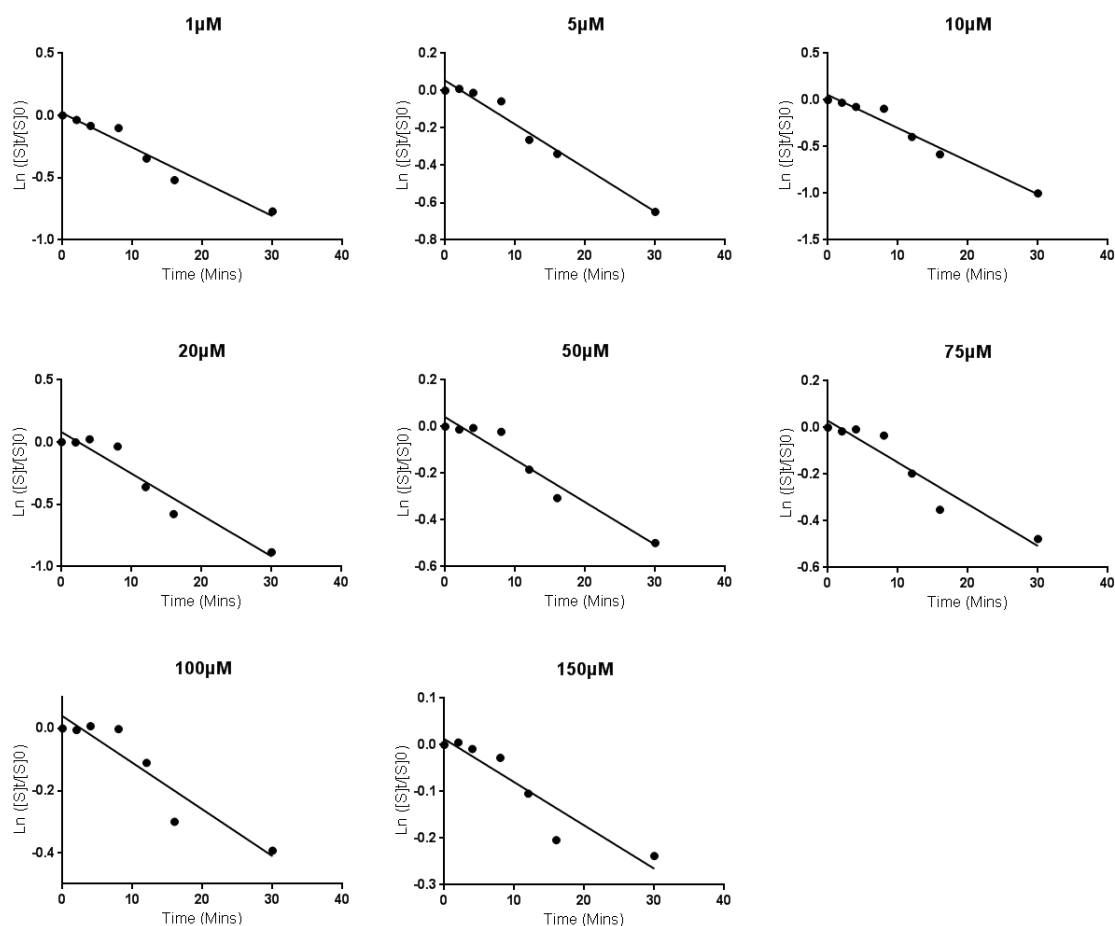
Figure 6.8.2: Metabolism of flunixin by equine rCYP2C92. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. Kdep is represented by the slope of the line (refer to table 6.8.1). Data was generated from one experiment.

Flunixin Concentration (µM)	K_{dep} (/min)	v_0 (uM/min)	% drug remaining after 60 mins
1	0.028	0.028	40
5	0.023	0.12	48
10	0.035	0.35	33
20	0.033	0.67	35
50	0.018	0.91	48
75	0.018	1.34	50
100	0.015	1.5	51
150	0.0093	1.4	73

Table 6.8.1: Initial rate and K_{dep} values for rCYP2C92 with flunixin. Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).
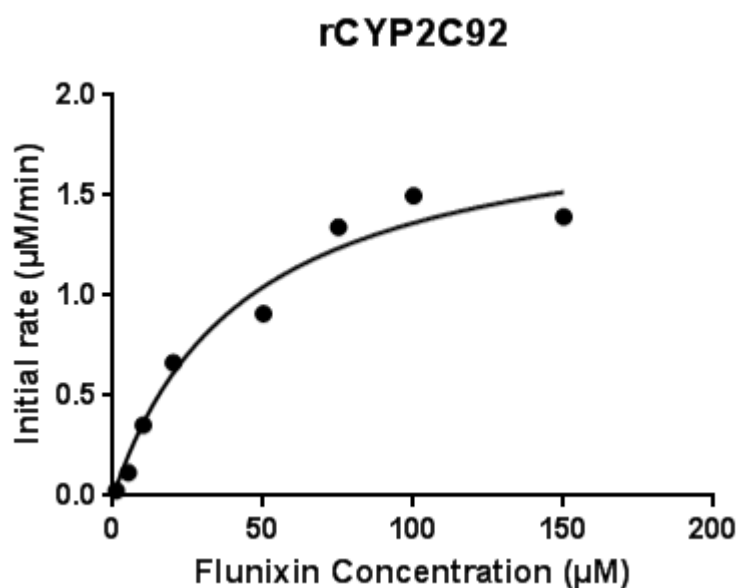

Figure 6.8.3: Michaelis-Menten curve for rCYP2C92 with flunixin. Initial rate is plotted against substrate concentration.

6.8.2: Flunixin metabolism by equine liver microsomes

Metabolism of flunixin was observed with equine microsomes (figure 6.8.4). With increasing substrate concentration, the initial rate firstly increased followed by a decline, therefore the data was fitted to a substrate inhibition curve as in equation 6 in section 6.1.5 (figure 6.8.5). The K_m and V_{max} values could not be deconvoluted so equation 2 in section 6.1.3 was used to estimate a CL_{int} value of 20 µL/min/pmol P450.
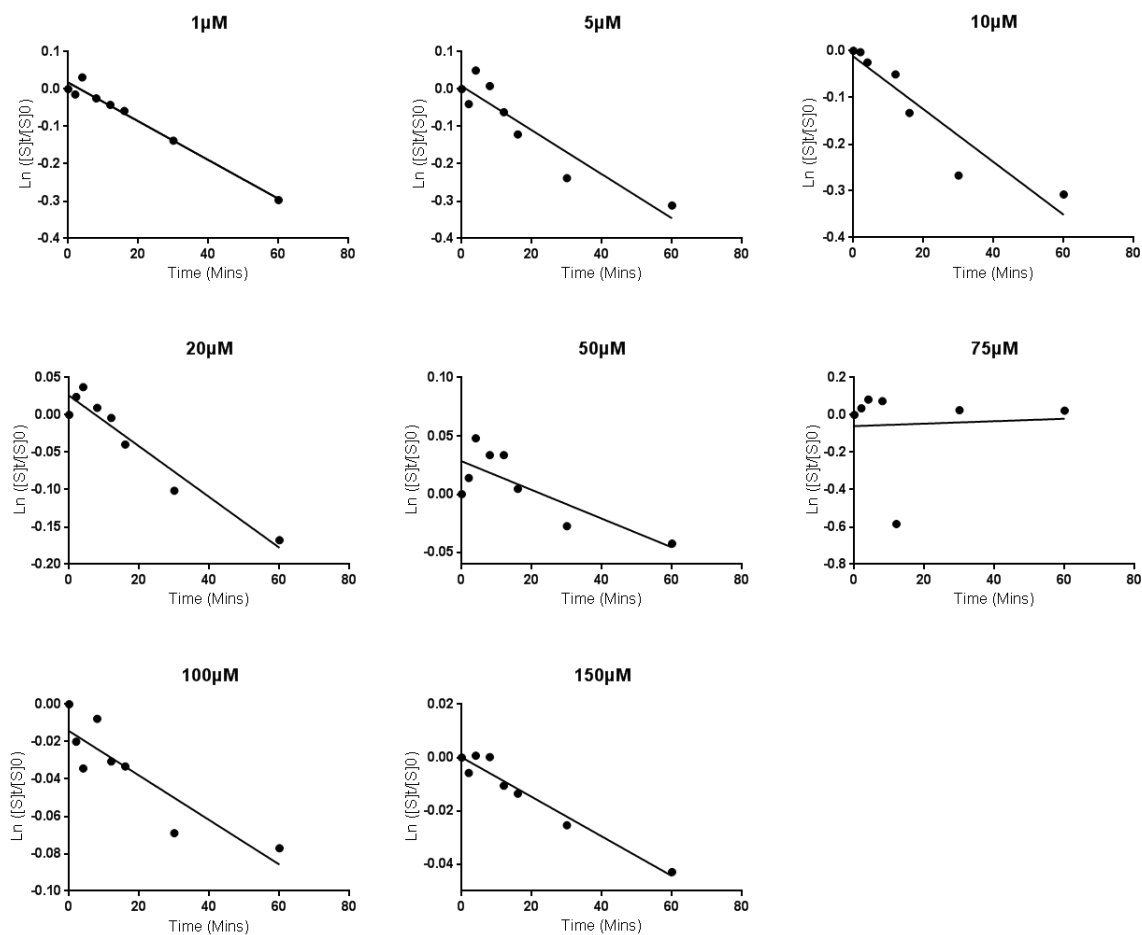

Figure 6.8.4: Metabolism of flunixin by equine liver microsomes. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.8.2). Data was generated from one experiment.

Flunixin Concentration (µM)	K_{dep} (/min)	v_0 (uM/min)	% drug remaining after 60 mins
1	0.0052	0.0052	74
5	0.0059	0.030	73
10	0.0057	0.057	73
20	0.0034	0.068	84
50	0.0012	0.060	95
75	0.00070	0.053	100
100	0.0012	0.12	92
150	0.00070	0.11	95

Table 6.8.2: Initial rate and K_{dep} values for equine liver microsomes with flunixin. Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).
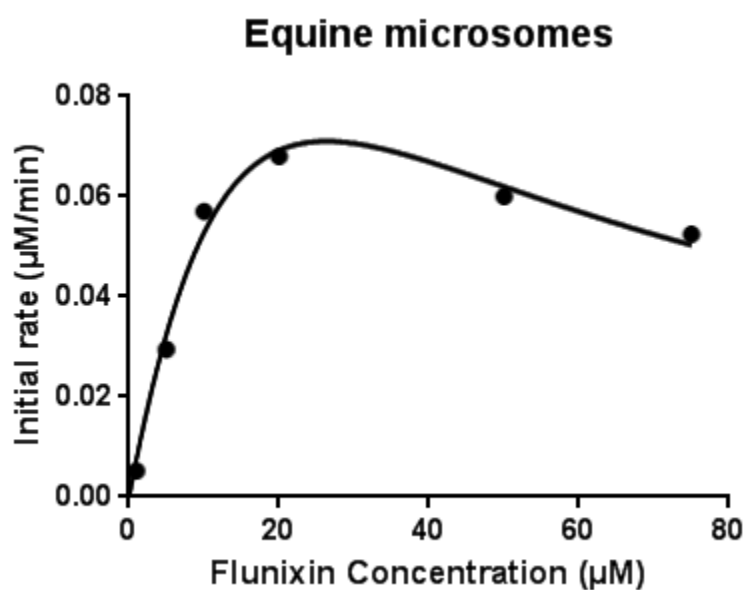

Figure 6.8.5: Substrate inhibition curve for equine liver microsomes incubated with flunixin.

Initial rate is plotted against substrate concentration.

6.8.3: Metabolism of flunixin by human rCYP2C9

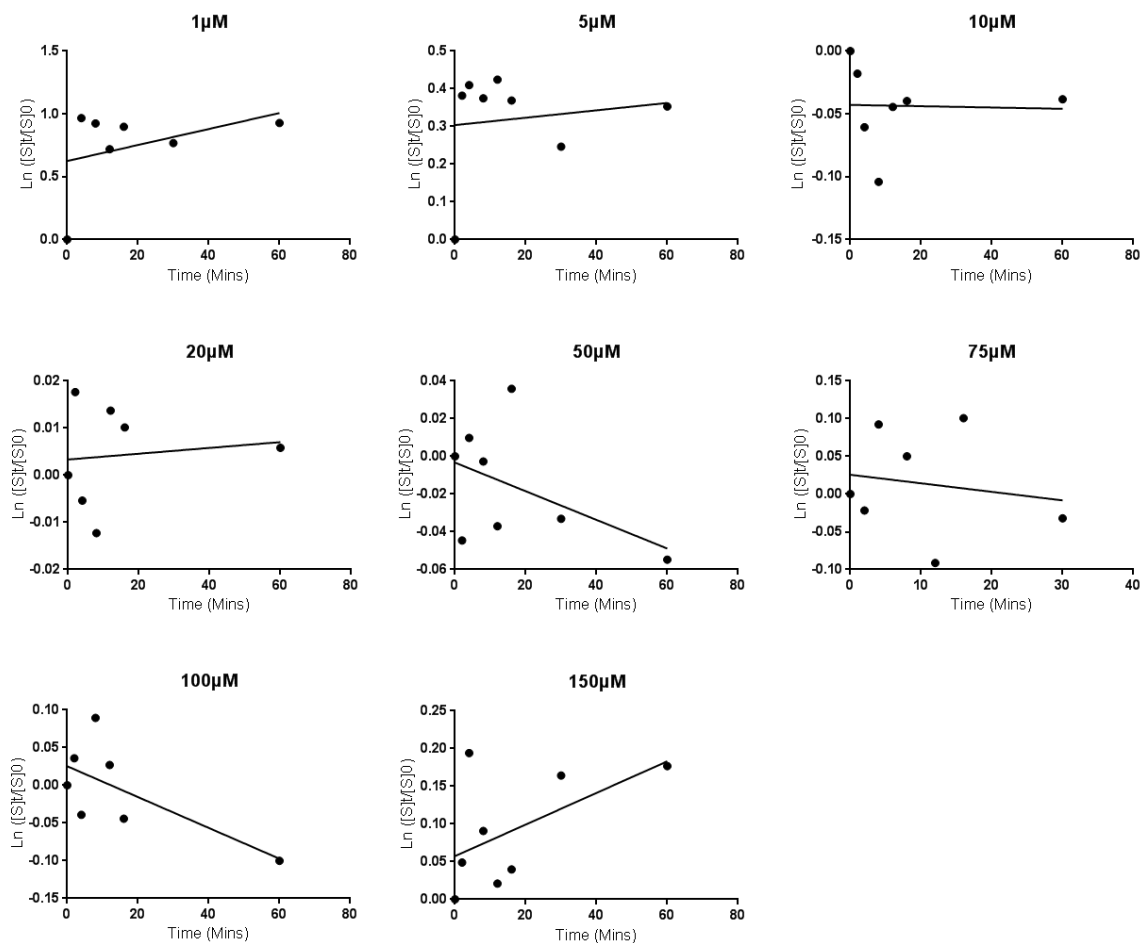No metabolism of flunixin was observed by human CYP2C9 (figure 6.8.6).

Figure 6.8.6: Metabolism of flunixin by human rCYP2C9. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. Kdep is represented by the slope of the line (refer to table 6.8.3). Data was generated from one experiment.

Flunixin Concentration (μM)	K_{dep} (/min)	v_0 (uM/min)	% drug remaining after 60 mins
1	0.0064	0.0064	250
5	-0.0010	0.0050	140
10	-5.000050	0.00050	96
20	0.000060	0.0012	100
50	0.00080	0.040	94
75	0.0011	0.083	9.6
100	0.0020	0.20	90
150	-0.0021	0.32	119

Table 6.8.3: Initial rate and K_{dep} values for human rCYP2C9 with flunixin. Substrate concentrations ranged from 1-150 μM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

6.8.4: Summary

Table 6.8.4 summarises the data gathered for all three enzyme systems with flunixin. Parameters could not be determined for human recombinant CYP2C9 due to absence of apparent metabolism in this system. Only CL_{int} could be determined for equine liver microsomes due to the presence of substrate inhibition.

Enzyme system	K_m [1]	K_m SE [1]	V_{max} [2]	V_{max} SE [2]	CL_{int} [3]
rCYP2C92	43.89	12.99	1.96	0.22	297.7
rCYP2C9	N/A	N/A	N/A	N/A	N/A
Equine Microsomes	N/A	N/A	N/A	N/A	20

Table 6.8.4: K_m, V_{max} and Intrinsic clearance (CL_{int}) values for all three enzyme systems with flunixin. Units = [1] µM, [2] µM/min, [3] µL/min/pmol P450 (µL/min/mg protein for CYP2C92). N/A signifies the value could not be obtained due to no metabolism (CYP2C9) or data being fit to a substrate inhibition curve (equine microsomes). For the microsomal system, the inhibition constant was used to calculate the CL_{int}. SE = standard error.

6.9: Phenylbutazone metabolism

6.9.1: Introduction

Phenylbutazone is one of the most well studied equine medications and is the most frequently prescribed equine NSAID (Soma *et al*, 2012). Consequentially, much is known about its pharmacokinetics. It can be administered orally or intravenously, with both routes having been extensively characterised (Scarth *et al*, 2011). For routine drug testing, the metabolite oxyphenylbutazone is generally used to monitor metabolism, although gamma-hydroxyphenylbutazone is another major metabolite of phenylbutazone, produced in addition to the minor metabolites shown in figure 6.9 (Lees and Toutain, 2013).
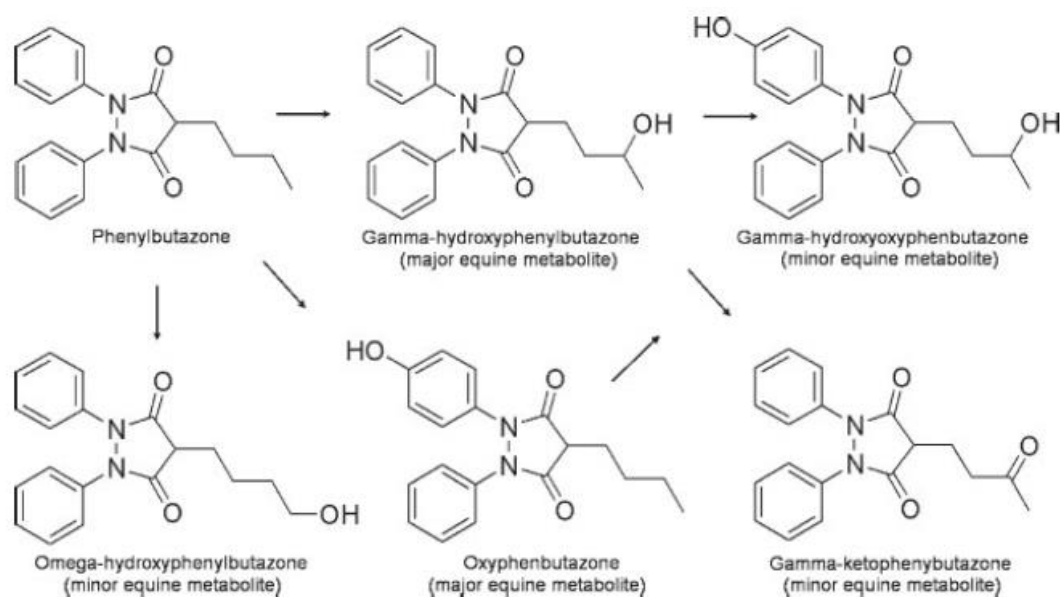
Figure 6.9: Phase I metabolism of phenylbutazone in the horse. The major metabolites are oxyphenbutazone and gamma-hydroxyphenybutazone while the minor metabolites are omega-hydroxyphenybutazone, gamma-hydroxyphenbutazone and gamma-ketophenybutazone. Adapted from "Drug metabolism in the horse" by Scarth, J.P, Teale, P, Kuuranne, T, 2011, *Drug Testing and Analysis*: 3(1):19-53.

Controversy has surrounded the use of phenylbutazone within the horseracing industry, due to its particularly high levels of use within this species (Soma *et al*, 2012). Adequate screening procedures for phenylbutazone have therefore become essential for effective medication control. Despite the levels of use and research on phenylbutazone in the horse, there is no data to date about the specific P450 isoforms responsible for its metabolism. In human, phenylbutazone is an inhibitor and substrate of CYP2C9 (Rendic, 2002). For the current study, recombinant equine CYP2C92 was used to determine whether this isoform is responsible for phenylbutazone metabolism in the horse. Activity was monitored by looking for both substrate depletion and metabolite appearance, which is the most frequently used method in equine phenylbutazone monitoring (Peck *et al,* 1996).

6.10: Results

6.10.1: Phenylbutazone LCMS analysis

The ion of mass 309.19 was used to monitor phenylbutazone depletion and the ion of mass 325.25 was used to monitor the appearance of its metabolite, oxyphenbutazone (figure 6.10.1).
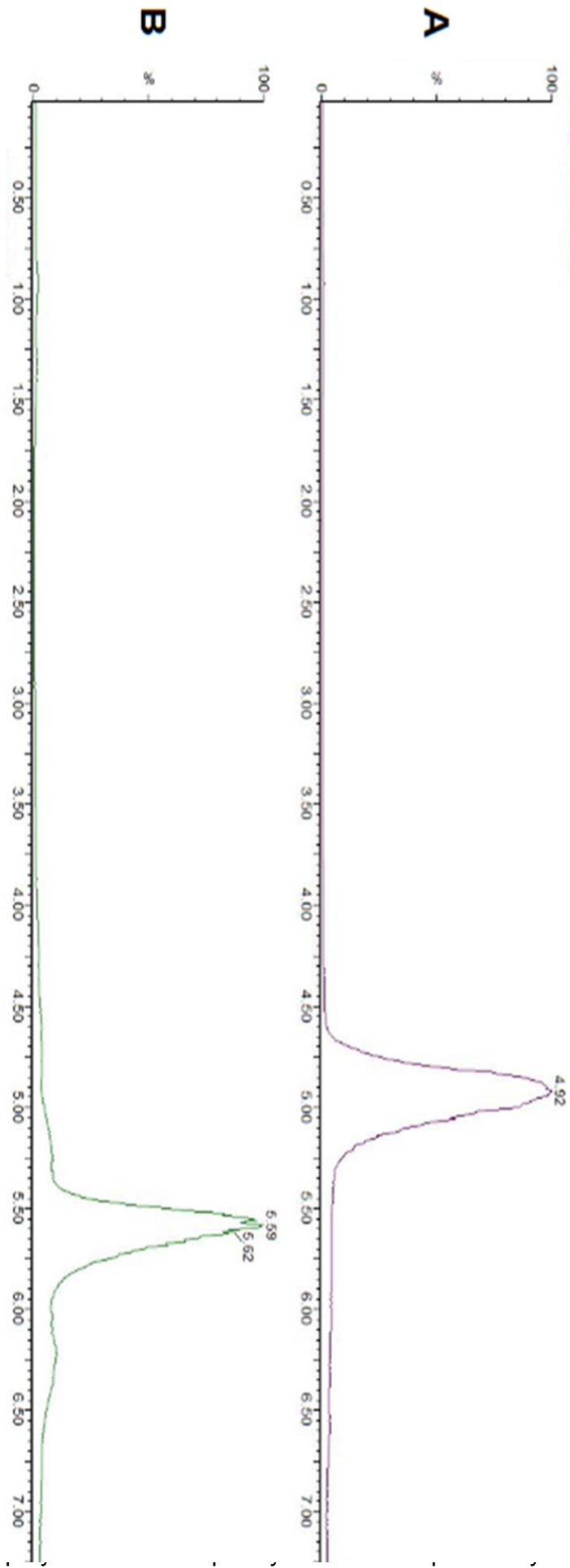
Figure 6.10.1: Chromatogram for monitoring phenylbutazone disappearance and oxyphenbutazone appearance. Oxyphenbutazone (A) had a retention time of 4.92 mins and phenylbutazone (B) had a retention time of 5.59 mins.

rCYP2C92. From 50 to 150 µM substrate auto inhibition appeared to be occurring with increasing concentration of substrate, due to slope of the line decreasing. A substrate inhibition curve was fitted to the data (figure 6.10.4) and consequentially V_{max} and K_m approximations could not be deconvoluted with any certainty and therefore the initial slope of the Michaelis-Menten curve was used to estimate a CL_{int} value of 6 µl/min/mg protein.
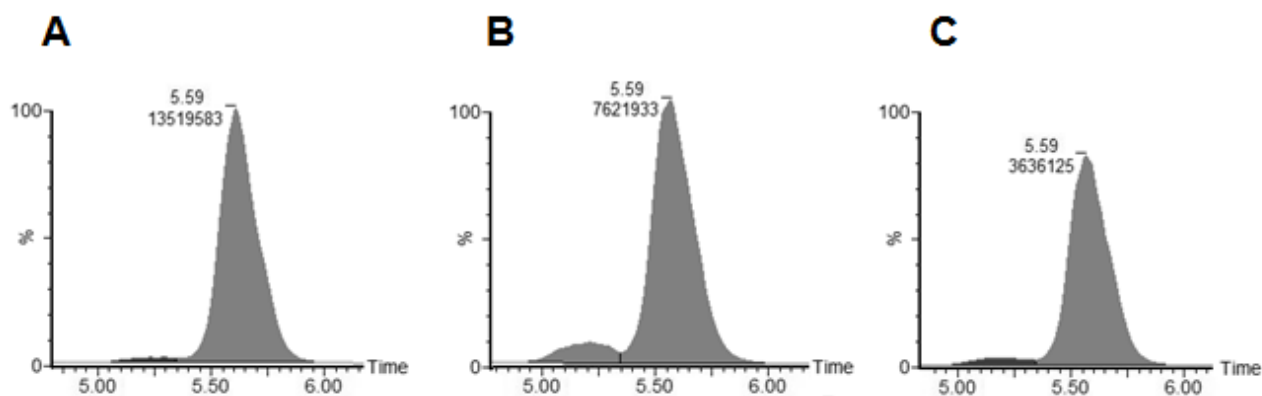

Figure 6.10.2: Chromatograms showing depletion of phenylbutazone by rCYP2C92 with time (A: 0 mins, B: 30 mins, C: 60 mins). Renention time is displayed on the Y axis of each chromatogram and the mass spectrometry response was calculated as the area under the curve. The retention time was 5.59 (mins).
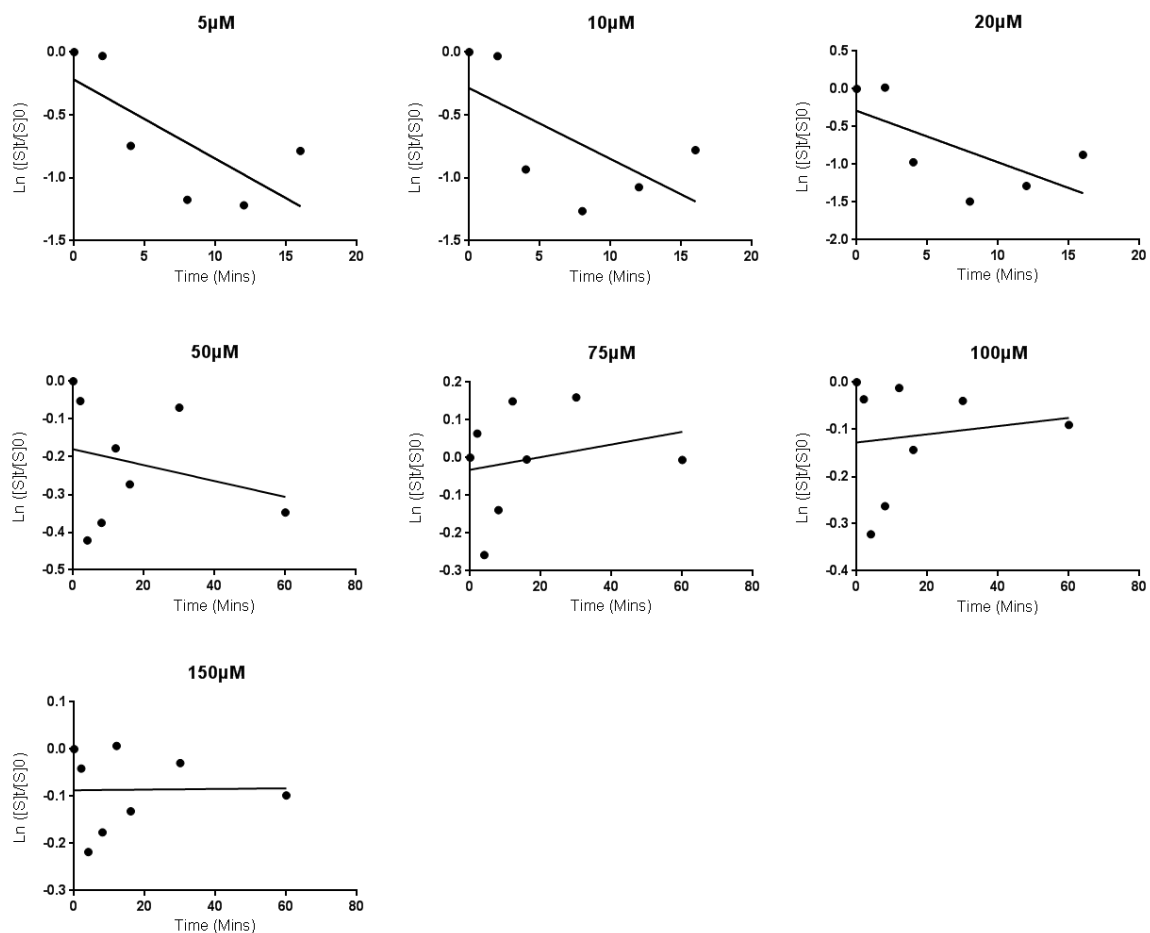
Figure 6.10.3: Data gathered using various phenulbutazone concentrations with equine rCYP2C92. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.10.1). Data was generated from one experiment.

Phenylbutazone Concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	N/A	N/A	N/A
5	0.0087	0.044	41
10	0.0096	0.096	37
20	0.011	0.22	32
50	0.0021	0.11	70
75	-0.0017	0.13	99
100	-0.00090	0.09	91
150	0.000070	0.011	90

Table 6.10.1: Initial rate and K_{dep} values for rCYP2C92 with phenylbutazone. Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

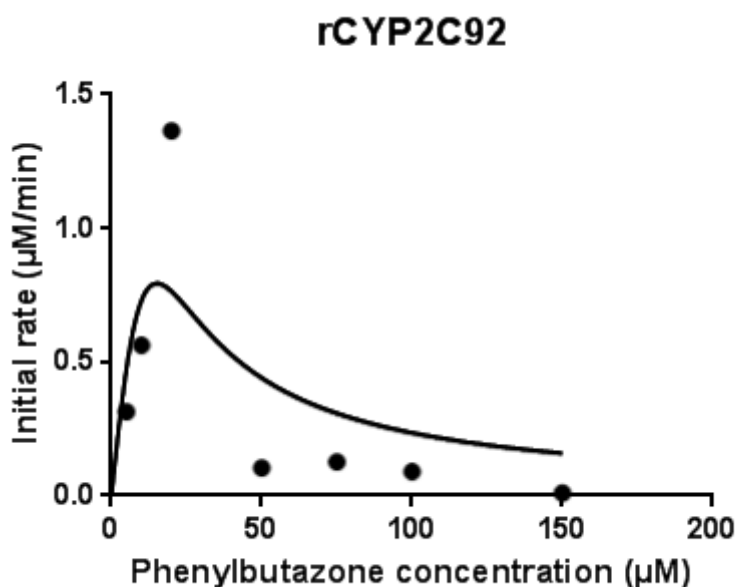**Figure 6.10.4: Substrate inhibition curve for rCYP2C92 and phenylbutazone.** Initial rate (µM/min) is plotted against substrate concentration (µM).

In addition to substrate depletion, the major metabolite of phenylbutazone, oxyphenbutazone, was monitored for however results were inconclusive – no distinct chromatographic peaks were visible.

6.10.3: Phenylbutazone metabolism by equine liver microsomes

Metabolism of phenylbutazone by equine liver microsomes was evident across all concentrations (figure 6.10.5). A Michaelis-Menten curve was successfully fitted to the data (figure 6.10.6) with K_m and V_{max} values estimated at 15.45 µM and 1.09 µM/min respectively. This was used to generate a Cl_{int} value of 0.16 µl/min/pmol p450.
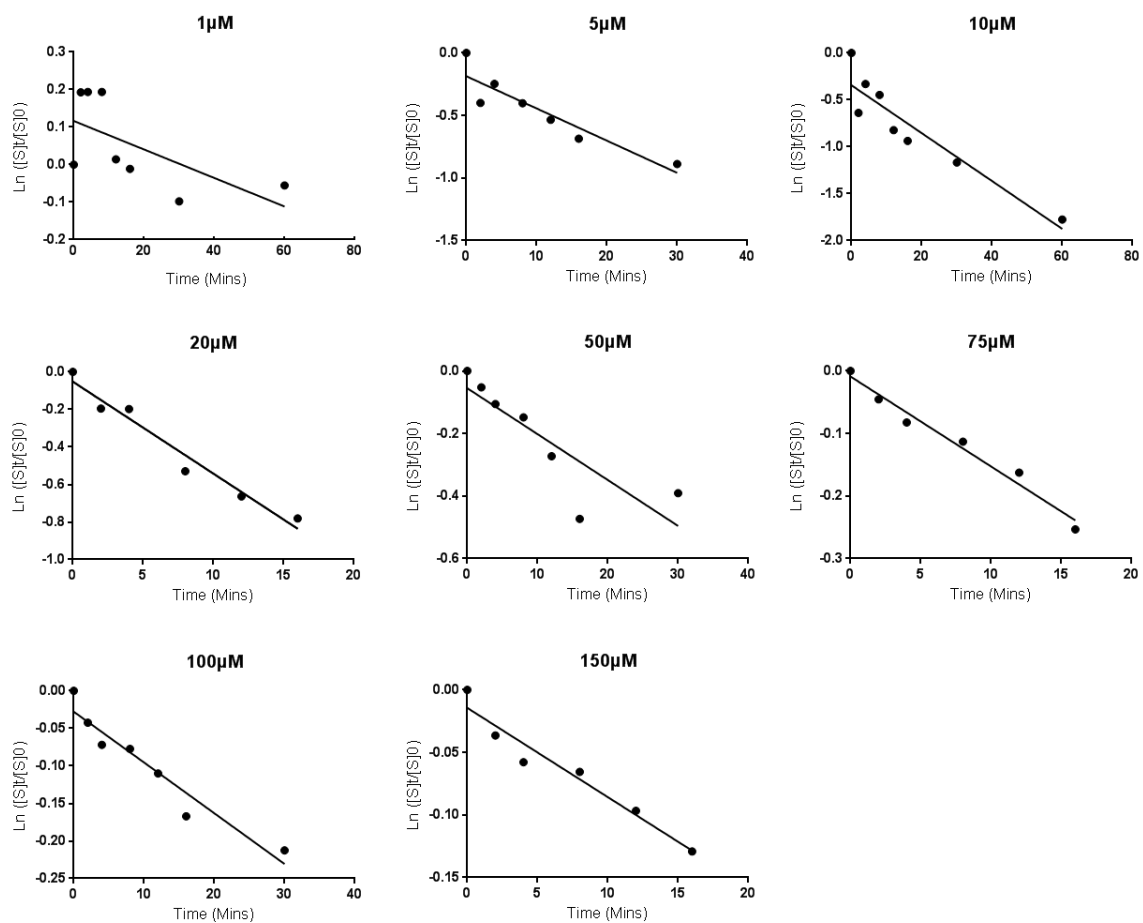

Figure 6.10.5: Data gathered using various phenulbutazone concentrations with equine microsomes. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.10.2). Data was generated from one experiment.

Phenylbutazone Concentration (µM)	K_{dep} (/min)	v_0 (µM/min)	% drug remaining after 60 mins
1	0.0038	0.0038	94
5	0.026	0.13	34
10	0.026	0.26	16
20	0.025	0.50	37
50	0.015	0.74	57
75	0.014	1.1	73
100	0.0068	0.68	81
150	0.0071	1.1	84

Table 6.10.2: Initial rate and K_{dep} values for equine liver microsomes with phenylbutazone.

Substrate concentrations ranged from 1-150 µM and v_0 was calculated as per equation 4 in section 6.1.3. Also highlighted is the percentage of drug remaining at the end of the assay (60 minutes).
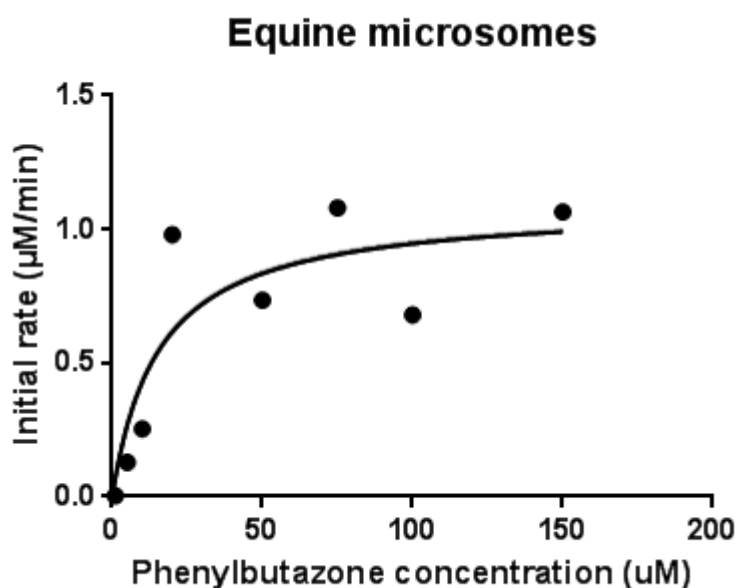
Figure 6.10.6: Michaelis-Menten curve for equine microsomes and phenylbutazone. Initial rate (µM/min) is plotted against substrate concentration (µM).

In addition to substrate depletion, the appearance of the metabolite, oxyphenbutazone, was monitored (figure 6.10.7). At concentrations 1 to 10 µM no detectable metabolite was produced however from 20-150 µM significant oxyphenbutazone production was detected (figure 6.10.8). V_{max} was determined to

be 0.7 µM/min while K_m could not be deconvoluted due to lack of data for lower

concentrations.

Figure 6.10.7: Data for oxyphenbutazone production with equine liver microsomes. Data was
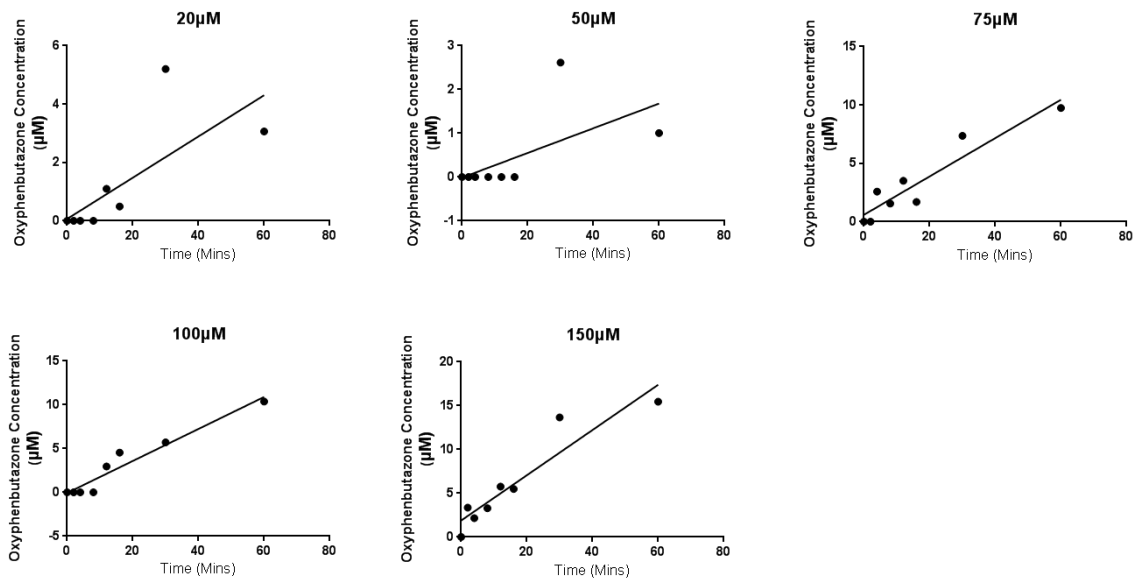
plooted as the concentration (in µM) against time.
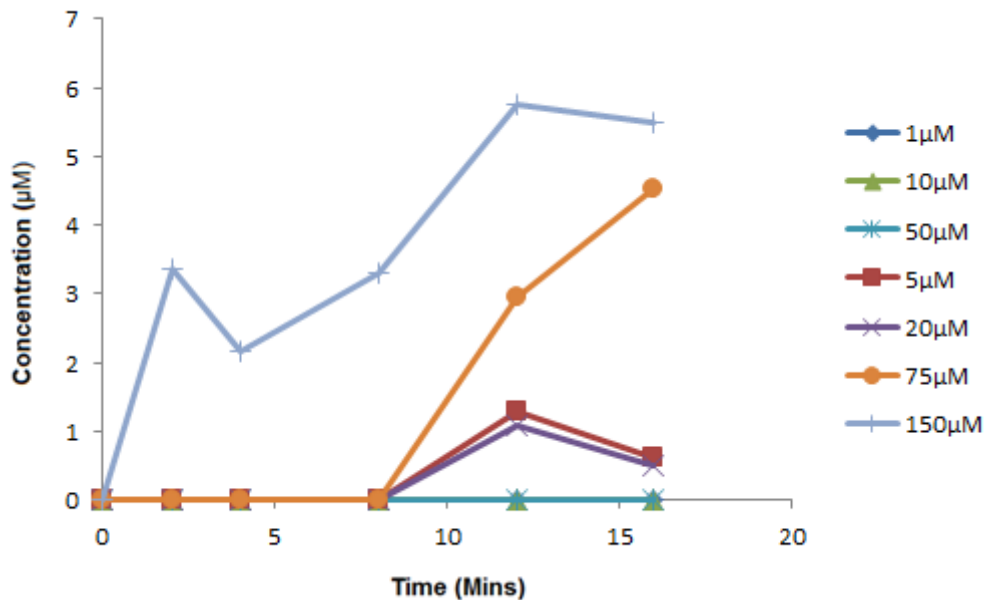
Figure 6.10.8: Oxyphenbutazone production by equine liver microsomes. Concentration was

determined using a stock solution of oxyphenbutazone of known concentration to covert mass

spectrometry response into µM.

6.10.4: Phenylbutazone metabolism by human recombinant CYP2C9

Substrate depletion was evident with human rCYP2C9 up until a substrate concentration of 150µM (figure 6.10.9). A Michaelis-Menten curve was successfully fitted to the data (figure 6.10.10). The estimated K_m and V_{max} were 18.79µM and 0.94 µM/min, respectively. CL_{int} was calculated as 5.002 µl/min/pmol P450.
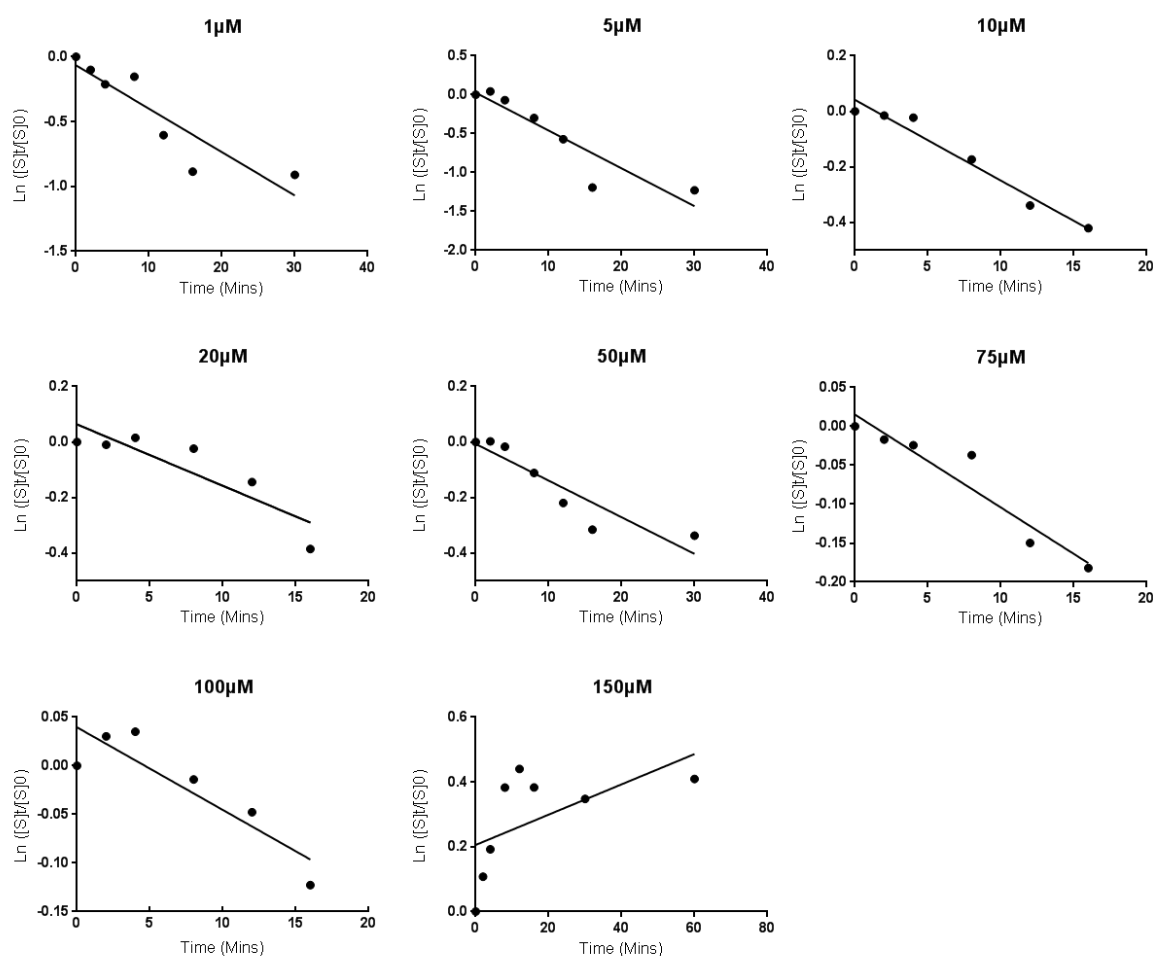
Figure 6.10.9: Data gathered using various phenulbutazone concentrations with human rCYP2C9. Data was plotted as the natural logarithm (Ln) of substrate concentration against time. K_{dep} is represented by the slope of the line (refer to table 6.10.3).
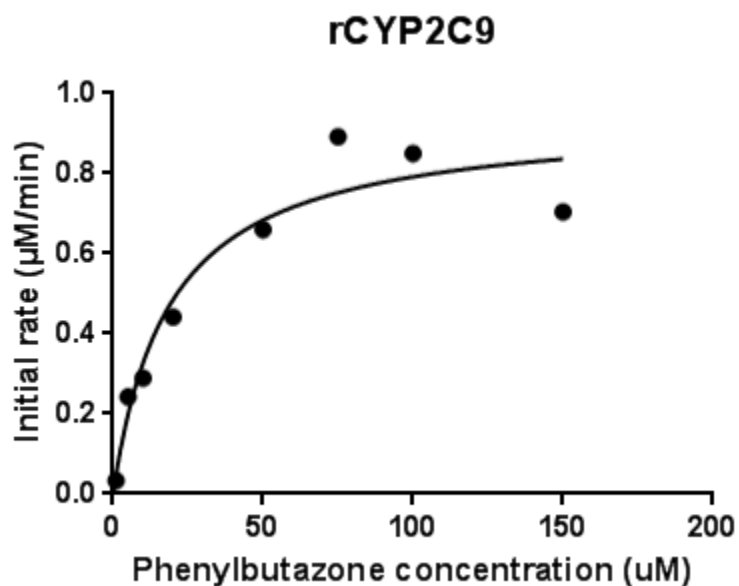
Figure 6.10.10: Michaelis-Menten curve for rCYP2C9 and phenylbutazone. Initial rate (μM/min) is

plotted against substrate concentration (μM).

Phenylbutazone Concentration (μM)	K_{dep} (/min)	v_0 (μM/min)	% drug remaining after 60 mins
1	0.03	0.03	47
5	0.05	0.24	59
10	0.03	0.29	81
20	0.02	0.44	86
50	0.01	0.66	79
75	0.01	0.89	86
100	0.0085	0.85	91
150	0.0047	0.71	150

Table 6.10.3: Initial rate and K_{dep} values for human rCYP2C9 with phenylbutazone. Substrate

concentrations ranged from 1-150 μM and v_0 was calculated as per equation 4 in section 6.1.3. Also

highlighted is the percentage of drug remaining at the end of the assay (60 minutes).

6.10.5: Summary

Table 6.10.4 summarises the data generated for all three enzyme systems with phenylbutazone. Only CL_{int} could be determined for rCYP2C92 due to presence of substrate auto-inhibition.

Enzyme system	K_m [1]	K_m SE [1]	V_{max} [2]	V_{max} SE [2]	CL_{int} [3]
rCYP2C92	N/A	N/A	N/A	N/A	6.0
rCYP2C9	18.79	6.6	0.94	0.09	5.0
Equine Microsomes	15.45	11.4	1.09	0.21	0.16

Table 6.10.4: Summary of K_m, V_{max} and Intrinsic clearance (CL_{int}) values for all three enzyme systems. Units = [1] µM, [2] µM/min, [3] µL/min/pmol P450 (µL/min/mg protein for CYP2C92). N/A signifies the value could not be obtained (do to data being fit with a substrate inhibition curve). SE = standard error.

6.11: Discussion

The primary aim of this research was to characterise equine recombinant P450s by using a range of probe substrates frequently used in equine medication.

Initially, drugs of various classes were investigated with rCYP2C92 and rCYP2D50. For rCYP2C92, the only drugs that displayed apparent metabolism were the NSAIDs. In other species, the vast majority of NSAID metabolism involves CYP2C isozymes, particularly CYP2C9 (Miners and Birkett, 1998, Leemann *et al*, 1993) so this result was as expected. The only substrate that rCYP2D50 showed activity with was dextromethorphan, which was as expected as this is a known CYP2D6 substrate in human (Yu and Haining, 2001). Many of the drugs used did not appear

to be substrates for either isoform. Hydroxyzine and fluphenazine are substrates of CYP2D6 in human; however equine CYP2D50 showed no activity towards either drug. P450 activity towards pyrilamine and glycopyrrolate is currently unknown in any species and neither drug appeared to be a substrate for CYP2D50 and CYP2C92. Overall, the only unexpected result was for the lack of metabolism by CYP2D50 of hydroxyzine and fluphenazine. This may imply CYP2D50 does not metabolise these drugs in the horse, however it may also be due to a lack of fully functional activity of CYP2D50 used in this research due to impaired post-translational modifications within the bacterial expression system. In future work, rCYP2D50 screening against other known CYP2D substrates will allow for a more comprehensive analysis of the activity of this expression method and further insight into differences between the equine and human forms. After initial screening, rCYP2C92 activity with the NSAIDs diclofenac, ketoprofen, flunixin and phenylbutazone was focused on, with attempts being made to determine full kinetic parameters along with comparisons to equine liver microsomes and human rCYP2C9.

For diclofenac, metabolism of this substrate on its own was analysed as well as metabolism in the presence of the potential inhibitor fluconazole. Disappearance of diclofenac was monitored along with apparent appearance of the known metabolite 4-hydroxydiclofenac. For rCYP2C92, a significant decrease in diclofenac concentration over time was observed, however substrate auto inhibition was noted in this system and not in the microsomal system, which could be due to the contribution of other P450 enzymes within the microsomal system, thereby masking any CYP2C92-specific inhibition. However, the apparent hydroxylated metabolite 4-

hydroxydiclofenac could not be conclusively detected with the recombinant system, which may imply the metabolic pathway of diclofenac with this recombinant enzyme may be altered. 4-hydroxydiclofenac was produced in both the recombinant human and equine microsomal systems and has previously been shown to be produced by equine CYP2C92 (DiMaio Knych *et al*, 2009). For the inhibition assay with fluconazole, rCYP2C92 and equine liver microsomes were used. This inhibition assay was carried out for two reasons – firstly to further study the activity of the recombinant enzyme, and secondly to investigate whether fluconazole is an inhibitor of CYP2C92 in the horse. Both the recombinant enzyme and microsomal systems showed no inhibition trend – data could not be fitted to a standard inhibition curve, implying that no significant inhibition was present. In human, the Ki of fluconazole when used with diclofenac is 17μM, much lower than the highest concentration used within this study (Hargreaves *et al*, 1994). Since inhibition was not seen in either equine system, it may be the case that fluconazole is not an inhibitor of diclofenac activity in the horse.

To date, there have been no published reports on which specific isoforms of P450 may be involved in ketoprofen metabolism in the horse. In human, P450s only play a minor role in the metabolism of ketoprofen while in mice and rats they hold a much more central importance (Yamasaki *et al*, 2010). From the results presented in the current study, CYP2C92 appears to have some activity towards ketoprofen, with the lower concentrations of drug (1 and 5 μM) showing a notable decrease over time. As substrate concentration increases however, the enzyme appears to have little to no effect on overall drug levels here. Likewise, equine liver microsomes showed a slight decrease in substrate concentration at 1μM and no change at higher concentrations.

This data implies P450s (CYP2C92 or other isoforms) only play a minor role in equine P450 metabolism of ketoprofen, although it should be noted that for the microsomal 1 µM assay (fig 6.6.4) the data was a poor fit to the graph, therefore repetition of this assay would be required before substrate depletion can be conclusively confirmed.

. Contrasting the equine data with the human data, there appears to be somewhat more activity towards the drug in the equine systems – no metabolism, even at 1µM, was detectable with human CYP2C9. CYP2C9 is known to have activity towards ketoprofen (Zhou *et al*, 2009a) however direct comparison between isoforms within the context of the current study is not possible due to the recombinant equine CYP2C92 being unable to be quantified via the carbon monoxide spectral assay (chapter 5). Further work will be required to fully ascertain whether the equine and human isoforms have different activities towards ketoprofen.

The first use of flunixin with recombinant equine CYP2C92 has been presented in the current research, and it provides an insight into isoform-specific P450 metabolism in the horse. As expected, both the recombinant enzyme system and equine liver microsomes showed turnover of flunixin. As a veterinary-only medication, flunixin is not permitted for use in humans and therefore data relating to its metabolism by human P450 enzymes is unavailable. Surprisingly, equine CYP2C92 and human CYP2C9 did not display similar activities with flunixin – no metabolism by the human isoform was detected. This was unexpected as CYP2C9 is the main P450 involved in the majority of NSAID metabolism (Rettie and Jones, 2005). Another difference between the systems was that equine microsomes

showed much more substrate inhibition than CYP2C92. This could be due to other

P450 isoforms contributing towards metabolism in the microsomal system and thus

producing metabolites that cause inhibition of flunixin depletion, or it could be due to

a conformational difference in the recombinant enzyme – since there is an amino

acid change in SRS6 (see chapter 4) which may affect the activity of the enzyme.

Further work will be required to determine the reason for these differences – higher

substrate concentrations will determine if the recombinant enzyme can be inhibited

in the same way as the microsomal system. Since the P450 content of the

recombinant system could not be quantified (chapter 5), the difference between the

microsomal and recombinant systems may be a higher P450 content in the latter,

thereby reducing the effect of inhibition at higher substrate concentrations. An

important next step will be identifying the hydroxylated metabolite of flunixin during

LCMS analysis. Nevertheless, these results give the first indication that CYP2C92 is

responsible for flunixin metabolism in the horse.

The last drug studied was phenylbutazone, which is the most highly-prescribed

NSAID in the horse (Soma *et al*, 2012). Phenylbutazone depletion and

oxyphenbutazone appearance were both monitored, since oxyphenbutazone is a

known metabolite in the horse (Lees and Toutain, 2013). For the microsomal system,

both approaches produced results however for the recombinant system no

significant metabolite formation was evident. This could imply that another metabolite

is the major product of CYP2C92 metabolism of phenylbutazone, however it is also

possible that the recombinant enzyme has conformational changes that have altered

its metabolic potential – which may also be the case for the lack of the major

metabolite of diclofenac metabolism, 4-hydroxydiclofenac. Additionally, substrate

auto-inhibition was evident with rCYP2C92 but not in the microsomal system, which as with diclofenac may be caused by other enzymes present in microsomes contributing towards clearance of phenylbutazone. Further studies will be required to determine if other P450s are involved in phenylbutazone metabolism while, for rCYP2C92, monitoring the full mass spectra of samples will be required to determine what metabolites are produced by this recombinant enzyme.

Intrinsic clearance values obtained *in vitro* can be scaled up to predict hepatic clearance and eventually used to predict the full pharmacokinetics of a drug *in vivo.* (Chiba *et al*, 2009). However, since rCYP2C92 could not be quantified within the current study this type of scaling up was not possible. Once recombinant P450s are quantified in future work, the CL_{int} values can be used to estimate hepatic clearance of each drug by determining the concentration of the P450 isoform present in the liver and then scaling up to take into account hepatic blood flow and the total weight of the liver (Chiba *et al*, 2009). Extrapolation of *in vitro* data is commonplace in the human pharmaceutical industry where concentration of a drug with multiple doses can be predicted in order to estimate potential drug-drug interactions (Fasinu *et al*, 2012). In the horse, *in vivo* drug metabolism studies are much more well-established than *in vitro* techniques however with increasing research in this area; accurate pharmacokinetic models can be developed and refined such that drug clearance and drug-drug interactions can be predicted in the laboratory.

Chapter 7: General discussion and concluding remarks

The aim of this thesis was to characterise equine P450s by identifying them via bioinformatics, detecting them in equine liver and by expressing and functionally testing them against probe substrates. Firstly, searching of the UCSC genome browser (https://genome.ucsc.edu/ and NCBI database (http://www.ncbi.nlm.nih.gov/) was carried out to identity potential P450 sequences, which were then analysed for phylogeny and grouped into clans (sequences with shared evolutionary divergence, Kirischian *et al*, 2011). Once *in silico* data had been compiled, RT-PCR was used to detect some of these P450s in equine liver which were then cloned using two systems – an insect cell system (well-established in P450 research and has previously been used to express equine P450s, DiMaio Knych *et al*, 2010) and a bacterial cold-induction system, selected due to its ability to produce eukaryotic proteins that are hard to express in *E coli* systems Sugiki *et al*, 2014. Once recombinant proteins were produced, their P450 status was assessed using the carbon monoxide spectral assay (Guengerich *et al*, 2009) and functional tests were carried out using various drugs as probe substrates. After this, the activity of rCYP2C92 against four NSAIDs (flunixin, phenylbutazone, diclofenac and ketoprofen) was focused on.

Analysis of the equine genome generated some novel results – in total 46 full length sequences were identified, and these spanned across all mammalian P450 clans. This is the first insight into how many P450s are present in the equine genome and sets the scope for future research to characterise equine P450s.

Once sequences had been identified, equine liver tissue was used to perform RT-PCR analysis, in order to detect which P450s are expressed in equine liver. Some

interesting results emerged from this analysis, with two isoforms that have high hepatic expression in other species being undetected (CYP1A2 and CYP2E1, which metabolise many important xenobiotics in human). Similarly, P450 isoforms which are not expressed in the liver of other species were detected in equine liver - CYP2R1 and CYP1B1. CYP2D14 and CYP2C26 were also detected via RT-PCR and are not well characterised in any species however both have been documented to have hepatic expression (Tsuneoka *et al*, 1992, Sakuma *et al*, 1994). CYP3A97 was used as a positive control for cDNA integrity (due to having previously been detected in equine liver, (DiMaio Knych *et al*, 2010, Tyden *et al*, 2012) and was also detected within the current study.

Two protein expression techniques were used to produce P450s in this research – sf9 insect cells and cold-induction bacteria. SF9 insect cells are the most well-established P450 expression system (Gonzalez, 1995) however protein expression here was minimal, with Western blot analysis of lysate generating consistently negative results. Conversely, the cold-induction bacterial system generated strong results – significant protein expression was detected for four isoforms – CYP2D50, CYP2C92, CYP1A1 and CYP2A13. Furthermore, for CYP2D50 and CYP2C92 (which are the most likely drug-metabolising P450s out of these four) showed activity towards some drug substrates, despite carbon-monoxide spectral analysis showing them as P420s instead of P450s. There are no reports of functional P420s in the literature so it is possible that with different conditions (such as use of a different lysis buffer, or application of some modifications to the sequences prior to expression) they may show the characteristic 450 nm spectral shift. *E coli* expression systems have been reported on many instances previously to produce P420 peaks with and without sequence modification (Guengerich *et al*, 2009). It has been

suggested that detergents in lysis reagents could at least be partially to blame for this (Luthra *et al*, 2013), however within the current study when sonication was used as an alternative lysis technique, neither a P420 nor a P450 peak was evident.

Four NSAID drugs were analysed in detail with rCYP2C92. These assays were run alongside ones involving equine liver microsomes and human recombinant CYP2C9. Although diclofenac has been studied with CYP2C92 before (DiMaio Knych *et al*, 2009) the other three (ketoprofen, phenylbutazone and flunixin) have never been used to characterise this isoform. Despite substrate depletion being evident for rCYP2C92 with phenylbutazone and diclofenac, the metabolites 4-hydroxydiclofenac and oxyphenbutazone were not produced at any significant levels. To contrast, 4-hydroxydiclofenac and oxyphenbutazone were detected in equine microsomes and the human rCYP2C9 systems. This finding could be used to imply the recombinant CYP2C92 is not a fully functional P450, although it is unknown what metabolite this enzyme produced instead of those that were monitored. Further analysis will be required to determine what the products of this recombinant CYP are by analysing the total ion current obtained from incubation samples.

All NSAIDs showed metabolism by rCYP2C92, although with ketoprofen this was only evident at lower concentrations. Interestingly, rCYP2C92 differed in its metabolism of ketoprofen and flunixin from the human rCYP2C9 isoform - ketoprofen and flunixin showed no metabolism with CYP2C9. Ketoprofen is known to be mostly metabolised by phase II enzymes in human (Skordi *et al*, 2004) however the metabolism of flunixin in human is unknown, since this drug is not used in human medicine. These findings further emphasize the importance of species differences in drug metabolism and the dangers of extrapolating data between species.

218

Addtionally, significant differences were noted between the recombinant equine and equine microsomal systems during the Michaelis-Menten analyses. For phenylbutazone and diclofenac, substrate auto-inhibition was evident for the recombinant equine system but not the microsomal system, and conversely with flunixin, substrate auto-inhibition appeared to be taking place in the microsomal system but not the recombinant system. This could have been caused by conformational differences in the recombinant enzyme – however since other P450 enzymes are present within microsomes, the concurrent metabolism by other isoforms of the probe substrates is also a possibility, as is it a possibility that other P450 isoforms produce metabolites that cause auto-inhibition with regard to the flunixin microsomal assay. Further studies using inhibitors of various P450 isoforms will be required to dismiss or confirm this possibility.

In future work, the P450s isolated in the current research can be further characterised against a greater range of substrates – drug and non-drug. Two P450s produced during the current study (CYP2A13 and CYP1A1) were not phenotypically characterised at all due to the fact these isoforms are not major drug-metabolising enzymes in other species (Galván *et al*, 2005, Fukami *et al*, 2007). Potential substrates of CYP2A13 and CYP1A1, such as PAHs, could be tested against these recombinant enzymes. Additionally, all P450 constructs could be expressed in *E. coli* with N-terminal modifications in order to assess whether this alters the CO spectral assay outcome or generates different metabolites with diclofenac and phenylbutazone. N-terminal modifications are not a requirement for *E. coli* production of all P450s but have been used to produced functional P450s when native forms were not successfully expressed (Yun *et al*, 2006). A greater range of known CYP2C

and CYP2D substrates could also be tested against the recombinant CYP2C92 and CYP2D50 enzymes produced during the current study.

Extrapolation of pharmacokinetic data between species is commonplace, however comparative studies have shown significant differences in drug metabolism between horse and other species (Chauret *et al*, 1997, Martignoni *et al*, 2006), Mössner *et al* (2011)). In the horse, *in vivo* metabolism studies are much more frequently used than *in vitro* studies, however *in vivo* research does not allow for specific P450 isoforms involved in the metabolism of a drug to be determined. Drug-drug interactions (DDIs) are a potential consequence of dosing an animal with drugs where P450-specific metabolism is unknown and it is only due to the thorough characterisation of P450 substrates, inhibitors and inducers in human that these are able to be avoided in human medicine (Rendic, 2002). For substrates and inhibitors, recombinant human P450s are routinely used for fast and effective screening of drug candidates (hepatocytes cultures are needed to test for induction of P450s), Fasinu *et al*, 2012. At this stage, drugs that may cause significant DDIs will be removed from the development process (Zhang *et al*, 2012). While human recombinant P450s have been commercially available (in the form of Supersomes) since the 1990s, equine P450s were only first recombinantly produced in 2008 (DiMaio Knych and Stanley, 2008) and no isoforms have commercial availability. Many adverse drug reactions are known to occur in the horse, enphasizing the importance of P450 research in this species (Scarth *et al*, 2010, Brumbaugh, 2001).

Due to the financial resources and motivations of the horseracing industry, the vast majority of pharmacokinetic data in the horse comes from routine drug testing of competing animals where samples (particularly urine) are taken on racedays in order to detect prohibited substances (Scarth *et al*, 2010). Although these methods are

highly developed, they cannot fully take into account the effect of DDIs on urinary

metabolite profiles since CYP-specific metabolism of medications is unknown. For

this reason, knowledge of P450 metabolism in the horse is important clinically as

well as within the horseracing industry. To date, only the tip of the iceberg has been

reached in equine P450 research and to fully understand drug metabolism in the

horse all major P450s will need to be determined with substrate and inhibitors

elucidated for each. As more research is carried out in this area, differences between

horse and other species may become more evident and may therefore influence

treatment regimens as well as allowing for greater sensitivity for doping control within

the horse racing industry.

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Appendices

Appendix A: Primer sites (bold) for primers as highlighted in table 2.2.1.

>Ecab.P450.1

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>Ecab.P450.4 chr1

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>Ecab.P450.5

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>Ecab.P450.17

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>Ecab.P450.18

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>P450.Equ.18
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>P450.Equ.23
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>P450.Equ.37 chr6
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>CYP1A1

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>CYP2U1-like

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>CYP3A97

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>CYP2C26-like

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>CYP1B1-like

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