The development of an *in vitro* system for the production of drug metabolites using microsomal enzymes from bovine liver

A thesis submitted in fulfillment of the degree of Master of Science of

**Rhodes University**

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Abstract

Drug metabolism is a specialised subset of xenobiotic metabolism, pertaining to the breakdown and elimination of pharmaceutical drugs. The enzymes involved in these pathways are the cytochrome P450 family of isozymes. Metabolism is an important factor in determining the pharmacological effects of drugs. The main aim of this study was to develop a system whereby the major metabolites of drugs can be produced in vitro.

An in vitro system was developed and optimised using commercially prepared microsomes from rat liver and coumarin (by monitoring its conversion to 7-hydroxycoumarin) as a model. The optimum running conditions for the incubations were 50 µM coumarin, 50 µg protein/ml microsomes, 1 mM NADP⁺, 5 mM G6P and 1U/ml G6PDH incubated for 30 minutes at 38°C. The HPLC method for the detection of coumarin and 7-hydroxycoumarin was also validated with respect to linearity, reproducibility, precision, accuracy and lower limits of detection and quantification.

The system developed was then tested using microsomes prepared from fresh bovine liver on these ten drugs of interest in doping control in horse racing: diazepam, nordiazepam, oxazepam, promazine, acepromazine, chlorpromazine, morphine, codeine, etoricoxib and lumiracoxib. The bovine liver microsomes were prepared using differential centrifugation and had activity on a par with the commercial preparations. This in vitro system metabolised the drugs and produced both phase I and II metabolites, similar to those observed in humans and horses in vivo. For example, the major metabolites of the benzodiazepine drug, diazepam, nordiazepam, temazepam and oxazepam as well as the glucuronidated phase II products were all found after incubations with the bovine liver microsomes. The metabolism of the drugs was also investigated in silico using the computational procedure, MetaSite. MetaSite was able to successfully predict known metabolites for most of the drugs studied. Differences were observed from the in vitro incubations and this is most likely due to MetaSite using only human cytochrome P450s for analysis.
Declaration

I acknowledge that this is original work completed by myself, the undersigned, and is submitted for the degree of Master of Science of Rhodes University.

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Roxann Morrison
February 2011
I am immensely grateful to my supervisor, Dr Brendan Wilhelmi, without whose help, guidance and unfailing support this research would not have been possible. Thank you so much for inspiring in me the confidence and motivation to successfully complete this research project and as I move forward in my studies I am assured that this will continue to be the case.

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# Table of Contents

**Chapter One: Review of Literature**

1.1 Introduction 1

1.2 Drug Metabolism 1

1.3 Mechanisms of Drug Metabolism 4

  1.3.1 Cytochrome P450s 4

  1.3.1.1 Cytochrome P450-Mediated Hydroxylation 8

1.3.2 Glucuronidation 10

1.3.3 Sulphation 10

1.3.4 Glutathione Conjugation 11

1.4 Techniques to Study Drug Metabolism 11

1.5 Drugs 13

  1.5.1 Diazepam, Nordiazepam and Oxazepam 13

  1.5.2 Opioids: Morphine and Codeine 14

  1.5.3 Phenothiazine derivatives: Promazine, Acepromazine & Chlorpromazine 15

  1.5.4 NSAIDs: Lumiracoxib and Etoricoxib 16

1.6 Problem Statement 18

  1.6.1 Aims and Objectives 18

**Chapter Two: Development and Optimisation of an in vitro system for drug metabolism using rat liver microsomes**

2.1 Introduction 19

  2.1.1 Microsomes 19

  2.1.2 Coumarin 19

  2.1.3 Non-steroidal Anti-inflammatory Drugs 20

2.2 Methods and Materials 23

  2.2.1 HPLC-UV method for the detection of coumarin and 7-hydroxycoumarin 23
# Table of Contents

2.2.1.1 Running Conditions 23  
2.2.1.2 Validation of the HPLC method 23  
2.2.2 Incubation studies using rat liver microsomes 24  
2.2.2.1 Experimental Conditions 24  
2.2.2.2 Extraction of Metabolites 24  
2.2.2.3 Optimisation of rat liver incubations 25  
2.2.3 Incubation studies using two NSAIDs, etoricoxib and lumiracoxib 25  
2.2.3.1 HPLC-UV method for the detection of etoricoxib and lumiracoxib 25  
2.2.3.2 Running Conditions 26  
2.2.3.3 Extraction of Metabolites 26  
2.2.3.4 Mass Spectrometry Confirmation of Metabolites 26  
2.3 Results and Discussion 28  
2.3.1 Validation of the HPLC method for coumarin and 7-hydroxycoumarin 28  
2.3.2 Extraction of Metabolites 31  
2.3.3 Optimisation of the in vitro rat liver microsomal system 31  
2.3.3.1 Time Optimisation 31  
2.3.3.2 Temperature Optimisation 32  
2.3.3.3 Microsome Concentration Optimisation 33  
2.3.3.4 Substrate Concentration Optimisation 34  
2.3.3.5 NADPH Generating System Concentration Optimisation 35  
2.3.4 Incubation studies using two NSAIDs, etoricoxib and lumiracoxib 36  
2.3.4.1 Incubation with lumiracoxib 37  
2.3.4.2 Incubation with etoricoxib 38  
2.4 Conclusion 40
# Table of Contents

Chapter Three: *The Production of Microsomes from Bovine Liver and the Investigation of the Phase I Metabolism of Ten Drugs*

3.1 **Introduction** 41
   3.1.1 Phase I Metabolism 41
   3.1.2 Predicting metabolism *in silico* 42
   3.1.3 Metabolism *in vitro* using liver microsomes 42

3.2 **Methods and Materials** 44
   3.2.1 Production of Microsomes from Bovine Liver 44
   3.2.2 Phase I metabolism study 45
      3.2.2.1 *In silico* predictive metabolism using MetaSite 45
      3.2.2.2 Incubations with bovine liver 46
      3.2.2.3 Extraction of Metabolites 46
      3.2.2.4 Mass Spectrometry Analysis of Metabolites 46

3.3 **Results and Discussion** 47
   3.3.1 Production of Microsomes from Bovine Liver 47
   3.3.2 Phase I metabolism study 47
      3.3.2.1 *In silico* predictive metabolism using MetaSite 47
      3.3.2.1.1 Diazepam, Nordiazepam and Oxazepam 48
      3.3.2.1.2 Phenothiazine derivatives: Promazine, Acepromazine & Chlorpromazine 49
      3.3.2.1.3 Opioids: Morphine and Codeine 50
      3.3.2.1.4 NSAIDs: Lumiracoxib and Etoricoxib 51
      3.3.2.2 *In vitro* metabolism using bovine liver microsomes 60
      3.3.2.2.1 Diazepam, Nordiazepam and Oxazepam 60
      3.3.2.2.1 Opioids: Morphine and Codeine 62
      3.3.2.2.1 Phenothiazine derivatives: Promazine, Acepromazine & Chlorpromazine 64
      3.3.2.2.1 NSAIDs: Lumiracoxib and Etoricoxib 68

3.4 **Conclusion** 70
# Table of Contents

**Chapter Four: The Investigation of the Phase II Metabolism of Ten Drugs**

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>71</td>
</tr>
<tr>
<td>4.2 Methods and Materials</td>
<td>73</td>
</tr>
<tr>
<td>4.2.1 Phase II Metabolism Study</td>
<td>73</td>
</tr>
<tr>
<td>4.2.1.1 Incubations with bovine liver</td>
<td>73</td>
</tr>
<tr>
<td>4.2.1.2 Extraction of Metabolites</td>
<td>73</td>
</tr>
<tr>
<td>4.2.1.3 Mass Spectrometry Analysis of Metabolites</td>
<td>73</td>
</tr>
<tr>
<td>4.3 Results and Discussion</td>
<td>75</td>
</tr>
<tr>
<td>4.3.1 In vitro metabolism using bovine liver microsomes</td>
<td>74</td>
</tr>
<tr>
<td>4.3.1.1 Diazepam, Nordiazepam and Oxazepam</td>
<td>75</td>
</tr>
<tr>
<td>4.3.1.2 Opioids: Morphine and Codeine</td>
<td>77</td>
</tr>
<tr>
<td>4.3.1.3 Promazine, Acepromazine &amp; Chlorpromazine</td>
<td>78</td>
</tr>
<tr>
<td>4.3.1.4 NSAIDs: Lumiracoxib and Etoricoxib</td>
<td>81</td>
</tr>
<tr>
<td>4.4 Conclusion</td>
<td>82</td>
</tr>
</tbody>
</table>

**Chapter Five: Conclusions and Future Work**

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Conclusions</td>
<td>83</td>
</tr>
<tr>
<td>5.2 Future Work</td>
<td>85</td>
</tr>
</tbody>
</table>

**Reference List**

<table>
<thead>
<tr>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
</tr>
</tbody>
</table>

**Appendix A: Standard Curves**

<table>
<thead>
<tr>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Gradient elution profile of the HPLC method used for MS analysis</td>
<td>27</td>
</tr>
<tr>
<td>2.2</td>
<td>Reproducibility of the HPLC method for coumarin and 7-hydroxycoumarin</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>Accuracy and precision of the HPLC method for coumarin and 7-hydroxycoumarin</td>
<td>30</td>
</tr>
<tr>
<td>2.4</td>
<td>LOD and LOQ for coumarin and 7-hydroxycoumarin</td>
<td>30</td>
</tr>
<tr>
<td>2.5</td>
<td>Extraction Recoveries for coumarin and 7-hydroxycoumarin</td>
<td>31</td>
</tr>
<tr>
<td>3.1</td>
<td>Activity of Microsomes prepared from bovine liver and rat liver</td>
<td>46</td>
</tr>
<tr>
<td>3.2</td>
<td>Site of metabolism hot-spots as predicted by MetaSite</td>
<td>52</td>
</tr>
<tr>
<td>3.3</td>
<td>Summary of Metabolites predicted by MetaSite</td>
<td>55</td>
</tr>
<tr>
<td>4.1</td>
<td>Summary of phase II metabolites identified using LC-MS/MS</td>
<td>74</td>
</tr>
</tbody>
</table>
List of Figures

1.1 Schematic representation of drug metabolism 2
1.2 Structure of human CYP2C9, one of the major drug metabolising isoforms of CYP 6
1.3 Schematic representation of the monooxygenase electron transfer system 8
1.4 General reaction mechanism for P450-mediated hydroxylation 9

2.1 Schematic representation of the conversion of coumarin to 7-hydroxycoumarin as catalysed by CYP2A6. 20
2.2 Structure of etoricoxib and lumiracoxib 21
2.3 HPLC Chromatogram of coumarin and 7-hydroxycoumarin 28
2.4 Time Optimisation Study 32
2.5 Temperature Optimisation Study 33
2.6 Microsome Concentration Optimisation Study 34
2.7 Substrate Concentration Optimisation Study 35
2.8 NADPH Generating System Optimisation Study 36
2.9 Incubation of lumiracoxib - HPLC chromatogram 37
2.10 Incubation of lumiracoxib - MS spectra 38
2.11 Incubation of etoricoxib - HPLC chromatogram 39
2.12 Incubation of etoricoxib - MS spectra 39

3.1 Structures of the drugs studied 45
3.2 Structures of temazepam and nordiazepam 49
3.3 Phase I Metabolism of diazepam 61
3.4 Phase I Metabolism of nordiazepam 62
3.5 Phase I Metabolism of morphine 63
3.6 Phase I Metabolism of codeine 64
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7</td>
<td>Phase I Metabolism of promazine</td>
<td>65</td>
</tr>
<tr>
<td>3.8</td>
<td>Phase I Metabolism of acepromazine</td>
<td>66</td>
</tr>
<tr>
<td>3.9</td>
<td>Phase I Metabolism of chlorpromazine</td>
<td>67</td>
</tr>
<tr>
<td>3.10</td>
<td>Phase I Metabolism of lumiracoxib</td>
<td>68</td>
</tr>
<tr>
<td>3.11</td>
<td>Phase I Metabolism of etoricoxib</td>
<td>69</td>
</tr>
<tr>
<td>4.1</td>
<td>Phase II Metabolism of diazepam</td>
<td>75</td>
</tr>
<tr>
<td>4.2</td>
<td>Phase II Metabolism of nordiazepam</td>
<td>76</td>
</tr>
<tr>
<td>4.3</td>
<td>Phase II Metabolism of oxazepam</td>
<td>76</td>
</tr>
<tr>
<td>4.4</td>
<td>Phase II Metabolism of morphine</td>
<td>77</td>
</tr>
<tr>
<td>4.5</td>
<td>Phase II Metabolism of codeine</td>
<td>78</td>
</tr>
<tr>
<td>4.6</td>
<td>Phase II Metabolism of promazine</td>
<td>79</td>
</tr>
<tr>
<td>4.7</td>
<td>Phase II Metabolism of acepromazine</td>
<td>79</td>
</tr>
<tr>
<td>4.8</td>
<td>Phase II Metabolism of acepromazine sulfoxide</td>
<td>80</td>
</tr>
<tr>
<td>4.9</td>
<td>Phase II Metabolism of chlorpromazine</td>
<td>80</td>
</tr>
<tr>
<td>4.10</td>
<td>Phase II Metabolism of chlorpromazine sulfoxide</td>
<td>81</td>
</tr>
</tbody>
</table>
# List of Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
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<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, Disposition, Metabolism and Excretion</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Adenosine phosphosulphate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C6G</td>
<td>Codeine-6-Glucuronide</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Enhanced Product Ion</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
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<td>G6P</td>
<td>Glucose-6-Phosphate</td>
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<td>G6PDH</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
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<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>GST</td>
<td>Glutathione-S-transferase</td>
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<td>HLB</td>
<td>Hydrophili-Lipophilic Balance</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
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<td>MGST</td>
<td>Microsomal Glutathione-S-transferase</td>
</tr>
<tr>
<td>MIM</td>
<td>Monoisotopic Mass</td>
</tr>
</tbody>
</table>
## List of Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP(H)</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
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<td>NSAID</td>
<td>Nonsteroidal Anti-inflammatory Drug</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>PAPS</td>
<td>3'-Phosphoadenosine-5'-phosphosulphate</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>RME</td>
<td>Relative Mean Error</td>
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<td>RSD</td>
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<td>SPE</td>
<td>Solid Phase Extraction</td>
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<tr>
<td>SULT</td>
<td>Sulphotransferase</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate glucuronide transferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>XIC</td>
<td>Extracted Ion Chromatogram</td>
</tr>
</tbody>
</table>
1.1 Introduction
Xenobiotic metabolism is the set of metabolic pathways that modify the chemical structure of xenobiotics, compounds foreign to an organism's normal biochemistry, such as drugs and poisons. These pathways are a form of biotransformation present in all major groups of organisms, and are considered to be of ancient origin (Nelson, 2002). Drug metabolism is a specialised subset of xenobiotic metabolism, pertaining to the breakdown and elimination of pharmaceutical drugs. The enzymes involved in these pathways are the cytochrome P450 family of isozymes. These enzymes, which form a large superfamily of heme-containing monooxygenases, play a major role in drug metabolism and are therefore of interest in both academic and commercial areas of research. The study of drug metabolism is important especially for new drugs and drugs of interest in doping control.

1.2 Drug Metabolism
The reactions of drug metabolism often act to detoxify poisonous compounds; however, in some cases, the intermediates can be the cause of toxic effects (Jakoby & Zeigler, 1990). For example, acetonitrile is metabolised to hydrogen cyanide and formaldehyde; these metabolites are mainly responsible for the toxic effects of actonitrile (Pozzani et al., 1959). Drug metabolism can result in toxification or detoxification – the activation or deactivation of the chemical. While both occur, the major metabolites of most drugs are detoxification products. The rate of drug metabolism is an important determinant of the duration and intensity of the pharmacological action of drugs (Eddershaw et al., 2000).

Drug metabolism is divided into two phases. In phase I, enzymes such as cytochrome P450s introduce reactive or polar groups into drug. These modified compounds are then conjugated to polar compounds by transferases in phase II reactions. A schematic overview of drug metabolism is shown in figure 1.1.
Phase I reactions usually occur before phase II, though this is not necessarily always the case (Rouini et al., 2008; Kaeferstein, 2009). During phase I metabolism, polar functional groups are either introduced or unmasked, which results in the production of metabolites that are more polar than the original compounds. Phase I reactions (also known as non-synthetic reactions) may occur by oxidation, reduction, hydrolysis, cyclization, and decyclization. Oxidation involves the enzymatic addition of oxygen or removal of hydrogen, catalysed by mixed function oxidases, often in the smooth endoplasmic reticulum (ER) of the liver (Gonzalez & Tukey, 2005).

Figure 1.1: Schematic representation of drug metabolism. Drugs are metabolised in three general ways: drug A undergoes only phase I oxidation catalysed by cytochrome P450s before excretion. Drug B undergoes phase I metabolism, followed by conjugation to a highly polar substrate during phase II metabolism, which is catalysed by a transferase. Drug C does not undergo phase I metabolism, but instead undergoes conjugation in phase II metabolism.
In the case of pharmaceutical drugs, phase I reactions can lead either to the activation or inactivation of the drug. A common phase I oxidation reaction sometimes converts a pharmacologically inactive compound (a prodrug) to a pharmacologically active one. An example would be psilocybin, a prodrug, which is converted to the active psychedelic, psilocin (Passie et al., 2002). Codeine is also considered a prodrug, as one of its major metabolites is morphine (Haffen et al., 2000). Similarly, phase I metabolism can transform a relatively nontoxic molecule into a toxic one, such as the demethylated metabolite of pethidine, norpethidine, which is responsible for many of the drug’s negative side effects (Söderlund et al., 1999).

If the metabolites of phase I reactions are sufficiently polar, they may be readily excreted at this point (Murphy, 2001). However, many phase I products are not eliminated rapidly and undergo a subsequent reaction in which an endogenous substrate combines with the newly incorporated functional group to form a highly polar conjugate.

These phase II reactions, known as conjugation reactions, are usually detoxifying in nature, and are catalysed by a large group of broad-specificity transferases (Zamek-Gliszczynski et al., 2006), which in combination can metabolise almost any hydrophobic compound that contains nucleophilic or electrophilic groups. Sites where conjugation reactions occur include carboxyl (-COOH), hydroxyl (-OH), amino (-NH2), and sulfhydryl (-SH) groups. Products of conjugation reactions have increased molecular weight and are usually inactive, unlike Phase I reactions which can produce active metabolites. However, in some cases (such as in the metabolism of opioids) phase II metabolites may also be active (Hidetoshi et al., 1969).

The transferases that catalyse phase II reactions generally have a higher catalytic rate than CYPs which catalyse phase I reactions. Thus, if a drug is targeted to undergo phase I oxidation followed by conjugation during phase II, its rate of elimination is determined by the rate of phase I metabolism. Since phase II conjugations have a higher rate and produce highly water soluble products, phase II metabolism generally assures rapid and efficient detoxification of most drugs (Gonzalez & Tukey, 2005).
The smooth ER of the liver cell is the principal organ of drug metabolism, although every biological tissue has some ability to metabolize drugs. The liver is the first organ perfused by chemicals absorbed in the gut, and contains high concentrations of most drug-metabolising enzyme systems relative to other organs. If a drug is taken into the GI tract, enters hepatic circulation through the portal vein and becomes well-metabolized, it shows the first pass effect. First-pass metabolism is a phenomenon whereby the concentration of a drug is greatly reduced before it reaches the systemic circulation (Eddershaw et al., 2000).

Other sites of drug metabolism include epithelial cells of the gastrointestinal tract, lungs, kidneys, and the skin. These sites are usually responsible for localized toxicity reactions (Gonzalez & Tukey, 2005).

1.3 Mechanisms of drug metabolism

1.3.1 Cytochrome P450s
Cytochrome P450s (CYPs) are a ubiquitous superfamily of heme containing mono-oxygenase enzymes found in all organisms from bacteria and yeasts to higher mammals, including humans. P450 enzymes are all designated ‘CYP’ for cytochrome P450, a number to denote the family, a letter indicating the subfamily and another number to denote the individual gene encoding the enzyme (Nelson, 2004).

These enzymes are involved in a diverse range of metabolic reactions. These include oxidative, peroxidative and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids and prostaglandins. This diversity in substrate specificity has been attributed to the presence of multiple forms of cytochrome P450 in the ER of liver and other tissues. P450s are highly abundant in livers of most mammals including rats, mice and humans. There are over 11 000 distinct CYP proteins known (Nelson, 2009).

The cytochrome P450 system which, in mammals, is located primarily in the ER of the liver and small intestine, is also important in the detoxification of xenobiotic compounds
by oxidative metabolism (Ortiz de Montellano, 2005). One of the most relevant functions
of the cytochrome P450 system to human beings, as well as livestock and sport animals, is
its role in drug metabolism. The duration of action of many medications depends on their
rate of inactivation by the P450 system.

Many animals have as many or more genes encoding CYP proteins than humans. For
example, mice have genes for 101 CYPs, and sea urchins even more, perhaps as many as
120 genes. Most CYP enzymes are presumed to have monooxygenase activity, and this is
the case for most mammalian CYPs that have been investigated (Nelson, 1999). However,
gene and genome sequencing is far outpacing biochemical characterisation of enzymatic
function, although many genes with close homology to CYPs with known function have
been found (Johnson et al., 2005). The classes of CYPs most often investigated in non-
human animals are those involved in development (such those involved in steroid hormone
metabolism) or involved in the metabolism of xenobiotics. CYPs have been extensively
examined in mice and rats in order to facilitate the use of these model organisms in drug
discovery and toxicology (Anandatheerthavarada et al., 1997).

The human genome encodes more than 50 members of the family, whereas the genome of
the plant Arabidopsis encodes more than 250 members. All members of this large family
arose by gene duplication followed by subsequent divergence that generated a range of
substrate specificity. Humans have 57 sequenced CYP genes and 58 pseudogenes (Nelson,
2009). A pseudogene is a defective gene that does not produce a functional protein.
Pseudogenes are relics of gene duplications where one of the copies has degenerated and
lost its function.

The first structure for a cytochrome P450 isozyme was elucidated in 1987 (Poulus et al.,
1987), although the structure of a mammalian CYP was no known until 2001 (Podust et
al., 2001). Most CYPs are triangular in shape with a helix rich region and a beta sheet rich
region. They all have a long helix that spans the length of the molecule known as the I-
helix (Figure 1.2A). In the hydrophobic centre is the active site, containing a heme
prosthetic group. The substrate binding site is above the heme and below the I-helix.
Below the heme is a negatively charged cysteine. This thiolate anion interacts directly with
the heme iron, giving cytochrome P450 its strong UV absorption peak at 450 nm (Figure 1.2B). This cysteine and several flanking residues are highly conserved in CYPs (Williams et al., 2003). Because of the vast variety of reactions catalyzed by CYPs, the activities and properties of the many CYPs may differ.

**Figure 1.2:** Structure of human CYP2C9, one of the major drug metabolising isoforms of CYP (Williams et al., 2003). (A): I-helix highlighted in purple, (B) exploded view of the active site showing the co-ordination between the heme iron and the highly conserved cysteine residue. Images produced using RasMol.
Almost all mammalian CYPs are membrane-bound. They are found primarily at the ER and mitochondria. P450 enzymes usually act as the terminal oxidase in multicomponent electron-transfer chains. All known P450-containing monooxygenase systems share common structural and functional domains. Apart from the CYP itself, these systems contain one or more fundamental redox domain such as FAD-containing flavoproteins, FMN domains, ferredoxin and cytochrome \(b_5\). These ubiquitous redox domains, in various combinations, are widely distributed in biological systems. FMN domain, ferredoxin or cytochrome \(b_5\) transfer electrons between the flavin reductase (either a protein or domain) and P450. While P450-containing systems are found throughout all kingdoms of life, some organisms lack one or more of these redox domains.

One of the most important functions of the cytochrome P450 system is the metabolism of xenobiotics, especially pharmaceutical drugs. The most common action catalysed by P450s is a monooxygenase reaction in which one atom of molecular oxygen (\(O_2\)) is incorporated into the substrate, while the other is reduced to water:

\[
RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O
\]

Typically, the lone pair electrons required for this reaction are provided by the electron carrier molecule NAD(P)H via an associated reductase enzyme such as cytochrome P450 reductase (Denisov et al., 2005). Eukaryotic microsomal P450 enzymes and some bacterial P450s receive electrons from a FAD- and FMN-containing enzyme known as cytochrome P450 reductase (CPR). Microsomal CPR is membrane-bound protein that interacts with different P450s. A schematic representation of the electron transfer in the CPR-CYP system is shown in figure 1.3. The general scheme of electron flow in this system is:

\[
NADPH \rightarrow FAD \rightarrow FMN \rightarrow P450 \rightarrow O_2
\]
1.3.1.1 Cytochrome P450-Mediated Hydroxylation

Hydroxylation reactions play an important role in the synthesis of compounds such as steroids and bile salts, as well as the phase I metabolism of most drugs and toxins. The hydroxylation reactions of drug metabolism are catalysed by a large group of heme-containing monooxygenase enzymes, known as cytochrome P450s, and require NADPH and oxygen. In the case of P450-mediated hydroxylation, the oxygen atom of the newly incorporated hydroxyl group comes from molecular oxygen (O₂) rather than from water (Ortiz de Montellano, 2005). While one oxygen atom is incorporated into the substrate, the other is reduced to water.

Hydroxylation requires the activation of oxygen which is accomplished by the heme group of cytochrome P450. A general mechanism for P450-mediated hydroxylation is shown in figure 1.4. The hydroxylation reactions promoted by P450 enzymes are oxidation reactions, but they also consume the reductant NADPH. NADPH transfers its high-potential electrons to a flavoprotein, which transfers them, one at a time, to cytochrome P450 reductase (CPR). CPR transfers one electron to reduce the ferric (Fe³⁺) form of P450 to the ferrous (Fe²⁺) form. Without the addition of this electron, P450 will not bind oxygen.

**Figure 1.3:** Schematic representation of the monooxygenase electron transfer system (reproduced from Gonzalez & Tukey, 2005).
as only the ferrous form is able to bind oxygen. The binding of oxygen to the heme is followed by the acceptance of a second electron from CPR. The acceptance of this second electron leads to cleavage of the oxygen bond. One of the oxygen atoms is then protonated and released as water. The remaining oxygen atom forms a highly reactive ferryl intermediate. This intermediate removes a hydrogen atom from the substrate to form a radical. This free radical captures the hydroxyl group from the heme iron to form the hydroxylated product, returning the iron atom to the ferric state.

Figure 1.4: General reaction mechanism for P450-mediated hydroxylation (adapted from Ortiz de Montellano, 2005). (a): Substrate (RH) binds, (b): CPR transfers an electron to reduce the heme iron, (c): Oxygen is now able to bind to the heme, (d): a second electron is transferred from CPR, (e): cleavage of the oxygen bond, (f): release of water, (g): formation of ferryl intermediate, (h): removal of hydrogen from substrate to form radical, (i): radical captures hydroxyl from heme iron to form product, (j): product released and heme restored
1.3.2 *Glucuronidation*

Glucuronidation involves the conjugation of the substrate to the highly soluble glucuronic acid. Glucuronic acid is a structural analogue of glucose. Glucuronidation is catalysed by a group of enzymes, uridine disphosphate-glucuronyltransferases (UGTs). The site of glucuronidation is generally the liver, although the enzyme responsible can also be found in other organs such as the brain, heart, kidneys and adrenal gland. Within the cell, UGTs are localised to the ER membrane. These proteins are always membrane bound, with the enzyme portion face the lumen of the ER and are anchored in the membrane at the C-terminal end (Zamek-Gliszczynski *et al.*, 2006). The reaction proceeds via the intermediate UDP-glucuronic acid, glucuronic acid linked via a glycosidic bond to uridine diphosphate.

\[
\text{UDP-glucuronic acid} + \text{ROH} \rightarrow \text{R-glucuronide} + \text{UDP}
\]

The resulting glucuronides (or glucuronosides) are typically more water-soluble, in preparation for excretion. The conjugation of glucuronic acid results in a product with a higher molecular mass, a modification that increases its preference for biliary excretion. Hormones, such as estrone, may also be glucuronidated to allow for easier transport around the body (Luu-The *et al.*, 2006). Pharmacologists have linked drugs to glucuronic acid to allow for more effective delivery of a broad range of substances (Hollinger, 2003).

1.3.3 *Sulphation*

Sulphation complements glucuronidation in that it occurs at many of the same sites on the substrate as glucuronide conjugation (Pang *et al.*, 1983). The sulphation of drugs occurs mainly in the cytosol and is catalysed by a group of transferases called sulphotransferases (SULTs). SULTs catalyse the conjugation of sulphonate (SO₃⁻) to sites on drugs such as hydroxyl and monoamide groups. Inorganic sulphate is provided by 3’-phosphoadenosine 5’-phosphosulphate (PAPS) which is the sulphonate (SO₃⁻) donor in all sulphate conjugation reactions (Zamek-Gliszczynski *et al.*, 2006). PAPS is rapidly formed in the cytosol when inorganic sulphate is conjugated to adenosine monophosphate (AMP) to form adenosine phosphosulphate (APS). APS is then phosphorylated by APS kinase to form PAPS.
PAPS + ROH → R–O–SO₂–OH + PAP

Sulphation is reversible; sulphate can be removed from sulphate conjugates by sulphatases and subsequently reconjugated, resulting in ‘futile cycling’ between the parent and the sulphate conjugate (Pang et al., 1994).

1.3.4 Glutathione conjugation

Compounds susceptible to glutathione conjugation may be electrophilic parent compounds, phase I metabolites and even some phase II products. Glutathione is a tripeptide that is synthesised from γ-glutamic acid, cysteine and glycine. It is found in cell in both an oxidised and reduced state. The ratio of oxidised: reduced glutathione is important in maintaining the cell in a reduced state (Ponpella, 2003). The main enzymes responsible for glutathione conjugation belong to a family of enzymes known as glutathione S-transferases, which occur predominantly in the cytosol. Some GSTs may also be membrane bound ER or mitochondrial proteins (Zhang et al., 2004). Most mammals express three microsomal glutathione S-transferases, abbreviated as MGST1 - 3 (Zhang et al., 2004). GSTs catalyse the conjugation of reduced glutathione - via the sulphydryl group of the cysteine moiety - to electrophilic centers on a wide variety of substrates.

\[ \text{GSH} + \text{R} \rightarrow \text{R–GSH} \]

1.4 Techniques to study drug metabolism

Techniques used to study drug metabolism range from in vivo administration trials to in vitro assays and in silico predictive modelling.

Drugs are metabolised in the liver before being excreted in the urine or bile. Currently, most drug detection methods require knowledge of drug metabolism as urine is most often the matrix used for analysing metabolites. These administration trials are carried out on live animals in vivo. In vivo methods allow for the most accurate representation of real life situations as all biotransformations can be considered. However, these techniques require
animal experimentation which can give rise to ethical considerations as well as considerable cost and resources. The analyses are time consuming and drugs with unknown metabolism cannot be easily studied. In addition, the matrix, urine, often complicates the analysis as there are often interfering contaminants.

*In vitro* methods using microsomes is a useful approach to study the breakdown pathways of drugs with previously unknown metabolism, such as designer drugs. *In vitro* methods are now commonplace in the drug development industry and are often coupled with *in vivo* studies. Microsomes are a valuable tool for investigating the metabolism of compounds as well as for examining drug-drug interactions. Disadvantages of using *in vitro* methods include ethical issues regarding the supply of tissue, the lack of a whole biological system, and the lack of sufficient correlation between *in vivo* and *in vitro* analyses. *In vitro* technologies are advantageous in many ways: they do not require animal experimentation, often produce ‘cleaner’ extracts for analysis and can be adjusted to study the mechanistic aspects of drug metabolism (Scarth *et al.*, 2010b).

Microsomes contain P450 enzymes and are turbid suspensions of vesicle-like artifacts formed from the ER when eukaryotic cells are broken up. Thus, by definition, microsomes are not ordinarily present in living cells (Pearce *et al.*, 1996). They can be concentrated and separated from other cellular debris by differential centrifugation. A typical method for the production of microsomes involves the homogenisation of the source tissue before centrifugation at low speed to remove cell and nuclear debris followed by ultracentrifugation at high speeds (generally at speeds of 100 000 g) to sediment out ER fragments. This way, cytochrome P450 enzymes are isolated and concentrated while soluble enzymes remain in the supernatant. Microsomes prepared from the liver are the most commonly used, although microsomes from other sites of drug metabolism such as the lung, brain and small intestine have also been investigated (Scarth *et al.*, 2010a, Cox & Emili, 2006).

Another useful tool in the study of drug metabolism is predictive metabolism software, such as MetaSite, which is used in this study. *In silico* predictive modelling is useful in drug design, to predict the production of harmful products from a new drug as well as
determine the metabolic sites on the compound. It is important to predict the potential drug-drug interactions and metabolism of a potential drug candidate in order to ensure it will reach its intended target in sufficient concentrations to still be effective (Zhou et al., 2006). There are several parameters by which drug-likeness can be assessed. Of these is the ‘rule of five’, a rule of thumb guide used to assess drug-likeness. Christopher Lipinski classified a compound as drug-like if it satisfied the following parameters: a molecular weight less than 500 Da, a distribution coefficient of less than 5, no more than 5 hydrogen bond donor atoms and no more than 10 hydrogen bond acceptor atoms. The rule of five is so called as all the parameters are multiples of 5 (Lipinski et al., 2001).

1.5 Drugs

In this study the metabolism of ten drugs was investigated. The drugs used in this study were diazepam and its derivatives, nordiazepam and oxazepam, the opioids morphine and codeine, promazine and its derivatives, acepromazine and chlorpromazine, and two non-steroidal anti-inflammatory drugs, lumiracoxib and etoricoxib.

1.5.1 Benzodiazepines: Diazepam, Nordiazepam and Oxazepam

Diazepam is a benzodiazepine derivative drug. Nordiazepam is the demethylated active metabolite and oxazepam is demethylated and hydroxylated metabolite of diazepam. All share common mechanisms of action and pharmacokinetics. Benzodiazepines act by binding to the neurotransmitter receptor, GABA$_A$, which enhances the binding of $\gamma$-aminobuteric acid (GABA) and inhibits neuronal firing (Yakushiji et al., 1989).

Diazepam is commonly used for treating anxiety, and insomnia in humans. It possesses anticonvulsant, sedative, skeletal muscle relaxant, and amnestic properties. In veterinary medicine, diazepam is used as a short-term sedative and muscle relaxant for horses (Bettchart-Wolfensberger & Larenza, 2007), and as a treatment for seizures in cats and dogs (Sloan et al., 1991). Nordiazepam and oxazepam are also used as sedatives and for the treatment of anxiety, although less potent than diazepam (Shini, 2000; Rouini et al., 2008).
In humans, diazepam undergoes oxidative metabolism by demethylation, hydroxylation as well as glucuronidation to produce several pharmacologically active metabolites. The main active metabolite of diazepam is desmethyldiazepam (known as nordiazepam). There are also two minor metabolites, temazepam (a hydroxylated product) and oxazepam (a demethylated and hydroxylated product). These metabolites are conjugated with glucuronide during Phase II metabolism, and are primarily excreted in the urine (Rouini et al., 2008). Nordiazepam, the metabolite of diazepam, is only slightly metabolised. It is hydroxylated to oxazepam during phase I metabolism and both the parent, nordiazepam, and oxazepam undergo phase II conjugation to glucuronide before excretion in the urine. Oxazepam does not undergo phase I metabolism, but is conjugated to glucuronide (Gobbi et al., 1987).

Diazepam is highly lipophilic, and in horses, it has high bioavailability and is readily absorbed. It is rapidly metabolised in the liver to produce active metabolites (nordiazepam and oxazepam) and thus, the effects of the drug are longer lasting. The metabolism of diazepam in horses is similar to that in humans. The major metabolite observed is nordiazepam, with temazepam and oxazepam found in smaller quantities. Diazepam and its metabolites can be detected in the urine of horses for up to 58 hours after administration (Shini, 2000).

1.5.2 Opioids: Morphine and Codeine

Opioids, such as morphine and codeine, are among the oldest class of drugs and are used to treat acute pain. Opioids act directly on the central nervous system (CNS) to relieve pain. This is achieved by the binding of opioids to specific opioid receptors in the brain and spinal column.

Morphine is a potent analgesic and a fast-acting narcotic, and binds very strongly to μ-opioid receptors. Morphine is subject to extensive first-pass metabolism, with a large proportion being broken down in the liver. Approximately 87% of a dose of morphine is excreted in the urine within 72 hours of administration. In humans, morphine is primarily metabolized into morphine-3-glucuronide (M3G) and the active morphine-6-glucuronide
(M6G) via glucuronidation by phase II metabolism enzyme UDP-glucuronosyl transferase -2B7 (UGT2B7). M6G has analgesic effects greater than that of the parent morphine. P450s involved in phase I metabolism play a lesser role, although the dealkylated product, normorphine is observed (Anderson et al., 2003). In horses, morphine metabolism is primarily glucuronidation to produce M3G and M6G as in humans. Morphine is detectable in the urine for up to 144 hours after administration (Combie et al., 1983).

Codeine is an opiate used for its analgesic, antitussive, and antidiarrheal properties. Codeine is considered a prodrug, since it is metabolised in vivo to the primary active compounds morphine and codeine-6-glucuronide (C6G), which has analgesic activity similar to codeine. Following this, morphine and norcodeine are metabolised to normorphine, M3G and M6G and norcodeine glucuronide. Although metabolism of codeine to morphine and consequentlty to M6G is limited, it contributes to the overall effect of the drug as both products have a strong effect (He et al., 1998).

1.5.3 Phenothiazone derivatives: Promazine, Acepromazine and Chlorpromazine
Phenothiazine tranquilizers, such as promazine, are used in veterinary medicine as sedatives in horses (Dewey et al., 1981) and as antipsychotics and strong sedatives in humans (Saracino et al., 2008). Such phenothiazines are highly lipophilic and have a high oral availability. These drugs undergo phase I metabolism to produce several active and inactive metabolites. Due to the presence of the active metabolites, the plasma half-life of phenothiazine derivatives can range from 14 to 78 hours (Saracino et al., 2008).

Acepromazine is frequently used in animals as a sedative and antiemetic; its main function is to calm anxious animals. Acepromazine is a prohibited drug, and its use is restricted by many equestrian organizations. In horses, the major metabolites of acepromazine include 7-hydroxy acepromazine and 2-(1-hydroxyethyl)-7-hydroxy-promazine and 2-(1-hydroxyethyl)promazine sulphoxide (Dewey et al., 1981).

Promazine is used for both equine and human administration. In humans, it has been used to treat psychosis and schizophrenia (Saracino et al., 2008) and is used as a sedative in
horses (Dewey et al., 1981). Promazine is metabolised in the liver of horses to several metabolites, including 3-hydroxydesmethyl promazine and 3-hydroxypromazine and two minor metabolites, promazine N-oxide and promazine N-oxide sulphoxide (Dewey et al., 1981). In humans the major metabolites of promazine are reported as desmethyl promazine and promazine sulphoxide (Wójcikowski et al., 2003).

Chlorpromazine is also used as a sedative in horses as well as a treatment for schizophrenia in humans. It primarily metabolised to desmethyl chlorpromazine, chlorpromazine sulphoxide and 7-hydroxy chlorpromazine (Wójcikowski et al., 2010). Chlorpromazine can be used as an antiemetic and muscle relaxant in horses however, it is not commonly prescribed due to negative side effects such as ataxia.

Phase II glucuronide and sulphate conjugates of promazine, acepromazine and chlorpromazine have also been identified as metabolites in horses (Weir & Sanford, 1972).

1.5.4 NSAIDs: Lumiracoxib and Etoricoxib
Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation, pain and fever by blocking the action of cyclooxygenases (COXs). Although such drugs are an important component of therapy for these diseases, overuse and misuse of NSAIDs can result in gastrointestinal injury and kidney damage in horses (Kvaternick et al., 2007). NSAIDs are considered drugs of abuse in horseracing as they can be used to mask the pain and inflammation of injuries in race horses.

Most NSAIDs act as nonselective inhibitors of the enzyme cyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes (Blandizzi et al., 2009). COX catalyzes the formation of prostaglandins and thromboxane from arachidonic acid. Arachidonic acid is the precursor substrate for the production of prostaglandins F, D & E which act as messenger molecules in the process of inflammation. NSAIDs also have antipyretic activity and can be used to treat fever, caused by elevated levels of prostaglandin E2 (PGE2). PGE2 signals to the hypothalamus to
increase the body’s thermal set point by altering the firing rate of neurons in the hypothalamus which control thermoregulation (Botting, 2006).

Both lumiracoxib and etoricoxib are NSAIDs that act as COX-2 selective inhibitors (Nageswara Rao et al., 2005). Cyclooxygenase 2 (COX-2) is an enzyme responsible for inflammation and pain, and is inducible, becoming abundant in activated macrophages and other cells at sites of inflammation. The classical COX inhibitors are not selective (i.e. they inhibit all types of COX), and the main adverse effects of their use are peptic ulceration and dyspepsia. Because COX-2 is usually specific to inflamed tissue, there is much less gastric irritation associated with COX-2 inhibitors, leading to a decreased risk of peptic ulceration (Botting, 2006).

Etoricoxib is a relatively new COX-2 selective inhibitor that has been developed to treat pain and the symptoms of osteoarthritis and rheumatoid arthritis. It has a high oral bioavailability and in humans, metabolism is reported to be extensive and 6'-methyl-hydroxylated and 1'-N-oxidation metabolites are excreted in the urine (Rodrigues et al., 2003). The major metabolites of etoricoxib observed in horses are hydroxy etoricoxib, etoricoxib-1’-N-oxide and hydroxyetoricoxib-1’-N-oxide. Hydroxy etoricoxib glucuronide is a prominent phase II metabolite (de Kock et al., 2008a).

Lumiracoxib is a new generation NSAID with improved gastrointestinal tolerance due to its highly selective COX-2 inhibition. It binds to a different site on the COX-2 receptor to other COX-2 inhibitors and has the highest COX-2 selectivity of any NSAID (Nageswara Rao et al., 2005). In human plasma the major metabolites were identified as 5-carboxylumiracoxib, 4'-hydroxy-5-carboxylumiracoxib and 4'-hydroxylumiracoxib. It was shown that 4-hydroxylumiracoxib also possess COX-2 inhibitory effects with similar potency to that of the parent compound. Several glucuronic acid conjugates of lumiracoxib metabolites have also been observed (Mangold et al., 2004). In horses, lumiracoxib is metabolised to 4'-hydroxylumiracoxib, 5-hydroxymethylumiracoxib, 5-carboxy lumiracoxib, 4'-hydroxy- 5-carboxylumiracoxib, 5-carboxylumiracoxib lactam and 4'-hydroxy-5-carboxy-lumiracoxib lactam (de Kock et al., 2008b). Several glucuronide conjugates of lumiracoxib have also been identified (Kalbag et al., 2004).
1.6 Problem Statement

The study of drug metabolism is often hindered by a lack of knowledge of the metabolites produced. This is of particular concern in new ‘designer’ drugs and drugs of abuse. Enzymes of the cytochrome P450 family catalyse more than 90% of the oxidative metabolic reactions of drug metabolism. The involvement of these enzymes in the biotransformation of a new drug is usually thoroughly investigated before it can be marketed. However, for drugs of the illicit market or drugs of abuse, such data is not available. Often, ethical concerns prevent the investigation of such drugs using in vivo techniques. The same problem is also encountered when studying the metabolism of drugs in animals, for which little data is reported as compared to humans. This study endeavoured to develop a system whereby the major metabolites of drugs of interest in horse racing doping control can be produced in vitro using animal microsomal enzymes.

1.6.1 Aims and Objectives

With this problem in mind, the aims for this research project were:

1. The development of an in vitro system for the production of drug metabolites using animal liver microsomal enzymes.
2. The analysis of these metabolites using HPLC.
3. The determination of the key metabolites of drugs of interest produced in vitro and in silico.

In order to achieve these aims, the following objectives were set:

1. Optimisation of production, extraction and analytical techniques, including HPLC-UV, SPE and LC-MS/MS.
2. Development and optimisation of a microsomal system using commercially prepared rat liver microsomes and coumarin.
3. Preparation of microsomes from bovine liver.
4. Investigation of phase I metabolism of drugs using the bovine liver microsomes.
5. Investigation of phase II metabolism of drugs using the bovine liver microsomes.
Chapter Two

Development and Optimisation of an *in vitro* system for drug metabolism using rat liver microsomes

2.1 Introduction

2.1.1 Microsomes

Microsomes are a valuable tool for investigating the metabolism of compounds, examining drug-drug interactions and can be used to produce drug metabolites *in vitro*. *In vitro* technologies are advantageous in many ways: they do not require animal experimentation, and often produce ‘cleaner’ extracts for analysis (Scarth *et al.*, 2010b).

In this study, commercially prepared microsomes from rat liver were used for the development and optimisation of an *in vitro* system for the production of drug metabolites. A commercial microsomal system would serve as a positive control for microsome activity while running parameters and methodology were developed and optimised. An animal model (opposed to the commercially prepared human microsomes) was used as this study is primarily concerned with the metabolism of drugs in race horses. Rat liver was chosen as the source tissue as the commercial preparations are a readily available and cost effective tool for development purposes. The use of rat liver microsomes and coumarin was able to provide a positive control system for the production of drug metabolites.

2.1.2 Coumarin

In this study, the activity of microsomes was monitored using coumarin as a control compound for microsomal activity during development and optimisation of the running parameters for the liver microsomal system.

Coumarin is a benzopyrone found in many plants, and has been used in perfumes since 1882. It is a bitter-tasting appetite suppressant, and is probably produced by plants as a defense chemical to discourage predation (Yamazaki *et al.*, 1996). Coumarin is used in the
pharmaceutical industry as a precursor molecule in the synthesis of a number of synthetic anticoagulant pharmaceuticals similar to dicoumarol, notably warfarin (Holbrook et al., 2005).

In humans, coumarin is hydroxylated to 7-hydroxycoumarin by CYP2A6 (Craeven et al., 1959). The equivalent CYP isoforms in other mammals are also able to catalyse this reaction (Chauret et al., 1997). CYP2A6 is localised in the cell to the ER and is thus present in microsome fractions. The formation of the 7-hydroxycoumarin is used to monitor microsome activity as it is a biomarker for monitoring CYP2A6 activity (Draper et al., 1997).

![Coumarin 7-Hydroxycoumarin](image)

**Figure 2.1:** Schematic representation of the conversion of coumarin to 7-hydroxycoumarin as catalysed by CYP2A6.

### 2.1.3 Non-steroidal Anti-inflammatory Drugs

Once the initial experimental conditions were established, the liver microsomal system was tested using two non-steroidal anti-inflammatory drugs, lumiracoxib and etoricoxib.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat inflammation, pain and fever by blocking the action of cyclooxygenases (COX) and are important in the treatment of painful conditions, such as arthritis. Most NSAIDs act as nonselective inhibitors of cyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isozymes. COX enzymes catalyse the formation of prostaglandins from arachidonic acid. Prostaglandins act as messenger molecules in the process of inflammation. COX-2 is inducible, becoming abundant in activated
macrophages and other cells at sites of inflammation. Because COX-2 is usually specific to inflamed tissue, there are fewer negative side effects associated with COX-2 selective inhibitors.

Etoricoxib (figure 2.2 A) was developed for the treatment of pain and the symptoms of osteoarthritis and rheumatoid arthritis. In humans, metabolism is reported to be extensive and 6'-methyl-hydroxylated and 1'-N-oxidation metabolites are excreted in the urine (Rodrigues et al., 2003). In horses, etoricoxib is metabolised to hydroxyethyletoricoxib and hydroxyethyletoricoxib-1’-N-oxide (de Kock et al., 2008a).

Lumiracoxib (figure 2.2 B) is a new generation NSAID which has highly selective COX-2 inhibition. In human plasma the major metabolites were identified as 5-carboxylumiracoxib, 4'-hydroxy-5-carboxylumiracoxib and 4'-hydroxylumiracoxib (Mangold et al., 2004). It was shown that 4-hydroxylumiracoxib also possess COX-2 inhibitory effects with similar potency to that of the parent compound. Several glucuronic acid conjugates of lumiracoxib metabolites have also been observed. In horses, lumiracoxib is metabolised to 4'-hydroxylumiracoxib, 5-hydroxymethylumiracoxib, 5-carboxylumiracoxib, 4'-hydroxy-5-carboxylumiracoxib, 5-carboxylumiracoxib lactam and 4'-hydroxy-5-carboxylumiracoxib lactam (de Kock et al., 2008b).
This chapter details the development and optimisation of an *in vitro* liver microsomal system for the production of drug metabolites using commercially prepared rat liver microsomes and coumarin as a model. The system was optimised for various running parameters such as time and substrate concentration. The solid phase extraction (SPE) and HPLC-UV method for the extraction and detection of the metabolites produced were also optimised and validated.
2.2 Methods and Materials

2.2.1 HPLC-UV method for the detection of coumarin and 7-hydroxycoumarin

2.2.1.1 Running Conditions
Coumarin and 7-hydroxycoumarin were detected on a reverse phase C18 column (15 cm x 4.6 mm, 5 µm Supelco ® Discovery HS column with a 2 cm x 4.0 mm, 5 µm Discovery HS C18 guard column), using a Beckman System Gold® System with a 166 UV Detector System. An isocratic elution of 60% acetonitrile and 40% water at a flow rate of 1 ml/minute was used. The injection volume was 20 µl for all samples and coumarin and 7-hydroxycoumarin were detected at 274 nm.

2.2.1.2 Validation of the HPLC method
The HPLC method was validated for coumarin and 7-hydroxycoumarin separately, with respect to the following parameters: linearity, accuracy and precision, reproducibility, and limits of detection and quantitation.

Standards of a concentration range 0 - 1000 µM for both coumarin and 7-hydroxycoumarin were prepared in 60% acetonitrile from stock solutions. Calibration curves for both compounds were prepared using these standards.

Accuracy and precision were determined by assaying each standard concentration in triplicate over three days. Concentrations of the samples were calculated from the resulting peak areas using the regression equation from the calibration curves. Precision was given as relative standard deviation (RSD), and accuracy as relative mean error (RME) of calculated concentrations.

Reproducibility of the method was assessed by performing repeated analyses of selected coumarin and 7-hydroxycoumarin standards and comparing the retention times. Intra-day reproducibility was determined by assaying each sample concentration in triplicate on the same day. The inter-day reproducibility was determined by assaying each sample concentration in triplicate over three days. Reproducibility was given as relative standard deviation (RSD) of the retention times.
The lower limits of detection and quantitation were determined using 32Karat analytical software. The limit of detection was defined as the concentration of the standards where the peak area yielded a signal-to-noise ratio of three. The limit of quantitation was defined as the concentration of the standards where the peak area yielded a signal-to-noise ratio of ten.

All statistical analyses were performed using the R statistical software.

2.2.2 Incubation studies using rat liver microsomes

The in vitro microsome incubation method was developed using commercially prepared microsomes from male rat liver (Sigma, product number: M9066) to convert coumarin to 7-hydroxycoumarin.

2.2.2.1 Experimental Conditions

Coumarin was incubated with the commercial microsomes and an NADPH generating system. The method used was adapted from Draper et al. (1997). Initial incubations of 1 ml were set up containing: 50 µg microsomes, 50 mM phosphate buffer (pH 7.4), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM G6P, 1 U/ml G6PDH. The reaction was initiated by the addition of the NADPH generating system (G6P, G6PDH and NADP⁺) and then incubated at 38°C for 20 min. The reaction was then terminated by the addition of 125 µl 15% (w/v) trichloroacetic acid.

2.2.2.2 Extraction of Metabolites

The metabolites generated (in this case, 7-hydroxycoumarin) were extracted from the incubation mixture using Oasis® HLB 3cc (60 mg) solid phase extraction cartridges. The cartridges were preconditioned with 2 ml acetonitrile, then 2 ml deionised water. The total incubation mix (1 ml) was added and allowed to elute under vacuum. The cartridges were washed with 1 ml 5% acetonitrile and the samples eluted with 1 ml acetonitrile.

The extraction efficiency was measured as percentage of coumarin and 7-hydroxycoumarin recovered. Incubation mixes were set up without microsomes or the NADPH generating system. The test samples were extracted using solid phase extraction and assayed using
HPLC as described in section 2.2.1.1. The percentage recovery was calculated by comparing the peak area of these samples to a pure standard of the same concentration as initially added to the test samples.

2.2.2.3 Optimisation of rat liver incubations

The *in vitro* microsomal system was optimised using commercially produced rat liver microsomes and coumarin as a positive control. The method was optimised for the following parameters: time, temperature, concentration of microsomes, concentration of substrate (coumarin) and the concentration of the components of the NADPH generating system.

For optimisation with respect to time, the reaction was carried out using a time range of 0 - 6 h. The range of temperatures around the given optimal temperature (38°C) was tested. A range of microsome concentrations was tested from 5 µg/ml to 200 µg/ml. The concentration of coumarin (the substrate) was also tested using the range 25 - 100 µM. The concentrations of the constituents of the NADPH generating system (NADP+, glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PDH)) were optimised in the same way as the substrate concentration. The concentration range was 0 - 5 mM, 0 - 10 mM and 0 - 2 U/ml for NADP+, G6P and G6PDH respectively.

The samples were extracted and assayed using HPLC. The activity of the microsomes was calculated as amount of 7-hydroxycoumarin produced per millilitre of reaction mix.

2.2.3 Incubation studies using two NSAIDs, etoricoxib and lumiracoxib

The rat liver microsome incubation method developed using coumarin was applied to the metabolism of two non-steroidal anti-inflammatory drugs, etoricoxib and lumiracoxib.

2.2.3.1 HPLC methods for the detection of etoricoxib and lumiracoxib

Lumiracoxib and Etoricoxib were detected on a reverse phase C18 column (15 cm x 4.6 mm, 5 µm Supelco ® Discovery HS column with a 2 cm x 4.0 mm, 5 µm Discovery HS C18 guard column), using a Beckman System Gold® System with a 166 UV Detector System. An isocratic elution of 60% acetonitrile and 40% water, and 60% acetonitrile and
40% water at pH 3.5, at a flow rate of 1 ml/minute was used for lumiracoxib and etoricoxib respectively. The injection volume was 20 µl for all samples and lumiracoxib and etoricoxib were detected at 274 nm.

2.2.3.2 Running Conditions
Both lumiracoxib and etoricoxib (50 µM) were incubated separately with the commercial rat microsomes and an NADPH generating system as described in section 2.2.2.1. Incubations of 1 ml were set up containing 10 µg microsomes, 50 mM phosphate buffer (pH 7.4), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM G6P, 1 U/ml G6PDH. The reaction was initiated by the addition of the NADPH generating system (G6P, G6PDH and NADP⁺) and the mixes incubated at 38°C for 20 minutes. The reaction was then terminated by the addition of 125 µl 15% (w/v) trichloroacetic acid.

2.2.3.3 Extraction of Metabolites
The lumiracoxib and etoricoxib metabolites generated were extracted from the incubation mixtures using Oasis® HLB 3cc (60 mg) solid phase extraction cartridges. The cartridges were preconditioned with 2 ml acetonitrile, then 2 ml deionised water. The total incubation mix (1 ml) was added and allowed to elute under vacuum. The cartridges were washed with 1 ml 5% acetonitrile and the samples eluted with 1 ml acetonitrile. Extracts were stored at -20°C prior to analysis.

2.2.3.4 Mass Spectrometry Confirmation of Metabolites
Mass spectrometry analysis of the NSAIDs incubations was conducted by the Laboratory of the National Horseracing Authority of Southern Africa, Turffontein.

The HPLC method used a Gemini C18 column (150 x 2 mm) and a gradient elution with a flow rate of 250 µl/min. Solvent A was 5 mM ammonium acetate and 0.1% formic acid in water and solvent B was 5 mM ammonium acetate and 0.1% formic acid in acetonitrile. The gradient elution profile is summerised in table 2.1.
Table 2.1: Gradient elution profile of the HPLC method used for MS analysis

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>0.50</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>3.00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>11.00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>13.00</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>15.00</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

The instrument employed was a MDS Sciex API 4000 QTrap (Applied Biosystems, California, USA) equipped with LightSight metabolic software. It was operated in either positive or negative turboionspray mode. In the positive ion mode the source voltage was 5 kV and in negative ion mode it was -4.5 kV. The source temperature was 400°C while the curtain gas, nebuliser flow gas and the heater gas were 20, 40 and 60 units of nitrogen respectively.
2.3 Results and Discussion

2.3.1 Validation of HPLC method for coumarin and 7-hydroxycoumarin

Coumarin and 7-hydroxycoumarin were separated and their retention times were 2.97 minutes and 1.96 minutes respectively (figure 2.3). The linear range for both compounds was found to be 25 - 1 000 µM. Calibration curves for coumarin and 7-hydroxycoumarin were constructed using this range for both compounds. A high correlation between the peak area and the concentration of coumarin and 7-hydroxycoumarin was observed. These calibration curves are shown in appendix A (p. 93).

Reproducibility (table 2.2) for both coumarin and 7-hydroxycoumarin was measured by calculating the RSD for all concentrations in the linear range (25 - 1 000 µM). Analyses were conducted in triplicate over three days. The RSD was 0.56% and 0.39% for coumarin and 7-hydroxycoumarin respectively, indicating reproducibility of the HPLC method.
Table 2.2: Reproducibility of the HPLC method for coumarin and 7-hydroxycoumarin, measured for a concentration range (25 - 1 000 µM) in triplicate over three days.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention Time (mean ± SD) (minutes)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>2.974 ± 0.028</td>
<td>0.5645</td>
</tr>
<tr>
<td>7-Hydroxycoumarin</td>
<td>1.962 ± 0.011</td>
<td>0.3963</td>
</tr>
</tbody>
</table>

Precision (table 2.3) for both coumarin and 7-hydroxycoumarin was measured by calculating the RSD for selected concentrations in the linear range. These were 25, 50, 100 and 500 µM for both coumarin and 7-hydroxycoumarin. Analyses were conducted in triplicate over three days. The relative standard deviation was less than 5% for all concentration of both coumarin and 7-hydroxycoumarin, indicating satisfactory precision.

Table 2.3 also summarises the accuracy for both coumarin and 7-hydroxycoumarin. Accuracy was measured by calculating the relative mean error (RME) for selected concentrations in the linear range. These were 25, 50, 100 and 500 µM for both coumarin and 7-hydroxycoumarin. Analyses were conducted in triplicate over three days. The relative mean error was less than 5% for all concentration of both coumarin and 7-hydroxycoumarin. This is to be expected as the samples analysed were pure standards. Some of the relative mean errors have negative values which indicates that the calculated concentration is higher than the nominal concentration. All RME are within ± 5% indicating a high degree of accuracy.

These data (for both accuracy and precision) fall well within the defined values for biological samples (Shah et al., 1992).
Table 2.3: Accuracy and precision of the HPLC method for coumarin and 7-hydroxycoumarin measured in triplicate over three days.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (μM)</th>
<th>Calculated Concentration (mean ± SD) (μM)</th>
<th>RSD (%)</th>
<th>RME (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>25</td>
<td>25.14 ± 0.09</td>
<td>0.35</td>
<td>-0.54</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.53 ± 0.13</td>
<td>0.26</td>
<td>-1.05</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99.96 ± 0.73</td>
<td>0.73</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>498.54 ± 26.72</td>
<td>4.93</td>
<td>0.29</td>
</tr>
<tr>
<td>7-Hydroxycoumarin</td>
<td>25</td>
<td>25.95 ± 0.37</td>
<td>1.44</td>
<td>-3.78</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>51.56 ± 0.08</td>
<td>0.16</td>
<td>-3.18</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>105.93 ± 0.19</td>
<td>0.86</td>
<td>-5.93</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>517.27 ± 0.27</td>
<td>0.05</td>
<td>-3.45</td>
</tr>
</tbody>
</table>

The limit of detection was calculated using the 32Karat software to be 511.86 ± 0.57 nM for coumarin and 736.31 ± 0.42 nM for 7-hydroxycoumarin. The limits of quantification were 1 536.59 ± 0.25 and 2 209.93 ± 0.34 nM for coumarin and 7-hydroxycoumarin respectively. These data are summarised in table 2.4. The lower limits for 7-hydroxycoumarin are higher than that of coumarin, indicating that the method is less sensitive for 7-hydroxycoumarin than for coumarin.

Table 2.4: LOD and LOQ for coumarin and 7-hydroxycoumarin

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD (nM) (a)</th>
<th>LOQ (nM) (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>511.86 ± 0.57</td>
<td>1 536.59 ± 0.25</td>
</tr>
<tr>
<td>7-Hydroxycoumarin</td>
<td>736.31 ± 0.42</td>
<td>2 209.93 ± 0.34</td>
</tr>
</tbody>
</table>

(a) LOD was defined as the concentration with a signal-to-noise ratio of 3. (b) LOQ was defined as the concentration three times the LOD (a signal to noise ratio of 10:1).
2.3.2 Extraction of Metabolites

The extraction efficiency of the solid phase extraction method was measured as the percentage recovery of coumarin and 7-hydroxycoumarin. The percentage recovery was calculated by comparing the peak areas of these samples to the peak area of a pure standard of the same concentration as initially added to the test samples. The percentage recovery for both coumarin and 7-hydroxycoumarin was approximately 75%. There was no significant difference between the percentage recoveries for coumarin and 7-hydroxycoumarin as well as between the different concentrations used (25 µM and 50 µM).

Table 2.5: Extraction Recoveries for coumarin and 7-hydroxycoumarin

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Initial Concentration (µM)</th>
<th>Calculated Concentration (mean ± SD) (µM), n = 5</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td>25</td>
<td>18.97 ± 0.26</td>
<td>75.89 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>37.50 ± 0.11</td>
<td>74.80 ± 0.11</td>
</tr>
<tr>
<td>7-Hydroxycoumarin</td>
<td>25</td>
<td>18.91 ± 0.33</td>
<td>75.62 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>38.06 ± 0.07</td>
<td>76.24 ± 049</td>
</tr>
</tbody>
</table>

2.3.3 Optimisation of the in vitro rat liver microsomal system

Initial running conditions for the microsome incubations were optimised with respect to time, temperature, amount of microsomes (as protein concentration), concentration of substrate (coumarin), and the concentration of the NADPH generating system.

2.3.3.1 Time Optimisation

The rat liver microsomal system was optimised with respect to time by performing incubations (as described in section 2.2.2.1) and varying the time of the incubation. Figure 2.4 shows the total amount of coumarin converted to 7-hydroxycoumarin (in µmol.ml⁻¹). At time zero, there was no discernible conversion (the conversion of coumarin to 7-hydroxycoumarin was 5.01%), while after 20 minutes 46.67% conversion has occurred.
There was no significant difference in the amount of 7-hydroxycoumarin produced even after longer lengths of time, such as three and six hours ($p < 0.05$, calculated using the student $t$-test).

2.3.3.2 Temperature Optimisation

The rat liver microsomal system was optimised with respect to temperature by performing incubations (as described in section 2.2.2.1) and varying the temperature of the incubation. Figure 2.5 shows the total amount of coumarin converted to 7-hydroxycoumarin (in $\mu$mol.ml$^{-1}$). At room temperature 0.003 $\mu$mol.ml$^{-1}$ 7-hydroxycoumarin was produced (the conversion rate of coumarin to 7-hydroxycoumarin was 9.28%) while 38°C (31.77% conversion) was found to be the optimum temperature for the production of 7-hydroxycoumarin.

Figure 2.4: Time Optimisation Study. The rat liver microsomal system was optimised for time by performing incubations at various times; time zero was a negative 7-hydroxycoumarin control. The activity of the microsomes was expressed as $\mu$mol of coumarin converted to 7-hydroxycoumarin per ml incubation volume ($\mu$mol.ml$^{-1}$). Thirty minutes was the optimum time for the reaction.
2.3.3.3 Microsome Concentration Optimisation

The rat liver microsomal system was optimised with respect to microsome concentration by performing incubations (as described in section 2.2.2.1) and varying the protein concentration of the incubation. Figure 2.6 shows the total amount of coumarin converted to 7-hydroxycoumarin (in µmol.ml⁻¹). When microsomes were absent, there was no discernible conversion. There was a slight decrease in coumarin, which could be attributed to loss during the extraction or other chemical processes. The conversion of coumarin increased as protein concentration increased until it leveled off at 50 µg protein/ml microsomes. There was no significant difference ($p < 0.05$, calculated using the student $t$-test) in the amount of 7-hydroxycoumarin produced. The optimal protein concentration for the conversion of coumarin to 7-hydroxycoumarin was 50 µg protein/ml microsomes.

![Figure 2.5: Temperature Optimisation Study](image)

**Figure 2.5: Temperature Optimisation Study.** The rat liver microsomal system was optimised for temperature by performing incubations at various temperatures for 30 minutes. The activity of the microsomes was expressed as µmol of coumarin converted to 7-hydroxycoumarin per ml incubation volume (µmol.ml⁻¹). 38°C was the optimum temperature for the reaction.
2.3.3.4 Substrate Concentration Optimisation

The rat liver microsomal system was optimised with respect to substrate concentration by performing incubations (as described in section 2.2.2.1) and varying the substrate concentration of the incubations. Figure 2.7 shows the total amount of coumarin converted (in µmol) as well as the appearance of 7-hydroxycoumarin. This result is deceptive as the total amount of 7-hydroxycoumarin produced is higher given higher initial substrate concentration. For this reason, this result is presented as the percentage conversion. Percentage conversion is highest for 50 µM.

Figure 2.6: Microsome Concentration Optimisation Study. The rat liver microsomal system was optimised for microsome concentration by performing incubations at various microsome concentrations. The activity of the microsomes was expressed as µmol of coumarin converted to 7-hydroxycoumarin per ml incubation volume (µmol.ml⁻¹). 50 µmol/ml was the optimum protein concentration for the reaction.
Figure 2.7: Substrate Concentration Optimisation Study. The rat liver microsomal system was optimised for coumarin concentration by performing incubations at various coumarin concentrations for 20 minutes. The activity of the microsomes (-----) was expressed as μmol of coumarin converted to 7-hydroxycoumarin per ml incubation volume (μmol.ml⁻¹). Percentage conversion is also shown (---). 50 μM was found to be the optimum substrate concentration for the reaction.

2.3.3.5 NADPH Generating System Concentration Optimisation

The rat liver microsomal system was optimised with respect to the NADPH generating system concentration by performing incubations (as described in section 2.2.2.1) and varying the concentration of glucose-6-phosphate (G6P), NADP⁺ and glucose-6-phosphate dehydrogenase (G6PDH) of the incubation.

Figure 2.8 shows the total amount of coumarin converted to 7-hydroxycoumarin (in μmol.ml⁻¹). When NADP⁺ is absent, there is no discernible conversion. The conversion of coumarin increases as NADP⁺ concentration increases. There is no significant difference in the amount of 7-hydroxycoumarin produced at 1 mM than at 5 mM. When G6P is absent, there is no discernible conversion. The conversion of coumarin increases as G6P concentration increases. There is no significant difference in the amount of 7-hydroxycoumarin produced at 5 mM than at 10 mM. When G6PDH is absent, there is no discernible conversion. The conversion of coumarin increases as G6PDH concentration
increases. There is no significant difference in the amount of 7-hydroxycoumarin produced at 1 U/ml than at 2 U/ml.

These results indicate that all three components of the NADPH generating system are required for the conversion of coumarin to 7-hydroxycoumarin. The optimal concentrations for the conversion of coumarin to 7-hydroxycoumarin are 1 mM, 5 mM and 1 U/ml for NADP⁺, G6P and G6PDH respectively. This supports the parameters defined by Draper et al., (1997).

![Figure 2.8: NADPH Generating System Concentration Optimisation Study](image)

**Figure 2.8: NADPH Generating System Concentration Optimisation Study.** The rat liver microsomal system was optimised for NADPH generating system concentration by performing incubations at various concentrations for 20 minutes. The total amount of coumarin converted to 7-hydroxycoumarin was expressed as μmol.ml⁻¹. The optimum concentrations for the reaction were 1 mM, 5 mM and 1 U/ml for NADP⁺, G6P and G6PDH respectively.

**2.3.4 Incubation studies using two NSAIDs, etoricoxib and lumiracoxib**

Incubations were performed using two NSAIDs, lumiracoxib and etoricoxib (figure 2.1) at 50 µM using the method described in section 2.2.3.1 and extracted using the described method (section 2.2.3.2). The extracts were then analysed using the HPLC-UV method described in section 2.2.3.1. The concentration of the remaining parent compound was determined using calibration curves constructed from a range of drug standards (0 - 2.5 mM for both drugs) prepared in mobile phase. These calibration curves are shown in figure A.3 and A.4 (pp. 94).
2.3.4.1 Incubation with lumiracoxib

The chromatogram shown in figure 2.9 describes a typical extraction following an incubation with lumiracoxib. By comparing retention times with that of standards, the large peak (b) is the parent, lumiracoxib, and the other smaller peaks (a and c) could possibly represent metabolites. Some conversion had taken place; the concentration of the remaining parent drug was 39.76 μM (a conversion rate of approximately 20%). Mass spectrometry was used to identify any metabolites present.

![Chromatogram of 50 μM lumiracoxib incubated with 10 μg/ml microsomes. Peaks: (a) and (c): unidentified possible metabolites, (b): parent drug, lumiracoxib.](image)

**Figure 2.9**: Chromatogram of 50 μM lumiracoxib incubated with 10 μg/ml microsomes. Peaks: (a) and (c): unidentified possible metabolites, (b): parent drug, lumiracoxib.

2.3.4.1.1 Mass spectrometry analysis of lumiracoxib incubations

The extracted metabolites were identified using LC-MS/MS. Key metabolites identified included oxidation and carboxylation metabolites as well as a carboxy-lactam product. The MS/MS spectra of the mass ions of these metabolites are shown in figure 2.10.
2.3.4.2 Incubation with etoricoxib

The chromatogram shown in figure 2.11 shows a typical extraction following an incubation with etoricoxib. The large peak (C) is the parent, etoricoxib, and the other smaller peaks (A and B) could possibly represent metabolites. The concentration of the remaining parent drug was 40.13 µM, indicating a conversion rate of approximately 20%. Mass spectrometry was used to identify any metabolites present.

Figure 2.10: Mass spectrum of 50 µM lumiracoxib incubated with 10 µg/ml microsomes. Metabolites were identified by LC-MS/MS operated in the positive ion mode. (A): MS/MS of ion 294.1 m/z, corresponding to the parent, lumiracoxib, (B): MS/MS of ion 310.1 m/z, corresponding to an oxidised metabolite, (C): MS/MS of ion 324.1 m/z, corresponding to a carboxylated metabolite, (D): MS/MS of ion 340.1 m/z, corresponding to a carboxylated and oxidised metabolite, (E): MS/MS of ion 322.1 m/z, corresponding to an oxidised and carboxylated lactam metabolite. The structures of these metabolites are inset.
2.3.4.2.1 Mass spectrometry analysis of etoricoxib incubations

No metabolites were found after MS/MS analysis. The MS/MS spectra of the mass ion of the parent (etoricoxib) is shown in figure 2.12.

**Figure 2.11:** Chromatogram of 50 μM etoricoxib with 10 μg/ml microsomes as described in section 2.2.3.2 and analysed using the HPLC method described in section 2.2.1.1. Peaks: (A) and (B): unidentified possible metabolites, (C): parent drug, etoricoxib. These peaks are not observed in blank samples.

**Figure 2.12:** Mass spectrum of 50 μM etoricoxib incubated with 10 μg/ml microsomes. Metabolites were identified by LC-MS/MS operated in the positive ion mode. MS/MS of ion 359.0 m/z, corresponding to the parent, etoricoxib (structure shown inset).
2.4 Conclusion

The HPLC method for the detection of coumarin and 7-hydroxycoumarin was successfully validated with respect to linearity, reproducibility, precision, accuracy and lower limits of detection and quantification. The values obtained fell within acceptable limits for biological systems (Shah et al., 1992). Initial experimental conditions for the microsome incubations were optimised using commercially prepared rat liver microsomes with respect to time, temperature, amount of microsomes (as protein concentration), concentration of substrate (coumarin), and the concentration of the NADPH generating system. The optimum experimental conditions for the incubations were 50 µM coumarin, 50 µg protein/ml microsomes, 1 mM NADP+, 5 mM G6P and 1U/ml G6PDH incubated for 30 minutes at 38°C.

Metabolites of lumiracoxib were identified by LC-MS/MS. These include oxidation and carboxylation metabolites as well as a carboxy-lactam product. Two hydroxylated metabolites and a carboxylated metabolite are the same as metabolites observed in vivo in horses (de Kock et al., 2008b). However, hydroxylated, carboxylated and lactam metabolites were observed which are not observed in horses. The difference in metabolism are most likely due to the difference in the P450s expressed by rats as compared with horses. Differences could also result from the incubations in vitro versus administration trials conducted in vivo. In vivo studies may differ from in vitro studies as they represent a complete system and take all possible transformations into account. No metabolites were found after the MS/MS analysis of etoricoxib. The lack of metabolites may have been due to the nature of the metabolites: if they are very polar, the metabolites may be eluted in the void volume; any unstable metabolites may have been broken down prior to analysis. Metabolites may have been lost during extraction, if they were eluted in one of the wash steps.

With the optimisation using rat liver microsomes and coumarin, and preliminary tests using two NSAIDs, completed, the parameters for the liver microsomal system were used with microsomes prepared from bovine liver to investigate the phase I metabolism of the drugs of interest, which is described in Chapter 3.
Chapter Three

Production of Microsomes from Bovine Liver and the Investigation of the Phase I Metabolism

3.1 Introduction

In this study, the phase I metabolism of ten drugs, including diazepam, nordiazepam, oxazepam, morphine, codeine, promazine, acepromazine, chlorpromazine, etoricoxib and lumiracoxib, was investigated both in silico, using the predictive metabolism software MetaSite, and in vitro using a bovine liver microsomal system.

3.1.1 Phase I metabolism

During Phase I metabolism, polar functional groups are either introduced or unmasked, which results in metabolites which are more polar than the original compounds. In the case of pharmaceutical drugs, Phase I reactions can lead either to activation or inactivation of the drug. Phase I reactions may occur by oxidation, carboxylation, reduction, hydrolysis, cyclization, and decyclization reactions. If the metabolites of these reaction are sufficiently polar, they may be more readily excreted. However, many phase I products undergo subsequent phase II metabolism in which an endogenous substrate, such as glucuronic acid, combines with the newly incorporated functional group to form a highly polar conjugate (Gonzalez & Tukey, 2005).

The most common phase I transformations are hydroxylation and carboxylation. Hydroxylation reactions play a very important role in the metabolism of many drugs. Most hydroxylations require electrons from NADPH, passed via the enzyme cytochrome P450 reductase, and oxygen and are catalysed by cytochrome P450 isozymes. In these reactions, the oxygen of the incorporated hydroxyl group comes from molecular oxygen, and water is released as a by-product (Ekins et al., 2000). Carboxylation is another common phase I reaction and is catalysed by carboxylyases. Carboxylyases, also known as decarboxylases,
are carbon-carbon lyases that add or remove a carboxyl group from organic compounds (Gonzalez & Tukey, 2005).

3.1.2 Predicting metabolism in silico

The use of computer modelling and predictive software are useful tools for determining the potential metabolism of a compound. Programmes, such as MetaSite, are used to predict the biotransformation of new compounds during drug discovery. MetaSite is a computational procedure used to predict the metabolism of compounds as mediated by cytochrome P450 enzymes. MetaSite compares the three dimensional structure of the ligand with the known structures of the active sites of cytochrome P450s. The different reaction mechanisms catalysed by the different CYP isoforms is also included. The software is able to estimate the chemical reactivity of each atom in the given substrate pre-calculated data on many chemical fragments. MetaSite then provides the structures of the metabolites and ranks them according to the site of metabolism predictions (Cruciani et al., 2005).

MetaSite is able to give information about the predicted metabolites, such as the monoisotopic mass (MIM) and the partition (log P) and distribution (log D) coefficient values. The partition and distribution coefficients are variables used in pharmacology as assessments of drug-likeness. Both are measures of hydrophobicity and effect a drug’s absorption, distribution, metabolism and excretion (ADME) properties (Bahl et al., 2007).

The drug-likeness of a particular compound or metabolite can be roughly evaluated using the rule of five. These rules were developed by Christopher Lipinski to describe important pharmacokinetic drug properties. In order for a drug target to be considered drug-like it must satisfy the following criteria: a molecular mass of less than 500 Da, a partition coefficient less than 5, no more than 5 hydrogen bond donor groups and no more than 10 hydrogen bond acceptor groups (Lipinski et al., 2001).

3.1.3 Metabolism in vitro using liver microsomes

In this study, the metabolism of drugs was studied using liver microsomes prepared from bovine liver. The microsomes contain P450 enzymes and were used to study drug
metabolism in vitro. In vitro methods using microsomes are a useful approach to the study of the breakdown pathways of drugs with previously unknown metabolism, such as designer drugs. In vitro methods are now commonplace in the drug development industry and are often coupled with in vivo studies (Scarth et al., 2010b).

In this chapter, bovine liver was used to produce microsomes. These microsomes were used to study the metabolism of the drugs of interest. The resulting metabolites were extracted using SPE and analysed using LC-MS/MS. The metabolism of the drugs investigated was also predicted in silico using the computational software MetaSite.
3.2 Methods and Materials

3.2.1 Production of Microsomes from Bovine Liver

Microsomes were produced from bovine liver by differential centrifugation. Liver was obtained from the Rosedale Abattoir (Southwell, South Africa) approximately 30 minutes after the death of the animal. The liver was chopped and homogenised in 1 volume cold phosphate buffer (100 mM, pH 7.4, 1 mM EDTA, 0.1 mM DTT and 20% (v/v) glycerol). The homogenate was filtered through cheesecloth and stored at -70°C or used directly.

The homogenate was then centrifuged at 800 g for 30 min in a Beckman Avanti J-E centrifuge. The supernatant was then centrifuged again at 13 500 g for 20 min. The second supernatant was then centrifuged at 105 000 g for 90 min in a Beckman Optima L-90K ultracentrifuge.

The resultant pellets were resuspended in 100 mM phosphate buffer, pH 7.4, and stored at -70°C. The protein concentration of the microsome fractions was evaluated using the Bradford method. Bovine serum albumin (BSA) was used as a standard in a concentration range of 0 - 2 000 µg/ml to construct a calibration curve. BSA standards and microsome samples of unknown concentrations were incubated with a equal volumes Bradford’s reagent for 5 min at room temperature before being analysed in a PowerWave plate reader at 595 nm.

The bovine microsome activity was evaluated using coumarin as a standard and the optimised incubation parameters described in section 2.2.2.1. The amount of 7-hydroxycoumarin produced was determined using HPLC-UV as per section 2.2.1.
3.2.2 Phase I metabolism study

The phase I metabolism of ten drugs was investigated both in silico (using the predictive metabolism software MetaSite) and in vitro (using the microsome assay developed in chapter 2). The drugs were diazepam, nordiazepam, oxazepam, morphine, codeine, promazine, acepromazine, chlorpromazine, etoricoxib and lumiracoxib. Their structures are shown in figure 3.1.

Figure 3.1: Chemical structures of drugs studied. (a) Diazepam, (b) nordiazepam, (c) oxazepam, (d) morphine, (e) codeine, (f) etoricoxib, (g) lumiracoxib, (h) acepromazine, (i) chlorpromazine and (j) promazine.

3.2.2.1 In silico predictive metabolism using MetaSite

In silico predictive metabolism was conducted using MetaSite (Molecular Discovery Ltd., Middlesex, UK). MetaSite is a computational procedure that predicts metabolic transformations related to cytochrome-mediated reactions in Phase I metabolism.
MetaSite was used to predict the site of metabolism as well as identify the first 5 metabolites and their respective log P and log D values for each of the ten drugs.

3.2.2.2 Incubations with bovine liver
The Phase I metabolism of the drugs was investigated in vitro by performing incubations with the bovine liver microsomes. The drugs (at a concentration of 50 µM) were incubated separately with the bovine microsomes and an NADPH generating system as described in section 2.2.2.1: incubations of 1 ml were set up in duplicate containing: 50 µg microsomes, 50 mM phosphate buffer (pH 7.4), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM G6P, 1 U/ml G6PDH. The reaction was initiated by the addition of the NADPH generating system (G6P, G6PDH and NADP⁺) and the reaction mixtures incubated at 38°C for 30 min, 1 h and 2 h. Microsome concentration was increased from 10 µg used in section 2.2.3.2 to 50 µg as this was found to be optimal with bovine liver. The reaction was then terminated by the addition of 125 µl 15% (w/v) trichloroacetic acid. A positive drug control, containing all constituents of the incubation mix but lacking microsomes and a blank sample, lacking drug, were also set up for each drug studied. The blank sample was used to establish a baseline reading for the subsequent LC-MS/MS analysis, detailed below.

3.2.2.3 Extraction of Metabolites
The metabolites generated were extracted from the incubation mixtures using Oasis® HLB 3cc (60 mg) solid phase extraction cartridges as described in section 2.2.2.2. The cartridges were preconditioned with 2 ml acetonitrile, then 2 ml deionised water. The total incubation volume (1 ml) was added and allowed to elute under vacuum. The cartridges were washed with 1 ml 5% acetonitrile and the samples eluted with 1 ml acetonitrile. Extracts were stored at -20°C prior to analysis.

3.2.2.4 Mass Spectrometry Analysis of Metabolites
Mass spectrometry analysis of the drug incubations was conducted as described in section 2.2.3.4.
3.3 Results and Discussion

3.3.1 Production of microsomes from Bovine Liver

Liver microsomes were prepared from fresh bovine liver using ultracentrifugation. The activity of the prepared microsomes was assessed using coumarin as a standard as described in section 2.2.2. This activity (shown in table 3.1) was then compared to that of the commercially prepared rat liver microsomes. There was no significant difference in the activities from either source \( (p > 0.05, \text{ calculated using the student } t\text{-test}) \).

Table 3.1: Activity of Microsomes prepared from bovine liver and rat liver

<table>
<thead>
<tr>
<th>Source</th>
<th>Activity (mean ± SD) ( (\mu\text{mol.min}^{-1}.\mu\text{g}^{-1})^{(a)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine liver</td>
<td>23.77 ± 0.28 (^{(b)})</td>
</tr>
<tr>
<td>Rat liver</td>
<td>24.15 ± 0.21 (^{(b)})</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Activity for each microsome fraction is reported as \( \mu\text{mol of 7-hydroxycoumarin produced per minute of reaction per } \mu\text{g protein.} \)

\(^{(b)}\) 50 \( \mu\text{g microsomes in a 1 ml incubation.} \)

Following the above result, the study of the phase I metabolism of four drug classes was investigated, both \textit{in silico}, using MetaSite and \textit{in vitro} using the incubation parameters developed in chapter 2.

3.3.2 Phase I metabolism study

3.3.2.1 \textit{In silico} predictive metabolism using MetaSite

MetaSite was used to predict the five most likely metabolites of each of the ten drugs. The sites of metabolism were also evaluated and are summarised in table 3.2. The structure of each drug with the major sites of metabolism are highlighted in red. The darker the red colour, the more likely metabolism will occur at that position. The highest ranking atom is
further highlighted with a blue ring. The structures of the predicted metabolites as well as their molecular masses and the log P and log D for each, are summarised in table 3.3.

3.3.2.1.1 Diazepam and its derivatives, nordiazepam and oxazepam

For diazepam, the highest ranking metabolism ‘hot spot’ is a methyl group on the aliphatic ring. This corresponds to the major metabolite known in vivo, nordiazepam (Friedericy & Bovill, 1998). The other site of metabolism identified with an equal ranking was on the benzene ring. This site was identified as the main metabolism hot spot for both nordiazepam and oxazepam.

The major metabolite predicted for diazepam was an N-dealkylated product, nordiazepam. Nordiazepam and temazepam have been identified as major metabolites in humans (Friedericy & Bovill, 1998) and in horses (Shini, 2000). The structures of nordiazepam and temazepam are shown in figure 3.2.

The four other metabolites predicted are all aromatic hydroxylation products. These metabolites are hydroxylated at different positions on the benzene ring. These metabolites are generally not observed in vivo either in humans or horses (Yasumori et al., 1993, Chauret et al., 1997). Aromatic hydroxylated metabolites are more likely to be predicted as products of cytochrome P450 mediated oxidation due to the high electron density of benzene rings.

All the predicted metabolites satisfy Limpinski’s rule of five, with molecular masses less than 500 Da and log P less than 5. This is expected for nordiazepam, as it is pharmacologically active and marketed as a drug.
The metabolites predicted for nordiazepam show some similarities to those of diazepam and are identical to those of oxazepam. The only difference in the metabolites of nordiazepam and oxazepam is the structure of the parent base. The first three are aromatic hydroxylations of the benzene ring in the para, meta and ortho positions. The forth metabolite is an N-oxidation of the aliphatic nitrogen and the fifth an aromatic hydroxylation of the chlorine-containing benzene.

These metabolites, as for diazepam, all satisfy the rule of five and can be considered to be drug-like. The metabolites for oxazepam show much lower $\log P$ and $\log D$ values than those for nordiazepam. The oxazepam and nordiazepam metabolites all have the same molecular masses: 302.05 and 286.05 for oxazepam and nordiazepam respectively.

### 3.3.2.1.2 Promazine, Chlorpromazine and Acepromazine

The main sites of metabolism predicted for promazine, chlorpromazine and acepromazine are the same: the two methyl groups of the amide group. Both atoms were equally ranked and are thus each as likely to be metabolised.

Consequently, the metabolites predicted for each drug are similar. The first metabolite for all three drugs is a demethylated product. For promazine, acepromazine and chlorpromazine this represents known *in vivo* metabolites, desmethyl promazine, desmethyl acepromazine and desmethyl chlorpromazine respectively. Desmethyl
chlorpromazine is the most prevalent metabolite observed in vivo (Wójcikowski et al., 2010).

The second metabolite has the amide group replaced with a carboxyl group, making the metabolite acidic.

The forth and fifth promazine metabolites identified are minor metabolites identified in vivo, promazine sulphoxide and promazine disulphoxide. Promazine sulphoxide is a major metabolite of promazine in horses. Acepromazine sulphoxide and acepromazine disulphoxide were predicted as metabolites of acepromazine (metabolites 3 and 4); acepromazine sulphoxide is a major metabolite of acepromazine in vivo. Sulphoxide metabolites of chlorpromazine were not identified, although they are known in vivo (Wójcikowski et al., 2010). The fifth metabolite of chlorpromazine, where the aliphatic side chain has been removed, is also a known metabolite in vivo (Coccia & Westerfeld, 1967).

All the metabolites predicted for promazine, acepromazine and chlorpromazine can be considered drug-like as they satisfy Lipinski’s rule of five.

Several hydroxylated products of promazine, acepromazine and chlorpromazine are known in vivo, including 7-hydroxy acepromazine, 3-hydroxypromazine and 7-hydroxy chlorpromazine (Dewey et al., 1981; Wójcikowski et al., 2010), but were not predicted by MetaSite.

3.3.2.1.3 Opioids: morphine and codeine

Morphine and codeine are structurally similar, and the sites of metabolism for both drugs are similar. The terminal end of the amide group is the highest ranking site of metabolism for both drugs. Other sites include the ketone group of codeine.

The metabolites predicted by MetaSite for opioids generally do not represent an in vivo situation. Morphine and codeine are conjugated to glucuronide to produce several pharmacologically active metabolites (Anderson et al., 2003, He et al., 1998). These
metabolites are not predicted by MetaSite as the software is able to predict CYP-mediated phase I transformations only.

The first metabolite for morphine is sometimes observed \textit{in vivo}: an N-dealkylated product, normorphine (Watson, 1995). MetaSite also predicted a dealkylated product for codeine, norcodeine, and morphine, which is produced by the O-dealkylation of codeine. Both of these metabolites are observed in low levels \textit{in vivo} (He \textit{et al.}, 1998).

The partition coefficients ($\log P$) for the opioids are all below 3; the $\log P$ values for morphine metabolites are lower, with all values less than 1. This shows that all the predicted metabolites are extremely hydrophilic and would be easily excreted.

\subsection*{3.3.2.1.4 NSAIDs: etoricoxib and lumiracoxib}

The highest ranking site of metabolism predicted for the NSAIDs was a terminal methyl group attached to a benzene ring for both etoricoxib and lumiracoxib. Other sites included aromatic nitrogen and carbon in both drugs.

The first metabolite identified for etoricoxib was a hydroxylated product, hydroxyetoricoxib, a metabolite that has been identified \textit{in vivo} in both horses (de Kock \textit{et al.}, 2008a) and humans (Rodrigues \textit{et al.}, 2003). MetaSite predicted the other metabolite also observed \textit{in vivo}, etoricoxib-1’-N-oxide as a result of the hydroxylation of an aromatic nitrogen.

4’-Hydroxylumiracoxib was identified as the first metabolite of lumiracoxib. This is a metabolite which has been identified as having COX-2 selective activity (Mangold \textit{et al.}, 2004). Another hydroxylated metabolite was also predicted, however this product is not prevalent \textit{in vivo}. Other known metabolites of lumiracoxib \textit{in vivo} include two carboxylated products, 5-carboxylumiracoxib and 4-hydroxy 5-carboxylumiracoxib (Mangold \textit{et al.}, 2004), which were not predicted by MetaSite.

All predicted metabolites of etoricoxib and lumiracoxib have a $\log P$ value of less than 5. They would most likely be water soluble and readily excreted.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Site of Metabolism by structure (^{(a)})</th>
<th>Main Hot-spot atoms (^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td><img src="image1.png" alt="Diazepam Structure" /></td>
<td>C15, C5, N14</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td><img src="image2.png" alt="Nordiazepam Structure" /></td>
<td>C17, C15, C16, C18, C19, C11</td>
</tr>
<tr>
<td>Oxazepam</td>
<td><img src="image3.png" alt="Oxazepam Structure" /></td>
<td>C2, C3 - 6, C17</td>
</tr>
<tr>
<td>Promazine</td>
<td><img src="image4.png" alt="Promazine Structure" /></td>
<td>C19, C20, C17, N18</td>
</tr>
</tbody>
</table>
### Table 3.2: Site of metabolism hot-spots as predicted by MetaSite

<table>
<thead>
<tr>
<th>Drug</th>
<th>Site of Metabolism by structure (a)</th>
<th>Main Hot-spot atoms (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acepromazine</td>
<td><img src="image" alt="Acepromazine" /></td>
<td>C22, C23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S8</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td><img src="image" alt="Chlorpromazine" /></td>
<td>C20, C21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N19</td>
</tr>
<tr>
<td>Morphine</td>
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<tr>
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<td></td>
<td>C10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N20</td>
</tr>
<tr>
<td>Codeine</td>
<td><img src="image" alt="Codeine" /></td>
<td>C23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N20</td>
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</tbody>
</table>
Table 3.2: Site of metabolism hot-spots as predicted by MetaSite

<table>
<thead>
<tr>
<th>Drug</th>
<th>Site of Metabolism by structure (a)</th>
<th>Main Hot-spot atoms (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etoricoxib</td>
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<tr>
<td></td>
<td></td>
<td>N20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C12</td>
</tr>
<tr>
<td>Lumiracoxib</td>
<td><img src="image" alt="Structure" /></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>C4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N9</td>
</tr>
</tbody>
</table>

(a) The site of metabolism prediction shows the position of the atoms in each molecule most likely to undergo metabolism. They are ranked from most likely (dark red) to least likely (pale red).
(b) The main hot-spot atoms are arranged by ranking. Atoms together on one line have equal rankings and are equally as likely to be metabolised.

The MetaSite software uses the site of metabolism predictions to produce a set of possible metabolites based on the number of possible reaction sites. These are given as a ranked list, along with their monoisotopic mass (MIM) and lipophilicity values ($\log P$ and $\log D$). The first five metabolites for each drug are summarised in table 3.4.
Table 3.3: Summary of Metabolites predicted by MetaSite

<table>
<thead>
<tr>
<th></th>
<th>Parent</th>
<th>Metabolite 1</th>
<th>Metabolite 2</th>
<th>Metabolite 3</th>
<th>Metabolite 4</th>
<th>Metabolite 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diazepam</strong></td>
<td><img src="image1" alt="Diazepam" /></td>
<td><img src="image2" alt="Diazepam" /></td>
<td><img src="image3" alt="Diazepam" /></td>
<td><img src="image4" alt="Diazepam" /></td>
<td><img src="image5" alt="Diazepam" /></td>
<td><img src="image6" alt="Diazepam" /></td>
</tr>
<tr>
<td><strong>log P</strong></td>
<td>2.92</td>
<td>2.95</td>
<td>2.38</td>
<td>2.37</td>
<td>2.47</td>
<td>2.40</td>
</tr>
<tr>
<td><strong>log D</strong></td>
<td>2.93</td>
<td>2.92</td>
<td>2.38</td>
<td>2.37</td>
<td>2.46</td>
<td>2.40</td>
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<td><strong>MIM</strong></td>
<td>300.05</td>
<td>270.05</td>
<td>300.07</td>
<td>300.07</td>
<td>300.07</td>
<td>300.07</td>
</tr>
<tr>
<td><strong>N-dealkylation</strong></td>
<td>Nordiazepam</td>
<td>Nordiazepam</td>
<td>Nordiazepam</td>
<td>Nordiazepam</td>
<td>Nordiazepam</td>
<td>Nordiazepam</td>
</tr>
</tbody>
</table>

| **Nordiazepam** | ![Nordiazepam](image7) | ![Nordiazepam](image8) | ![Nordiazepam](image9) | ![Nordiazepam](image10) | ![Nordiazepam](image11) | ![Nordiazepam](image12) |
| **log P**      | 2.95         | 2.52         | 2.62         | 2.53         | 1.97         | 2.56         |
| **log D**      | 2.92         | 2.52         | 2.60         | 2.53         | 1.96         | 2.54         |
| **MIM**        | 270.05       | 286.05       | 286.05       | 286.05       | 286.05       | 286.05       |
| **Aromatic hydroxylation** |             |             |             |             |             |             |

Chapter 3: Phase I Metabolism
Table 3.3: Summary of Metabolites predicted by MetaSite

<table>
<thead>
<tr>
<th>Parent</th>
<th>Metabolite 1</th>
<th>Metabolite 2</th>
<th>Metabolite 3</th>
<th>Metabolite 4</th>
<th>Metabolite 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxazepam</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>
| | log P: 2.32  
| | log D: 2.31  
| | MIM: 286.05  
| | Aromatic hydroxylation | Aromatic hydroxylation | Aromatic hydroxylation | Anilinic hydroxylation | Aromatic hydroxylation |
| Promazine | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) | ![Image](image) |
| | log P: 4.45  
| | log D: 0.43  
| | MIM: 284.13  
| | N-dealkylation | N-dealkylation | Alliphatic N-oxidation | Thioetheric S-oxidation | Thioetheric S-oxidation |
| | Desmethyl promazine | Promazine sulphoxide | Promazine disulphoxide |

Reported in vivo: Dewey et al., 1981

Chapter 3: Phase I Metabolism
<table>
<thead>
<tr>
<th>Parent</th>
<th>Metabolite 1</th>
<th>Metabolite 2</th>
<th>Metabolite 3</th>
<th>Metabolite 4</th>
<th>Metabolite 5</th>
</tr>
</thead>
<tbody>
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<td>Acepromazine</td>
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<td><img src="https://example.com/metabolite3.png" alt="Image" /></td>
<td><img src="https://example.com/metabolite4.png" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>log P: 4.19</td>
<td>log P: 3.27</td>
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<td>log D: 0.17</td>
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<td>log D: 0.17</td>
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<td>Desmethyl acepromazine</td>
<td>Acepromazine sulphoxide</td>
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<td>log P: 4.74</td>
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<tr>
<td></td>
<td>log D: 1.18</td>
<td>log D: 1.07</td>
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<td>Alliphatic N-oxidation</td>
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<td>Wójcikowski et al., 2010</td>
<td>Wójcikowski et al., 2010</td>
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Table 3.3: Summary of Metabolites predicted by MetaSite

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<th>Parent</th>
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<th>Metabolite 3</th>
<th>Metabolite 4</th>
<th>Metabolite 5</th>
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<td><img src="image" alt="Metabolite 2" /></td>
<td><img src="image" alt="Metabolite 3" /></td>
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<td>log D: -4.02</td>
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<td>log D: 0.26</td>
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<td>Alliphatic N-oxidation</td>
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<table>
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<td>N-dealkylation</td>
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Table 3.3: Summary of Metabolites predicted by MetaSite

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</tr>
<tr>
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<td>Mangold et al., 2004</td>
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</tbody>
</table>
3.3.2.2 *In vitro* metabolism using liver microsomes

The phase I metabolism of ten drugs was studied in vitro using the prepared bovine liver microsomes over three time intervals: 30 min, 1 h and 2 h. The extracted metabolites were identified using LC-MS/MS. Metabolites were identified by extracted ion chromatography (XIC), by scanning for diagnostic ions specific to each metabolite.

3.3.2.2.1 Diazepam, Nordiazepam and Oxazepam

Three metabolites of diazepam were identified as nordiazepam, temazepam and oxazepam. The XIC for these metabolites is shown in figure 3.3. The ions monitored were 284.92/193.20 m/z, 270.94/140.00 Da, 286.98/241.10 Da and 300.94/255.10 Da for diazepam, nordiazepam, temazepam and oxazepam respectively. External standards were also analysed for comparison.

Most metabolism of diazepam occurred between 30 min and 2 h; there was little change in the signal intensity of diazepam in the control lacking microsome compared to the sample after 30 minutes incubation. After 2 hours, 80% of the drug was left unmetabolised. The major metabolite identified was the oxidised product, temazepam, with nordiazepam and oxazepam identified as minor metabolites. Oxazepam, as an oxidised and demethylated metabolite, was likely produced by the further metabolism of temazepam. There were also 3 mono-oxidised products observed, but these could not be identified.
Metabolism of nordiazepam (figure 3.3) left 85% of the parent unmetabolised after 2 hours, with an increase in metabolism between 30 minutes and 2 hours. Oxazepam was identified as the major metabolite. This is an oxidised product of nordiazepam and a known metabolite of both diazepam and nordiazepam in vivo (Yasumori et al., 1993). A further oxidation with hydrogenation metabolite could not be identified. No known metabolites of oxazepam were identified.

Figure 3.3: Metabolism of diazepam after 2 hours. (A): XIC of diazepam, retention time: 9.02 min, ions scanned: 284.92/193.20 Da, (B): XIC of nordiazepam, retention time: 8.43 min, ions scanned: 270.94/142.00 Da, (C): XIC of oxazepam, retention time: 7.96 min, ions scanned: 286.98/241.10 Da, and (D): XIC of temazepam, retention time: 8.45 min, ions scanned: 300.94/255.10 Da. Structures inset.
Limited morphine metabolism was observed: there was no reduction in signal intensity between the control lacking microsomes and the test samples. Normorphine, was identified (figure 3.5 B) by monitoring the ions 272.20/152.00 Da. The lack of observed metabolism is possibly due to morphine metabolism being predominantly phase II glucuronide conjugations (Meng et al., 2000).

**Figure 3.4:** Metabolism of nordiazepam after 2 hours. (A): XIC of nordiazepam, retention time: 8.44 min, ions scanned: 270.94/142.00 Da, (B): XIC of temazepam, retention time: 7.98 min, ions scanned: 300.94/255.10 Da. Structures inset.

### 3.3.2.2.2 Opioids: Morphine and Codeine

Limited morphine metabolism was observed: there was no reduction in signal intensity between the control lacking microsomes and the test samples. Normorphine, was identified (figure 3.5 B) by monitoring the ions 272.20/152.00 Da. The lack of observed metabolism is possibly due to morphine metabolism being predominantly phase II glucuronide conjugations (Meng et al., 2000).
The metabolism of codeine produced several metabolites. The predominant peak (figure 3.6 B) was identified as both norcodeine and morphine, but they could not be separated to quantify. Normorphine was also identified as a minor metabolite. The ions monitored to identify these metabolites were 300.04/215.30 Da, 286.02/152.00 Da and 272.20/152.00 Da for codeine, norcodeine/morphine and normorphine respectively. After 2 hours, 88% of the parent was left unmetabolised, with an increase in metabolism between 30 minutes and 2 hours.

Figure 3.5: Metabolism of morphine after 2 hours. (A): XIC of morphine, retention time: 7.74 min, ions scanned: 286.02/152.00 Da, and (B): XIC of normorphine, retention time: 6.37 min, ions scanned: 272.20/152.00 Da. Structures inset.
3.3.2.2.3: Promazine, Acepromazine and Chlorpromazine

Despite the structural similarities between promazine, acepromazine and chlorpromazine, the metabolites identified in vitro are dissimilar. Both promazine and acepromazine were metabolised to sulphoxide products, while the major metabolite of chlorpromazine was a demethylated product.

**Figure 3.6**: Metabolism of codeine after 2 hours. (A): XIC of codeine, retention time: 8.52 min, ions scanned: 300.04/215.30 Da, (B): XIC of norcodeine/morphine, retention time: 7.81 min, ions scanned: 286.02/152.00 Da and (C): XIC of normorphine, retention time: 6.44 min, ions scanned: 272.20/152.00 Da. Structures inset.
In the incubation of promazine (figure 3.7), 90% of the parent was left unmetabolised after 2 hours. The major metabolite of promazine identified was promazine sulphoxide, with 3-hydroxypromazine being formed in smaller quantities. The ions used for the identification of promazine metabolites were 285.13/86.10 Da, 301.15/86.20 Da and 301.15/58.00 Da for promazine, 3-hydroxypromazine and promazine sulphoxide respectively.

![Figure 3.7: Metabolism of promazine after 2 hours. (A): XIC of promazine, retention time: 6.90 min, ions scanned: 285.13/86.10 Da, (B): XIC of 3-hydroxypromazine, retention time: 6.43 min, ions scanned: 301.15/86.20 Da and (C): XIC of promazine sulphoxide, retention time: 6.96 min, ions scanned: 301.15/58.00 Da.](image)

In the incubation of promazine (figure 3.7), 90% of the parent was left unmetabolised after 2 hours. The major metabolite of promazine identified was promazine sulphoxide, with 3-hydroxypromazine being formed in smaller quantities. The ions used for the identification of promazine metabolites were 285.13/86.10 Da, 301.15/86.20 Da and 301.15/58.00 Da for promazine, 3-hydroxypromazine and promazine sulphoxide respectively.

A cepromazine was metabolised faster than other drugs investigated; after 30 minutes 90% of the parent remained unmetabolised. This shows a quicker reaction than other drugs, such as promazine and codeine, which had similar degree of metabolism, but only after 2
hour incubations. The two metabolites identified were 2-hydroxyethylpromazine and 7-hydroxy-2-(1-hydroxyethyl) promazine sulphoxide (figure 3.8).

**Figure 3.8:** Metabolism of acepromazine after 2 hours. (A): XIC of acepromazine, retention time: 6.67 mins, ions scanned: 327.14/86.10 Da, (B): XIC of 2-(1-hydroxyethyl)promazine, retention time: 6.52 min, ions scanned: 329.20/86.10 Da, (C): XIC of 2-(1-hydroxyethyl)promazine sulphoxide, retention time: 5.73 min, ions scanned: 345.20/86.10 Da and (D): XIC of 7-hydroxy-2-(1-hydroxyethyl)promazine sulphoxide, retention time: 6.60 min, ions scanned: 361.20/86.10 Da.
For both promazine and acepromazine, there were many other similar metabolites. Oxidation also occurred at different positions within the molecule, although these metabolites could not be identified.

Chlorpromazine showed a very high degree of metabolism; after 2 hours only 12% of the parent remained unmetabolised. This is a higher degree of metabolism than any of the other drugs studied. Several metabolites were identified (figure 3.9), with desmethylchlorpromazine the most prominent. Others were 7-hydroxychlorpromazine and promazine.

**Figure 3.9:** Metabolism of chlorpromazine after 2 hours. (A): XIC of chlorpromazine, retention time: 7.20 min, ions scanned: 319.12/86.00 Da, (B): XIC of desmethyl chlorpromazine, retention time: 7.14 min, ions scanned: 305.12/72.20 (C): XIC of 7-hydroxy chlorpromazine retention time: 7.25 min, ions scanned: 335.13/86.10 Da and (D): XIC of promazine, retention time: 6.89 min, ions scanned: 285.13/86.10 Da.
3.3.2.2.4 NSAIDs: lumiracoxib and etoricoxib

The phase I metabolism of lumiracoxib and etoricoxib were investigated using bovine liver microsomes as well as rat liver microsomes (section 2.3.4.1). The major metabolite of lumiracoxib identified was hydroxylumiracoxib (ions scanned: 310.2/264.0 Da, figure 3.10) with carboxylumiracoxib and hydroxycarboxylumiracoxib being detected in smaller amounts.

Figure 3.10: Metabolism of lumiracoxib after 2 hours. (A): XIC of lumiracoxib, retention time: 8.61 min, ions scanned: 294.1/248.0 Da, (B): XIC of hydroxylumiracoxib, retention time: 6.98 min, ions scanned: 310.2/264.0 Da. Structures inset

Lumiracoxib was metabolised to several different products when metabolised by rat liver microsomes (section 2.3.4.1). These include oxidation, carboxylation and lactam products. The hydroxylated and carboxylated metabolites observed in both the incubations with rat and bovine liver microsomes are the same as those observed in horses in vivo (de Kock et al., 2008b). However, some metabolites observed after incubation with rat liver microsomes are different from both the bovine liver microsome incubations and in vivo horse trials such as the carboxy-lactam product.
Due to difficulties identifying diagnostic ions for the detection of etoricoxib and its metabolites, EPI (enhanced product ion) mode was used for the analysis. The parent, etoricoxib was identified (m/z: 356.9 Da) as well as one metabolite, hydroxymethyl etoricoxib (m/z: 375.0) as shown in figure 3.11.

Known phase I metabolites of etoricoxib in horses in vivo are 6’-hydroxymethyl etoricoxib and 6’-hydroxymethyl etoricoxib-1-N-oxide (de Kock et al., 2008a). In humans, metabolites of etoricoxib in vivo are the same as in horses, with the addition of etoricoxib-1-N-oxide (Rodrigues et al., 2003). The metabolite identified in vitro, hydroxymethyl etoricoxib is possibly 6’-hydroxymethyl etoricoxib, as identified in humans and horses in vivo as well as predicted by MetaSite (section 3.3.2.1.4).

**Figure 3.11:** Metabolism of etoricoxib after 2 hours. EPI of etoricoxib from time 5.66 min to 5.87 min. Peak with m/z of 356.9 Da corresponds to the parent, etoricoxib and peak with m/z of 375.0 corresponds to hydroxymethyl etoricoxib. Structures inset.
3.4 Conclusion

The metabolism of drugs of interest in doping control studied was investigated both in silico and in vitro. There are differences observed in the in silico predictive metabolism as compared to the in vitro microsome assays. Both these methods produced different results from known in vivo metabolism studies.

There are several possible explanations for these differences. The first is most probably due to the difference in the source tissue used to produce the microsomes used in this study. Bovine liver microsomes were used in this study as a substitute for equine liver, as there was difficulty obtaining fresh horse liver for the production of microsomes. Bovine liver provided a model similar to equine; both are large, herbivorous mammals. For example, the metabolites observed in the in vitro incubation of codeine and morphine produce the same metabolites observed in horses during in vivo studies (He et al., 1998). Many of the metabolites observed in vitro for the other drugs are the same as those observed in the equine.

The differences in metabolism observed are possibly due to the difference in the P450 isoforms expressed in bovine as compared with horse and human liver. Differences could also result from the incubations in vitro versus administration trials conducted in vivo. In vivo studies may differ from in vitro studies as in vivo techniques take all possible transformations into account.

The major differences between the in silico and in vitro systems is likely due to MetaSite using the known structures of human P450s. Currently, there are no computational procedures to predict drug metabolism in animals such as horses, as only few of the structures of the P450s expressed by horses and other animals are known.

The phase II metabolism of the ten drugs was also investigated and these results are detailed in chapter 4. The drugs were incubated with phase II conjugates using the bovine liver microsomal system used in this chapter.
4.1 Introduction

In this chapter, the phase II metabolism of the drugs of interest was investigated using an in vitro bovine liver microsomal system adapted from the method developed in Chapter 3.

Phase II metabolism involves the conjugation of highly polar substrates, such as glucuronic acid, glutathione and sulphate, to drugs or the phase I metabolites of drugs. The products of phase II metabolism are usually polar and readily excreted (Gonzalez & Tukey, 2005).

Phase II conjugation reactions are catalysed by a large group of broad-specificity transferases (Zamek-Gliszczynski et al., 2006). Products of conjugation reactions have increased molecular weight and are usually inactive, unlike phase I reactions which can produce active metabolites. In some cases, for example opioids, such as morphine and codeine, phase II metabolites may also be active (Hideyoshi et al., 1969).

Glucuronidation involves the conjugation of the substrate to the highly soluble glucuronic acid and is catalysed by the enzyme uridine diphosphate-glucuronyltransferase (UGT). UGTs are exclusively membrane bound, to the luminal side of the ER membrane via their C-terminal ends (Zamek-Gliszczynski et al., 2006). The conjugation reaction they catalyse proceeds via the intermediate, UDP-glucuronic acid, which is glucuronic acid linked via a glycosidic bond to uridine diphosphate.

Sulphation complements glucuronidation in that sulphation occurs at many of the same sites as glucuronide conjugation. The sulphation of drugs occurs mainly in the cytosol and is catalysed by a group of cytosolic transferases called sulphotransferases (SULTs). SULTs catalyse the conjugation of sulphonate (SO\(_3^-\)) to sites on drugs such as hydroxyl and monoamide groups. Inorganic sulphate is provided by 3'-phosphoadenosine 5'-
phosphosulphate (PAPS) which is the sulphonate (SO$_3^-$) donor in all sulphate conjugation 
reactions (Zamek-Gliszczynski et al., 2006).

Glutathione conjugation is not as prevalent as glucuronidation. The main enzymes 
responsible for glutathione conjugation belong to a family of mainly cytosolic enzymes 
known as glutathione S-transferases (GSTs). GSTs catalyse the conjugation of reduced 
glutathione, via a sulfhydryl group, to a wide variety of substrates (Jakoby & Ziegler, 
1990). Several classes of GSTs have been described, both membrane-bound and occurring 
in the cytosol.

The investigation of the phase II metabolism of the drugs of interest is described in this 
chapter. The drugs of interest were incubated with the bovine liver microsomes and the 
phase II conjugates. Thereafter, the metabolites were extracted and analysed using LC-MS/ 
MS.
4.2 Methods and Materials

4.2.1 Phase II metabolism study
The phase II metabolism of the drugs was investigated in vitro, using an adapted form of the microsome assay developed for the investigation of phase I metabolism in chapter 3.

4.2.1.1 Incubations with bovine liver
The phase II metabolism of the drugs of interest was investigated in vitro by performing incubations with the bovine liver microsomes. The drugs (at a concentration of 50 µM) were incubated separately with the bovine microsomes, phase II conjugate salts and an NADPH generating system as described in section 2.2.2.1. Incubations of 1 ml were set up in duplicate containing: 50 µg microsomes, 50 mM phosphate buffer (pH 7.4), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM G6P, 1 U/ml G6PDH as well as 50 µM L-glutathione, 50 µM adenosine 3′-phosphate 5′-phosphosulphate lithium salt and 50 µM uridine 5′-diphosphoglucuronic acid trisodium salt. The reaction was initiated by the addition of the NADPH generating system (G6P, G6PDH and NADP⁺) and the reaction mixtures incubated at 38°C for 30 min, 1 h and 2 h. The reaction was then terminated by the addition of 125 µl 15% (w/v) trichloroacetic acid. A positive drug control, containing all constituents of the incubation mix but lacking microsomes and a blank sample, lacking drug, were also set up for each drug investigated.

4.2.1.2 Extraction of Metabolites
The metabolites generated were extracted from the incubation mixtures using Oasis® HLB 3cc (60 mg) solid phase extraction cartridges. The cartridges were preconditioned with 2 ml methanol, then 2 ml deionised water. The total incubation volume (1 ml) was added and allowed to elute under vacuum. The cartridges were washed with 1 ml deionised water and the samples eluted with 1 ml methanol. Extracts were stored at -20°C prior to analysis.

4.2.1.3 Mass Spectrometry Confirmation of Metabolites
Mass spectrometry analysis of the drug incubations was performed, as described in section 2.2.3.4.
4.3 Results and Discussion

4.3.1 In vitro metabolism using liver microsomes

The phase II metabolism of ten drugs was studied in vitro using the prepared bovine liver microsomes over three time intervals: 30 min, 1 h and 2 h. The metabolites produced are summarised in table 4.1. The extracted metabolites were identified using LC-MS/MS. Metabolites were identified by enhanced product ion (EPI) scanning and by neutral loss scanning, with a neutral loss of 176 for glucuronides.

Most phase II metabolites are detected with LC-MS/MS using a neutral loss study. A neutral loss study can only be performed using tandem in space mass analysers. The first mass analyser scans all masses, while the second mass analyser scans a set different from the first. This offset corresponds to a neutral loss specific to a particular group of compounds.

Table 4.1: Summary of phase II metabolites identified using LC-MS/MS

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<th>Drug</th>
<th>Metabolite</th>
</tr>
</thead>
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<td>Diazepam</td>
<td>Temazepam glucuronide</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>Oxazepam glucuronide</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>Oxazepam glucuronide</td>
</tr>
<tr>
<td>Promazine</td>
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</tr>
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<td>Acepromazine</td>
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</tr>
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</tr>
<tr>
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<td>Chlorpromazine glucuronide</td>
</tr>
<tr>
<td>Morphine</td>
<td>Morphine glucuronide</td>
</tr>
<tr>
<td>Codeine</td>
<td>Codeine glucuronide</td>
</tr>
<tr>
<td></td>
<td>Morphine/Norcodeine glucuronide</td>
</tr>
</tbody>
</table>

(a) No conjugates were observed; data not shown.
4.3.1.1 Diazepam, Nordiazepam and Oxazepam

As discussed in chapter three (section 3.3.2.2.1), the major phase I metabolite identified was the oxidised product, temazepam, with nordiazepam and oxazepam identified as minor metabolites. The most prominent phase II metabolite identified was temazepam glucuronide. Figure 4.1 shows the enhanced product ion (EPI) spectrum. A prominent peak with a m/z of 301.1 Da corresponds to the aglycone temazepam and a smaller peak at a m/z of 477.2 Da which corresponds to temazepam glucuronide. Temazepam glucuronide was identified also using a neutral loss of 176. There was also a loss which corresponds to the aglycone of diazepam glucuronide.

The phase II metabolism of nordiazepam produced one metabolite, oxazepam glucuronide. The neutral loss scan showed the glucuronide of oxazepam in low levels. The EPI chromatogram (figure 4.2) shows a peak at a m/z 287.0 Da which corresponds to the aglycone oxazepam and a peak with a m/z 463.2 Da which corresponds to oxazepam glucuronide.
Oxazepam glucuronide was identified as a phase II metabolite of oxazepam. The neutral loss scan showed the glucuronide of oxazepam in low levels. The EPI chromatogram (figure 4.3) shows a peaks at a m/z 241.0 and 287.0 Da which are characteristic fragments of oxazepam and a peak with a m/z 463.2 Da which corresponds to oxazepam glucuronide.

**Figure 4.2:** Phase II metabolism of nordiazepam after 2 hours: EPI of time 6.92 min. Peak with m/z of 287.0 Da corresponds to the parent, oxazepam and peak with m/z of 463.2 corresponds to oxazepam glucuronide. Structure of oxazepam glucuronide shown inset.

Oxazepam glucuronide was identified as a phase II metabolite of oxazepam. The neutral loss scan showed the glucuronide of oxazepam in low levels. The EPI chromatogram (figure 4.3) shows a peaks at a m/z 241.0 and 287.0 Da which are characteristic fragments of oxazepam and a peak with a m/z 463.2 Da which corresponds to oxazepam glucuronide.

**Figure 4.3:** Phase II metabolism of oxazepam after 2 hours: EPI from time 6.82 min to 7.05 min. Peak with m/z of 287.0 Da corresponds to the parent, oxazepam and peak with m/z of 463.1 corresponds to oxazepam glucuronide. Structure of oxazepam glucuronide shown inset.
The phase II metabolism of diazepam in vivo yields glucuronides of all three major phase I metabolites, nordiazepam, temazepam and oxazepam (Kaeferstein, 2009). This in vitro study only showed one glucuronide metabolite, temazepam glucuronide, for diazepam.

4.3.1.2 Opioids: Morphine and Codeine

Limited morphine phase I metabolism was observed (section 3.3.2.2.2). The lack of observed metabolism is possibly due to morphine metabolism being predominantly phase II glucuronide conjugations. The conjugation of glucuronide to morphine is possible at positions 3 and 6, resulting in the two active metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) (Meng et al., 2000).

Morphine glucuronide and the aglycone, morphine, were observed in the enhanced product ion (EPI) spectrum (figure 4.4B). It was unclear whether the glucuronidation had occurred at position 3 or 6. The peak observed was a strong, broad peak, possibly indicating a high concentration of morphine glucuronide.

**Figure 4.4:** Phase II metabolism of morphine after 2 hours: EPI of morphine from time 4.67 min to 5.37 min. Peak with m/z of 286.2 Da corresponds to the aglycone, morphine and peak with m/z of 462.2 Da corresponds to morphine glucuronide. Structure of morphine-3-glucuronide and morphine-6-glucuronide shown inset.
The neutral loss study for the phase II metabolism of codeine identified codeine glucuronide as a metabolite of codeine. The EPI spectrum (figure 4.5) shows peaks which correspond to the aglycone codeine and codeine glucuronide. Norcodeine glucuronide was also identified by neutral loss scan.

4.3.1.3: Promazine, Acepromazine and Chlorpromazine

The metabolism of promazine yielded two glucuronidated products, promazine glucuronide and chlorpromazine glucuronide. Figure 4.6 shows the EPI spectrum of promazine glucuronide; the peak with a m/z of 285.1 Da corresponds to the aglucone promazine and the peak with a m/z of 461.1 Da corresponds to promazine glucuronide. This metabolite provided a strong MS/MS spectrum, implying that it is formed in significant amounts. 7-hydroxypromazine glucuronide has been identified in vivo (Dewey et al., 1981).
Phase II metabolite of acepromazine was acepromazine glucuronide (present in small quantities). The EPI spectrum (figure 4.7) shows a peak corresponding to the aglycone acepromazine and a peak corresponding to acepromazine glucuronide. Hydroxy acepromazine glucuronide and acepromazine sulphoxide glucuronide were also identified. 3'-Hydroxy acepromazine glucuronide and acepromazine sulphoxide glucuronide are known in vivo (Dewey et al., 1981).

Figure 4.6: Phase II metabolism of promazine after 2 hours: EPI of promazine from time 6.88 min to 6.92 min. Peak with m/z of 285.1 Da corresponds to the aglycone, promazine and peak with m/z of 461.1 Da corresponds to promazine glucuronide. Structure of promazine glucuronide shown inset.

Figure 4.7: Phase II metabolism of acepromazine after 2 hours: EPI of acepromazine at time 6.85 min. Peak with m/z of 327.1 Da corresponds to the aglycone, acepromazine and peak with m/z of 503.3 Da corresponds to acepromazine glucuronide. Structure of acepromazine glucuronide shown inset.
Chlorpromazine glucuronide was identified as a phase II metabolite of chlorpromazine. The EPI spectrum of chlorpromazine glucuronide is shown in figure 4.9; the peak with a m/z of 495.1 Da corresponds to chlorpromazine glucuronide.

Figure 4.9: Phase II metabolism of chlorpromazine after 2 hours: EPI of chlorpromazine from time 7.14 min to 7.26 min. Peak with m/z of 319.1 Da corresponds to the aglycone, chlorpromazine and peak with m/z of 495.1 Da corresponds to chlorpromazine glucuronide. Structure of chlorpromazine glucuronide shown inset.
Oxidised chlorpromazine glucuronide was also observed, although it was unclear whether it was the hydroxy, sulfoxide or N-oxide form. The EPI spectrum of oxidised chlorpromazine glucuronide is shown in figure 4.10. 7-Hydroxy chlorpromazine is the main phase II metabolite observed in vivo (Yeung et al., 1993).

Figure 4.10: Phase II metabolism of chlorpromazine after 2 hours: EPI of chlorpromazine sulphoxide at time 6.37 min. Peak with m/z of 335.1 Da corresponds to the aglycone, chlorpromazine sulphoxide and peak with m/z of 511.2 Da corresponds to chlorpromazine sulphoxide glucuronide. Structure of chlorpromazine sulphoxide glucuronide shown inset.

4.3.1.4: NSAIIDs: etoricoxib and lumiracoxib

No phase II metabolites of etoricoxib and lumiracoxib were observed, although the glucuronide conjugates of both drugs are known in vivo (Rodrigues et al., 2003; Kalbag et al., 2004)

It is important to note that the phase I products identified in chapter 3 would most likely also be present in these samples used to investigate the phase II metabolism of drugs. The same controls put in place for the phase I study were also used in this. A blank sample containing microsomes and no drug and a positive drug control containing drug but lacking microsomes were used.
4.4 Conclusion

Phase II metabolites were observed after the incubation with diazepam, nordiazepam and oxazepam, morphine and codeine, promazine, acepromazine and chlorpromazine.

Only glucuronide conjugates were observed despite the addition of glutathione and sulphate conjugate salts to the incubation mixes. The most likely reason for the lack of these conjugates is the lack of the enzymes responsible for the formation of these conjugates. Sulphotransferases and glutathione-S-transferases are almost always found in the cytosol, and may not be present in microsome fractions.

The production of glucuronide conjugates using the liver microsomal system correspond to those observed in vivo. For example, morphine glucuronide was observed after incubation with bovine liver microsomes; the major metabolites of morphine in vivo are morphine-3-glucuronide and morphine-6-glucuronide (Gerostamoulos & Drummer, 1995).
Chapter Five
Conclusions and Future Work

The aim of this research was to develop an in vitro screening system for drug metabolism, especially with respect to drugs of abuse in horse racing. This in vitro system used liver microsomal enzymes to produce metabolites of the drugs of interest.

At first, commercially prepared microsomes from rat liver were used to develop initial parameters. Coumarin was used as a control compound. The system was then tested on two NSAIDs, etoricoxib and lumiracoxib. This initial study also included the establishment of the analytical techniques for the extraction and identification and quantification of the test compounds. These analytical methodologies, including the incubation, solid phase extraction and HPLC-UV detection, were validated.

The ideal experimental parameters established were 50 µM coumarin, 50 µg protein/ml microsomes, 1 mM NADP+, 5 mM G6P and 1U/ml G6PDH incubated for 30 minutes at 38°C.

Once initial parameters and analytical protocols had been established, the method was used to study the metabolism of ten drugs of interest to doping control in horse racing. This study included in silico predictive metabolism using MetaSite and the preparation of a bovine liver microsomal system. Bovine liver was used as the source tissue for the production of microsomes due to availability and cost considerations.

The use of the in silico predictive tool, MetaSite gave a useful prediction of the metabolites produced. The major metabolites of all the drugs studied were predicted by MetaSite. The predicted metabolites varied slightly from those observed in vitro, most likely due to MetaSite utilising human cytochrome P450s for prediction. MetaSite was particularly useful in predicting the metabolism of promazine; 3 out of the 5 metabolites predicted have been reported in vivo. It has limitations as it only predicts the products of CYP-mediated
phase I metabolism. MetaSite could not predict the major metabolites of morphine and codeine, M3G, M6G and C6G, as these are the products of phase II conjugation with glucuronic acid. MetaSite is a valuable tool for the study of drug metabolism, especially in the study of drugs with unknown metabolism.

The in vitro system developed during this study was successfully able to metabolise drugs of different classes. The metabolites analysed by LC-MS were comparable with the metabolites observed during in vivo administration trials. The bovine liver microsomal system used was able to produce phase I metabolites of the drugs studied. The metabolites produced are consistent with those reported in vivo. For example, incubation with diazepam produced nordiazepam, temazepam and oxazepam as metabolites. These metabolites have been reported in vivo (Friendericy & Bovill, 1998). In the study of the phase I metabolism of chlorpromazine, promazine was detected as a metabolite. It was considered that it may be the result of fragmentation of chlorpromazine. However, Yeung et al. (1993) report promazine as a metabolite of chlorpromazine and for this reason it was included as a metabolite.

In order to account for a possible loss in CYP activity, positive controls using coumarin were performed prior to the analysis of the NSAIDs. This was also done to ensure a similar activity between days and among the microsome preparations used in each assay.

Microsomal systems such as the one developed in this study are advantageous as drug metabolites are produced and can be extracted with relative ease to produce clean samples for further analysis. The bovine liver system prepared was a cost effective, reliable and robust method for the production of drug metabolites in vitro.

The bovine liver system was also used to study the Phase II metabolism of the drugs of interest. The in vitro system produced only glucuronide conjugates; these conjugates corresponded to those reported in vivo. The observation of only glucuronide conjugates is most likely due to the cellular location of the enzymes that catalyse these reactions. UGTs, which catalyse the conjugation of glucuronide, are almost exclusively membrane bound and located on the ER, the site of phase I CYP-mediated metabolism. They are present in
microsome fractions, unlike GSTs and SULTs, which catalyse the conjugation of glutathione and sulphate, respectively. These enzymes are found in the cytosol, and are possibly not be present in microsome fractions. In the phase II metabolism study, the sulphate and glutathione conjugate salts were added. Zamek-Gliszczynski et al. (2006) report that some glutathione-S-transferases are membrane bound, while most sulphotransferases are cytosolic. The bovine liver microsomes used were relatively crude, and for this reason, the conjugate salts for these enzymes were investigated. Future work could include the development of the differential centrifugation method for the isolation of the transferase enzymes.

The use of bovine liver to produce the microsomes for these studies proved a reliable model for drug metabolism investigations. Most of the metabolites produced using bovine liver are observed in in vivo equine administration trials. For example, the main metabolites observed after incubation with diazepam were nor diazepam, oxazepam and temazepam. These metabolites are reported as metabolites of diazepam in vivo ((Yasumori et al., 1993, Friedericy & Bovill, 1998, Shini, 2000). Phase II glucuronide conjugates of these metabolites were also detected. These products have also been reported in vivo (Kaeferstein, 2009). The major metabolites of the opioids, morphine and codeine were the phase II glucuronides, M6G, M3G and C6G (Meng et al., 2000, He et al., 1998) and these were detected in vitro using the bovine liver microsomal system. The main metabolites of promazine, chlorpromazine and acepromazine reported in vivo (Wójcikowski et al., 2010, Scarth et al., 2010, Coccia & Westerfeld, 1967) were observed after incubation with the bovine liver microsomal system. The major metabolites of the NSAIDs, lumiracoxib and etoricoxib, observed in vitro have also been reported in vivo in equine administration trials by deKock et al. (2008a & 2008b).

Fresh horse liver for the production of microsomes for this study was unavailable and bovine liver was chosen as a cost effective and readily available alternative. Future work could include the study of drug metabolism in the equine using the microsomal system developed with microsomes isolated from equine liver. This would allow for direct comparison between in vitro microsomal incubations and in vivo administration trials.
The production of metabolites could be increased through the use of purified recombinant expressed equine CYPs, as reported by DiMaio Knych et al. (2009a). The major drug metabolising CYP isoforms, such as CYP3A4 and CYP2C9, could be expressed using a bacterial or yeast expression system. The use of purified isoforms would increase the specificity of the drug incubations. The genes that would be used to express these isoforms have been sequenced (DiMaio Knych et al., 2009b, Schmitz et al., 2010) and their sequences are available on Genbank.

The system developed in this study, both in silico predictions and in vitro microsome incubations, could be used to study the metabolism of drugs with unknown metabolism, such as designer drugs. The in vitro microsomal system has the potential application to produce drug metabolites in sufficient quantities that the products, once authenticated, could be used as standards. This would be especially useful in doping control for the testing of controlled or banned drugs.
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Appendix A: Standard Curves

Figure A.1: Standard Curve of Coumarin. Pure coumarin standards, prepared in mobile phase (ACN:H2O 60:40), were analysed using the HPLC-UV method described in section 2.2.1.1 (pg 23). The standard curve was constructed using a concentration range from 0 - 100 μM. Samples were analysed in triplicate on the same day.

Figure A.2: Standard Curve of 7-hydroxycoumarin. Pure 7-OH coumarin standards, prepared in mobile phase (ACN:H2O 60:40), were analysed using the HPLC-UV method described in section 2.2.1.1 (pg 23). The standard curve was constructed using a concentration range from 0 - 100 μM. Samples were analysed in triplicate on the same day.
Figure A.3: Standard Curve of Etoricoxib. Pure etoricoxib standards, prepared in mobile phase (ACN:H20 60:40), were analysed using the HPLC-UV method described in section 2.2.3.2 (pg 26). The standard curve was constructed using a concentration range from 0 - 2 mM. Samples were analysed in duplicate on the same day.

Figure A.4: Standard Curve of Lumiracoxib. Pure lumiracoxib standards, prepared in mobile phase (ACN:H20 60:40), were analysed using the HPLC-UV method described in section 2.2.3.2 (pg 16). The standard curve was constructed using a concentration range from 0 - 2.5 mM. Samples were analysed in duplicate on the same day.
**Figure A.5: Exemplar Protein Standard Curve.** Protein concentration was evaluated using the Bradford method. Equal volumes of Bradford reagent and BSA protein standard (standards were prepared in phosphate buffer, pH 7.4, containing 3 mM MgCl₂ and 1mM EDTA with a concentration range of 0 - 200 μg/ml) were incubated at room temperature for 10 minutes before the absorbance at 595 nm was read. A new standard curve was used for each analysis.