

**CHEMICAL AND BIOCHEMICAL ASPECTS  
OF DRUG-INDUCED LIVER INJURY**

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENTS OF  
THE UNIVERSITY OF LIVERPOOL FOR THE DEGREE OF DOCTOR OF  
PHILOSOPHY

BY

RACHEL JOANNE WALSH

SEPTEMBER 2010

## **DECLARATION**

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

**Rachel Joanne Walsh**

This research was carried out in the Department of Pharmacology and Therapeutics  
The University of Liverpool.

## ACKNOWLEDGMENTS

Firstly, I would like to thank both Professor Kevin Park and Dr Dominic Williams for their invaluable support and guidance over the last four years, throughout my time both in and out of the lab. Gratitude also goes out to Dr James Maggs for all his time and effort in helping with the LC-MS work. I'd also like to thank all at AstraZeneca, both for funding and for all the help and guidance they provided, especially Professor Raimund Peter and Dr Charles O'Donnell. I'd also like to thank Dr Mhairi Greer and Dr Gerry Kenna, also from AstraZeneca, for their efforts in providing help and resources for the THLE-CYP chapter.

Huge thanks go out to everyone in the lab who has helped to make my time there so enjoyable. I'd like to thank Rowena Sison for sharing my ups and downs with chapter five, and to Adam Lister and Alvin Chia for making me laugh. For sharing Friday lab cleaning, hepatocyte woes and joys all in the G.31 gang, especially Dan Antoine and Sophie Regan for all their advice and support. For lunchtime respite and being fantastic friends, I'd like to thank Hayley Webb, Craig Benson and Andrew Swale.

Lastly, I'm massively grateful to my parents whose unwavering support, patience and love has literally got me through the past four years. Thank You.

**CONTENTS**

<b>Abstract</b>	<b>5</b>
<b>Publications</b>	<b>7</b>
<b>Abbreviations</b>	<b>8</b>
<b>Chapter One: General Introduction</b>	<b>10</b>
<b>Chapter Two: Use of Mass Spectrometry to Define the Reactive Metabolite of Methapyrilene</b>	<b>47</b>
<b>Chapter Three: An Investigation of the Bioactivation and Toxicity of Thiophenes in Isolated Rat Hepatocytes</b>	<b>81</b>
<b>Chapter Four: An Investigation of the Bioactivation and Toxicity of Thiophenes in THLE-CYP cell lines</b>	<b>118</b>
<b>Chapter Five: Ophthalmic Acid as a Biomarker of Glutathione Depletion</b>	<b>144</b>
<b>Chapter Six: General Discussion</b>	<b>176</b>

## **Chemical and Biochemical Aspects of Drug Induced Liver Injury**

Rachel Walsh

Department of Pharmacology and Therapeutics, MRC Centre for Drug Safety Science,  
University of Liverpool, UK

Adverse drug reactions (ADRs) are a major obstacle for the development of new medicines. They are also a leading cause of patient morbidity and mortality. Although ADRs affect many different organs and bodily systems, drug induced liver injury has led to the withdrawal of several drugs at the post licensing stage, and is a key cause of drug attrition. Many of the drugs that cause liver injury are thought to do so through metabolism to a reactive metabolite, exposure to which can cause modification of cellular proteins, leading to loss of function, and can result in a loss of cellular homeostasis. It is therefore important to understand the chemistry and the downstream biochemical events associated with bioactivation. Information on the chemistry of metabolism coupled with mechanistic biomarkers reflective of certain pathways of hepatic injury would enable both researchers and physicians to predict and diagnose DILI, leading to the improvement of safe drug design.

This thesis focuses firstly on the use of in vitro models and mass spectrometry to provide integrated data on the metabolism and toxicity of xenobiotics, using thiophene containing molecules as a paradigm. The thiophene ring has the potential to be bioactivated via *S*-oxidation and epoxidation pathways, and several thiophene containing drugs have been associated with drug induced liver injury. The investigations described intended firstly to elucidate the chemistry of methapyrilene bioactivation using mass spectrometry and hydrogen-deuterium exchange. The following two chapters aimed to establish a link between bioactivation and toxicity of thiophenes and to evaluate two in vitro models as tool for predicting DILI. The final experimental chapter aims to investigate the potential of ophthalmic acid (OA) to act as a serum biomarker reflective of depletion of hepatic levels of the protective tripeptide, glutathione (GSH). Disturbance of GSH levels through quenching of reactive metabolites can lead to disturbance of its anabolism and catabolism pathways. Indeed, serum OA levels, a GSH analog, have been shown to rise following hepatic GSH depletion.

This work utilises GSH adduct formation as a marker of bioactivation of thiophenes in several different in vitro models. Rat liver microsomal incubations were analysed using hydrogen deuterium exchange and LC-MS to define the reactive metabolite of methapyrilene as an *S*-oxide of the thiophene ring. Freshly isolated rat hepatocytes or single P450 expressing THLE cell cultures were exposed to either methapyrilene, tienilic acid, ticlopidine or 2-phenylthiophene and subsequent LC-MS analysis confirmed GSH adduct formation for all compounds in the isolated rat hepatocyte model, but only for 2-phenylthiophene in the THLE cell model. Cytotoxicity was also investigated in both models, and all compounds were found to cause a greater degree of toxicity in the isolated rat hepatocyte molecule than in the THLE model.

By exposing rodents to depleters of hepatic GSH, such as acetaminophen and diethylmaleate, and monitoring the resultant serum OA levels, it has been determined that OA is not a reliable

mechanistic marker of hepatic GSH depletion. Kinetic studies of OA in rat serum have revealed that OA is subject to a similar metabolic and elimination pathway as GSH.

The overall scope of this work reveals the usefulness of LC-MS/MS to determine *S*-oxide and epoxide adducts in in vitro studies. The freshly isolated rat hepatocyte model was a useful tool for providing integrated metabolic and toxicological data of thiophene containing molecules and has the potential to be expanded to include data on covalent binding and levels of DILI biomarkers. The single CYP expressing THLE cell model was not as useful in this case, but has been used in other studies to explore the role of discrete P450 enzymes in toxicity and metabolism . Whilst it is unfortunate that serum OA did not reflect hepatic OA in such a way that it could be easily exploited as a biomarker, this does help us to understand that the plethora of potential biomarkers uncovered by proteomic, metabolomic and transcriptomic studies need to be investigated in depth in order to understand their applications across different species and systems.

## PUBLICATIONS

Graham, EE, **Walsh, R.J**, Hirst, CM, Maggs, JL, Martin, S, Wild, MJ, Wilson, ID, Harding, JR, Kenna, JG, Peter, RM, Williams, DP, Park, BK (2008) Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* **326**(2): 657-671.

**LIST OF ABBREVIATIONS**

2-PT, 2-Phenylthiophene

2-AB, 2-aminobutyrate

ABT, 1-aminobenzotriazole

ACN, acetonitrile

ADR, Adverse drug reaction

ALT, serum alanine transferase

APAP, acetaminophen

CYP450, cytochrome P450 mixed function oxidase

DEM, diethylmaleate; DILI, drug-induced liver injury

GCL, glutamate cysteine ligase

GSH, glutathione

HPLC, high performance liquid chromatography

LC-MS/MS, liquid chromatography tandem mass spectrometry

MeOH, methanol

MeOD, monodeuteromethanol

MP, methapyrilene; MRM, multiple reaction monitoring

NADPH,  $\beta$ -nicotinamide adenine dinucleotide phosphate (reduced)



OA, ophthalmic acid

RLM, rat hepatic microsomes

rt, retention time

S-hex, S-hexylglutathione

TA, tienilic acid

TC, ticlopidine

THLE-CYP, SV40 large tumour antigen-immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes.

## CHAPTER ONE

---

**GENERAL INTRODUCTION**

**Contents**

1. 1. Adverse Drug Reactions .....	12
1.2 Off Target Toxicity - Drug Induced Liver Injury .....	14
1.3. Xenobiotic Metabolism.....	16
1.4 The Role of Metabolism in DILI .....	17
1.5 Toxicophores and the Thiophene ring .....	19
1.6 Use of Model Hepatotoxins to Explore Mechanisms of DILI .....	24
1.7 Methods for trapping, identifying and defining reactive metabolites .....	25
1.9 Glutathione Conjugation as a Detoxification Mechanism .....	29
1.10 Development of Novel Biomarkers in DILI .....	30
1.11 Aims of this work.....	33

## 1. 1. Adverse Drug Reactions

Adverse drug reactions (ADRs) represent one of the major clinical challenges to patients' health, accounting for 2-6% of US hospital admissions (Bates *et al.*, 1995; Classen *et al.*, 1997; Einarson, 1993) and are a key reason for attrition in drug development. Mechanisms of drug induced toxicity have been reviewed and classified into five categories. (Liebler *et al.*, 2005). These categories are: on target or mechanism related toxicity; hypersensitivity and immunological reactions; bioactivation to reactive metabolites; off target toxicity and idiosyncratic toxicities. On target reactions account for 80% of all ADRS and can be predicted from the known primary or secondary pharmacology of the drug and often represent an exaggeration of the pharmacological effect of the drug. They show simple dose-response relationships and, therefore, can usually be avoided by dose reduction and are only rarely life-threatening. A good example of this toxicity is the risk of bleeding associated with anti-coagulant therapy. Indicated in venous thrombosis, the pharmacology of warfarin is to inhibit vitamin K epoxide reductase and prevent the coagulation cascade. Warfarin has a narrow therapeutic range, and patients can be monitored to prevent haemorrhage (Ng, 2009). Statins can also cause on target toxicity. The pharmacological action of this class of drug is to inhibit HMG-CoA and therefore to reduce levels of cholesterol and other products of the isoprenoid pathway. When large concentrations of statins accumulate outside of the liver, this inhibition of the isoprenoid pathway can lead to cases of muscle toxicity, and potentially proteinuria and even kidney failure due to release of proteins from overloaded muscles.

In contrast, off target reactions cannot be predicted from knowledge of the basic pharmacology of the drug, therefore the hypersensitivity, bioactivation and idiosyncratic categories could also be described as types of off target reaction. Whilst not as common as on target reactions, these can be serious and life-threatening. A type of off target reaction that is now regularly screened for in pre-clinical safety studies is inhibition of cardiac ion (hERG) channels. Cardiac arrhythmias caused by this mechanism were a side effect of antihistamine terfenadine (Honig *et al.*, 1992). Hypersensitivity reactions are common and range from mild skin rashes to organ failure. Formation of reactive metabolites and covalent binding are thought to play a part in the mechanism of this type of reaction (Pirmohamed *et al.*, 2002). An example of this mechanism is the hypersensitivity reaction associated with sulphamethoxazole. Metabolism of sulphamethoxazole to reactive nitroso compounds leads to protein adduct formation. These are then recognised by the immune system, resulting in an adverse reaction (Sanderson *et al.*, 2006). Idiosyncratic reactions are incredibly rare, and difficult to reproduce and are thought to be a function of both the chemistry of the drug and biology of the individual patient. Isoniazid, an anti-tuberculosis agent, causes fatal hepatitis in less than 1% of patients. There is evidence to indicate formation of reactive metabolites may be part of this idiosyncratic reaction (Nelson *et al.*, 1976), but it is not understood how these affect the hepatocyte. Bioactivation as a mechanism will be discussed at greater length, but there is evidence to suggest formation of reactive metabolites plays a part in the onset of many types of off target adverse drug reactions.

## 1.2 Off Target Toxicity - Drug Induced Liver Injury

ADRs are known to affect every organ system within the body. Dapsone causes methaemoglobinaemia (Shelley *et al.*, 1976), chronic exposure to benzene can lead to bone marrow hyperplasia (Ross *et al.*, 1996) and cephaloridine can cause acute renal failure (Rosenthal *et al.*, 1971). However, those involving the liver represent a significant proportion with around 52% of all cases of acute liver failure in the US attributed to drug-induced liver injury (DILI) (Ostapowicz *et al.*, 2002). DILI is the most frequent reason for the withdrawal of an approved drug from the market (Temple *et al.*, 2002). Of the 548 new drugs approved by the US Food and Drug Administration between 1975 and 1999, four were withdrawn from the market due to cases of DILI and 10 received a 'black box' warning due to their potential to cause liver injury (Lasser *et al.*, 2002). Several factors contribute to this. The liver is the largest solid organ of the body, constituting 2-5% of human adult body weight, and receives a dual blood supply from the portal vein and hepatic artery (Desmet, 2001). The liver is the principal site of drug metabolism; therefore it is often the target of toxicity. Typically DILI is off target, with most being unpredictable and poorly understood. The manifestations range from mild, asymptomatic changes in serum transaminases, which are relatively common, to fulminant hepatic failure, which although rare, is potentially life threatening and may necessitate a liver transplant (Park *et al.*, 1998).

DILI can present in various, complex manifestations and is therefore difficult to diagnose. Recommendations from the FDA suggested that the use of clinical chemistry should be used to define DILI (FDA, 2000), such as level of serum transaminase enzymes or bilirubin. The various types of injury present different

symptoms that may be used to differentiate. Mitochondrial injury may involve lactic acidosis, the observation of steatosis on biopsy and slight elevations of aminotransferase levels (Navarro *et al.*, 2006). This type of injury can be induced by tetracycline treatment (Freneaux *et al.*, 1988). Cholestatic injury refers to damage to bile ducts or canaliculi by xenobiotics such as anabolic steroids (Velayudham *et al.*, 2003). It is characterised in the clinic by a prominent elevation in alkaline phosphates, followed by increase in alanine transaminase. Many compounds directly damage the hepatocyte, and cause the onset of apoptosis and necrosis. This is identified by an increase in serum transaminase followed by elevated bilirubin levels (Navarro *et al.*, 2006). Drugs such as chlorpromazine and ketoconazole not only induce cholestasis, but also damage the hepatocyte, resulting in cholestatic hepatitis. As well as these types of injury, hypersensitivity or immune reactions can also occur, usually with delayed onset, or following repeated exposure, and can vary in severity from skin rash to autoimmune hepatitis (Liu *et al.*, 2002).

The complete mechanisms behind DILI present a complex challenge. There are many pathways which when disrupted could result in the types of injury described above, from disruption of the cell membrane, covalent binding to proteins, interruption of transport processes, mitochondrial disruption and induction of apoptosis (Lee, 2003). However, for many molecules involved in DILI, the initial factor sparking the onset of injury appears to be formation of a reactive metabolite (Park *et al.*, 2005)

### 1.3. Xenobiotic Metabolism

The biotransformation of lipophilic compounds into water-soluble derivatives that are more readily excreted is the physiological role of xenobiotic metabolism. The principal site of drug metabolism is the liver. The liver is exposed to drugs and other xenobiotics immediately after their absorption from the gastro-intestinal tract and has a high capacity for both phase I and phase II biotransformations. Phase I enzymes catalyse oxidative, reductive and hydrolytic reactions. Cytochrome P450 enzymatic reactions are the predominant phase I processes and although the most common transformation catalysed by these enzymes is oxidation, other less frequent reactions include epoxidation, dehydrogenation and *N* or *S*-oxygenations (Guengerich, 2001a; Guengerich, 2001b). The P450 superfamily describes a large number of related, endoplasmic reticulum bound, haem protein enzymes, with ancestral genes predating the prokaryote/eukaryote divergence (Nelson *et al.*, 1993). The P450 cycle is complex, involving the reduction of the ferric ion, incorporation of molecular oxygen and a proton and loss of water, resulting in the addition of oxygen to drug molecules to form a hydroxyl group. Seventy-four CYP families have so far been identified. The main families involved with drug metabolism are CYP1, CYP2 and CYP3 (Zimmerman, 1999). Substrate specificity is overlapping between different isoforms, but is dependent upon the topography of the active site, degree of steric hinderance impeding the haem-oxygen complex to sites of metabolism and ease with which electrons or protons can be abstracted from atoms within the substrate. Phase I reactions, however, are not confined to P450 catalysed reactions. Other enzymes involved include alcohol dehydrogenase, a soluble cytoplasmic enzyme which



metabolises ethanol, and monoamine oxidase which is responsible for inactivation of amines such as noradrenaline.

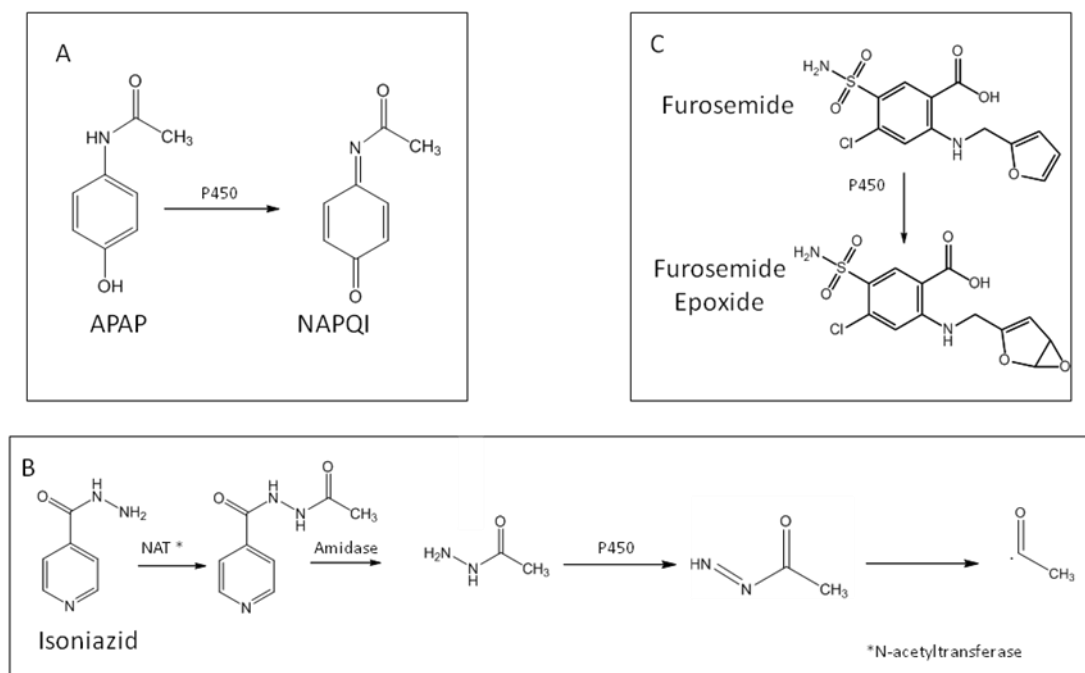
Phase II reactions utilise hydroxyl, thiol and amine groups, either innate in the molecule or added through Phase I reactions. Large, lipophobic groups are added to the molecule through conjugation reactions, usually resulting in a pharmacologically inactive, polar molecule which are more easily excreted (Kalgutkar *et al.*, 2005). Frequently observed conjugating groups are glucuronyl, sulphate, glycyl and glutathione. Conjugation is facilitated by dedicated enzymes for each conjugating agent. For example, glucuronidation is catalysed by uridine phosphate glucuronyl transferase (UGT) and glutathione conjugation sometimes involves glutathione transferase (GST). Like P450, UGT and GST proteins both comprise a family of enzymes. There are two families of UGT enzymes, UGT1 and UGT2, whereas there are eight families of GST isoforms, alpha, mu, theta, pi, zeta, sigma, kappa and omega.

#### **1.4 The Role of Metabolism in DILI**

Usually the conversion from a lipid to water soluble form results in loss of pharmacological/biological activity, but certain xenobiotics undergo biotransformation to toxic/reactive metabolites that can interfere with cellular functions and may have intrinsic chemical reactivity towards certain types of cellular macromolecules. These reactive metabolites have the ability to interact with cellular proteins, lipids and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress, potentially leading to an off target ADR. This impairment of cellular function can result in cell death and possible liver failure.

The propensity of a molecule to form either toxic and/or chemically reactive metabolites is simply a function of its chemistry. Such metabolites are typically short-lived, with half-lives of generally less than one second, and are not usually detectable in plasma. Formation of chemically reactive metabolites is mainly catalyzed by cytochromes P450, although products of phase-II metabolism can also lead to toxicity. Additionally, non-cytochrome P450 oxidative enzymes, such as myeloperoxidase and prostaglandin H synthetase, have been implicated in the bioactivation of drugs and other chemicals, the metabolites of which are thought to be responsible for observed toxicity, e.g. clozapine and agranulocytosis, benzene and aplastic anaemia (Fischer *et al.*, 1991; Mason *et al.*, 1992; Ross *et al.*, 1996; Smith *et al.*, 1989). Cytochrome P450 isoforms are present in different proportions in many organs, though most abundantly in the liver, and thus bioactivate the chemicals to cause organ-specific toxicity (Kao *et al.*, 1990; Pelkonen *et al.*, 1997; Uetrecht, 1992). The relationship between bioactivation and the occurrence of hepatic injury is not simple. For example, many chemicals undergo bioactivation in the liver but are not hepatotoxic. The best example is the absence of hepatotoxicity with therapeutic doses of acetaminophen. Tight coupling of bioactivation with bioinactivation may be one reason for this. Many enzymic and nonenzymic pathways of bioinactivation are present in the liver, which is perhaps the best equipped of all the organs in the body to deal with chemically reactive toxins. Typical examples of bioinactivation pathways include glutathione conjugation of quinones by glutathione *S*-transferases and hydration of arene oxides to dihydrodiols by epoxide hydrolases. The efficiency of a bioinactivation process is dependent on several factors including the inherent chemical reactivity of the substrate, substrate-selectivity of the enzymes, which is usually very broad, tissue expression of the enzymes, and the rapid-upregulation of

enzyme(s) and co-factors mediated by cellular sensors of chemical stress. It is only when a reactive metabolite can by-pass or saturate these defence systems of bioinactivation and thereby damage proteins and nucleic acids through covalent binding that it exerts significant toxic effects.



**Figure 1.1.** Bioactivation of (A) acetaminophen (APAP); (B) isoniazid; and (C) furosemide.

### 1.5 Toxicophores and the Thiophene ring

Certain chemical moieties have a predisposition to be transformed into reactive metabolites as described above. These toxicophores are vulnerable to oxidation by P450, or other enzyme, and the resulting metabolite is able to react with cellular proteins. Functional groups which are susceptible to formation of reactive metabolites include aromatic amines, such as the analgesic acetaminophen, which can form quinone imines (Fig 1.1A); hydrazines such as the anti-TB agent isoniazid,

which forms a free radical metabolite via *N*-acetyldiazine (Fig 1.1B) and furans such as the diuretic furosemide, which form epoxide intermediates (Fig 1.1C) (Kalgutkar *et al.*, 2005). Often, toxicophores consist of unsaturated aromatic ring moieties, where epoxidation can take place. The thiophene ring, a sulphur containing, 5-membered, unsaturated aromatic heterocycle, is an example of such a moiety. Thiophene derivatives are commonly found in nature and exposure as a consequence of human activity occurs when thiophenes are released into the environment through fossil fuel combustion (Huffman *et al.*, 2000) and generated in cooking processes (Bredie *et al.*, 2002; Methven *et al.*, 2007). In medicinal chemistry, thiophene rings can be used to improve stereoselectivity by acting as bioisosteres for benzo, phenyl, phenol and heterocyclic moieties (Broom *et al.*, 1995; Kilbourn, 1989; Press *et al.*, 1993; Rodenhuis *et al.*, 2000) and are also present in many drugs, incorporated to increase potency and efficacy (Broom *et al.*, 1995; Hagen *et al.*, 2001; Press *et al.*, 1993) as well as enhancing pharmacokinetic properties (Broom *et al.*, 1995; Hagen *et al.*, 2001; Rodenhuis *et al.*, 2000). Thiophene rings have been shown to undergo P450 bioactivation by several mechanisms (Fig 1.2). The first consists of oxidation of the ring to form an epoxide intermediate (Dansette *et al.*, 2005; O'Donnell *et al.*, 2003). This intermediate can then be detoxified by GSH conjugation, resulting in an hydroxyl GSH adduct (Dansette *et al.*, 2005), or can undergo rearrangement to form an  $\alpha,\beta$ -unsaturated aldehyde intermediate (O'Donnell *et al.*, 2003) (Fig 1.2). Another mechanism consists of oxidation of the sulphur atom to form a thiophene *S*-oxide intermediate (Belghazi *et al.*, 2001; Nishiya *et al.*, 2008; Shimizu *et al.*, 2009). These reactive metabolites can again be quenched by GSH conjugation (Fig 1.2). Epoxidation and *S*-oxidation of thiophene containing drugs has been linked with occurrences of toxicity. Tienilic acid, a diuretic, has been shown to form an *S*-oxide

metabolite (Belghazi *et al.*, 2001), which can covalently bind to human CYP2C9 (Lopez-Garcia *et al.*, 1994). This mechanism is thought to lead to the autoimmune hepatitis brought on by tienilic acid therapy (Pons *et al.*, 1991). Methapyrilene, a H<sub>1</sub> receptor antagonist, is a rat specific hepatotoxin. The periportal damage caused by methapyrilene is thought to be metabolism dependant (Ratra *et al.*, 1998) and due to bioactivation of the thiophene ring (Graham, 2007). Suprofen, a non-steroidal anti-inflammatory drug, has been shown to form a thiophene epoxide and consequently an  $\alpha,\beta$ -unsaturated aldehyde intermediate (O'Donnell *et al.*, 2003). Suprofen use has been associated with acute renal failure (Abreo *et al.*, 1986). S-oxide metabolites of the anti-platelet agent ticlopidine have also been discovered in in vivo and in vitro systems (Lim *et al.*, 2008; Shimizu *et al.*, 2009). Ticlopidine has been associated with cases of acute hepatitis, (Pizarro *et al.*, 2001), but more commonly causes aplastic anemia (Yeh *et al.*, 1998).

Ticlopidine belongs to a class of drugs for whom metabolism of the thiophene ring is essential for efficacy, which also includes clopidogrel and prasugrel. These thienopyridine drugs inhibit aggregation of platelets via their action as irreversible antagonists of the PY12 receptor. In order to covalently bind to this receptor, the thiophene ring must undergo bioactivation via a thiol acetone metabolite to the active thiol metabolites (Dansette *et al.*, 2009; Nishiya *et al.*, 2009a; Nishiya *et al.*, 2009b) (Fig 1.3). These metabolic pathways have not been linked as a cause of off target toxicity. Clopiogrel is still used safely in patients, but there are some reports of similar adverse reactions as ticlopidine (Goyal *et al.*, 2009). Prasugrel has only recently been introduced and there are currently no reports of off target adverse events (Huber *et al.*, 2009).

Indeed, whilst some thiophene containing drugs have been associated with DILI as described above, thiophenes have also been safely incorporated in many widely used drugs, such as the estrogen receptor modulator, raloxifene, whose major metabolites in vivo are glucuronides (Trontelj *et al.*, 2007), but has been shown to covalently bind to liver microsomes (Obach *et al.*, 2008). The fused ring structure of raloxifene prevents formation of an epoxide intermediate, but *S*-oxidation would still be possible. However, protein adduct formation by raloxifene is postulated to be due to epoxidation of a benzene ring (Yukinaga *et al.*, 2007).

Another thiophene containing drug not associated with DILI is the opioid analgesic, sufentanil, which is subject to *N*-dealkylation in vivo (Thevis *et al.*, 2005). The thiophene moiety in sufentanil is not fused, therefore both epoxidation and *S*-oxidation are possible pathways of metabolism, however no such intermediates have been identified. Investigations in liver microsomes of human, rat and dog discovered similar metabolic pathways in all species, including oxidative *N*-dealkylation on the piperidine nitrogen, oxidative *O*-demethylation and aromatic hydroxylation (Lavrijsen *et al.*, 1990).

The alternative metabolic pathways may in these cases be favoured over formation of reactive metabolites, or in the case that reactive metabolites are formed, detoxification pathways are able to deal sufficiently with the electrophilic species, providing protection from potential hepatocyte damage. It is therefore difficult for preclinical scientists to unravel the relationship between structure and toxicity. Whilst certain moieties may have a predilection for bioactivation, this does not always lead to the onset of DILI, whether this is due to other, more favoured pathways of metabolism, or effective detoxification pathways.

**Figure 1.2.** Potential pathways of thiophene bioactivation and detoxification by nucleophiles such as GSH. Pathways shown are *S*-oxidation, epoxidation and epoxidation followed by rearrangement to an  $\alpha,\beta$  unsaturated aldehyde.

**Figure 1.3.** Formation of active metabolite of thienopyridine P<sub>Y</sub>12 receptor antagonists, ticlopidine, clopidogrel and prasugrel via a thioacetone intermediate.

## 1.6 Use of Model Hepatotoxins to Explore Mechanisms of DILI

Several compounds can be used to further investigate DILI due to their actions as hepatotoxins in animal and cell models. The reliability of these models enables an understanding of idiosyncratic reactions which are difficult to reproduce. Such molecules include acetaminophen, bromobenzene, carbon tetrachloride, furosemide and methapyrilene. In each of these cases, the basis for injury appears to be metabolic. Acetaminophen (APAP) forms a quinone imine metabolite (Raucy *et al.*, 1989), bromobenzene is metabolised to an epoxide and a quinone (Fisher *et al.*, 1993), carbon tetrachloride is converted into a free radical, and furosemide forms an epoxide of the furan ring (Williams *et al.*, 2007). In the case of methapyrilene, bioactivation of the thiophene ring has been postulated (Graham, 2007), but the chemistry has not been defined. Use of model hepatotoxins in *in vivo* and *in vitro* experiments can begin to unravel some of the complex mechanisms involved. APAP overdose causes massive necrosis, and also results in an adaptive defence response in the hepatocyte (Copple *et al.*, 2008). Bromobenzene is postulated to cause injury via GSH depletion, covalent binding, lipid peroxidation and disruption of mitochondria. The formation of a free radical from carbon tetrachloride leads to lipid peroxidation. Covalent binding has been observed with furosemide, which causes necrosis in the mouse. Methapyrilene is rat specific hepatotoxin, causing periportal hepatotoxicity.

An understanding of the chemistry of bioactivation is essential in providing a full picture of how structure can influence the propensity of a molecule to illicit mechanisms resulting in liver injury. The mechanisms by which model hepatotoxins produce liver injury can provide us with more information regarding frequent targets of reactive metabolites, and therefore aid the design of new preclinical tests and biomarkers of DILI.



### **1.7 Methods for trapping, identifying and defining reactive metabolites**

In order to design safe drugs and elucidate how structure may affect toxicity due to propensity for bioactivation, it is imperative that companies are aware of the metabolism of any new chemical entity under investigation.

Traditionally, metabolic products of xenobiotics have been assessed by microsomal incubation followed by analysis by liquid chromatography. Metabolites can also be identified *in vivo*, either in urine and faeces, or from the bile of cannulated animals. Both these studies are generally undertaken from a pharmacokinetic standing, to identify metabolic ‘soft spots’ of compounds which have a low metabolic stability and therefore inform how structural alterations could be directed. Prior to toxicological studies in animals, both these *in vitro* and *in vivo* methods can be used to determine metabolite exposure and identify which molecules to take forward (Baranczewski *et al.*, 2006).

As reactive metabolites are usually short lived, it is difficult to identify them directly, therefore it is by looking for the presence of conjugates that we can infer the structure of reactive intermediates. These conjugates can be found in the *in vivo* studies mentioned above, either as GSH or N-acetylcysteine conjugates, or as products of these, such as mercapturic acid conjugates (Pascoe *et al.*, 1988). When using microsomal incubations to investigate bioactivation, it is necessary to supplement the incubation with a trapping agent due to the absence of co-factors restricting Phase II metabolism (Masubuchi *et al.*, 2007). Both hard and soft electrophiles can be determined by the use of different trapping agents; cyanide for hard electrophiles (Meneses-Lorente *et al.*, 2006), and thiols such as GSH, N-acetylcysteine and glutathione ethyl ester for soft electrophiles (Castro-Perez *et al.*, 2005).

Use of radiolabelled compounds allows for quantitative analysis via radiometric HPLC, but mass spectrometry is required in order to elucidate the chemistry of metabolites. Since the LC-MS technique was introduced in the 1990s (Bryant *et al.*, 1997), there have been several advances in technology and introduction of new techniques which have aided in the use of mass spectrometry to define structure of thiol conjugates, and therefore in the deduction of reactive metabolite chemistry. Tandem mass spectrometry has allowed more intricate investigation of the structure of metabolites, by enabling more information about fragmentation to be examined. Various types of instrument are currently available with different scan functions and use of different mass spectrometers in metabolite profiling has been reviewed (Zhang *et al.*, 2009), along with their abilities in screening for GSH adducts.

LC-MS methods of analysis do not always provide sufficient information to definitively determine a chemical structure, and therefore it is possible to complement these analyses with nuclear magnetic resonance (NMR). However, static NMR, whilst providing highly detailed information on the structure of molecules, requires an isolated sample of high purity. Sample and data processing techniques can therefore be introduced into LC-MS analysis to provide more information regarding the structure. These include hydrogen/deuterium exchange, in which labile hydrogen atoms are replaced with deuterium, enabling the number of labile hydrogen atoms to be deduced (Lam *et al.*, 2002; Liu *et al.*, 2001; Nassar, 2003).

Although formation of a reactive species does not necessarily translate into hepatocyte damage and onset of DILI, it is important to determine if a reactive metabolite is formed in order to not only provide evidence for careful safety

monitoring of that particular molecule, but also to provide information relevant when assessing other molecules containing similar structural qualities.

### **1.8 Use of Hepatocytes in Metabolism and Toxicity Studies**

Although microsomes are a useful tool for studying Phase I metabolism and covalent binding, their use is restricted with regards to Phase II metabolism due to the lack of co-factors present in the fraction. Because of this, *in vitro* studies in metabolism are often carried out using whole isolated hepatocytes. Isolated human and animal hepatocytes are recognised as a useful and relevant model for studying not only metabolism, but also transporter interactions (Liu *et al.*, 1999), and toxicological potential of molecules (Tirmenstein *et al.*, 2002). Once isolated, if suspended under adequate conditions, hepatocytes will maintain the entire hepatic metabolising system for up to six hours, and if cultured, will express functional hepatocyte traits (Gomez-Lechon *et al.*, 2004; Gomez-Lechon *et al.*, 2003; O'Brien *et al.*, 2005). Studies have shown that the results obtained *in vitro* using this system and results obtained *in vivo* are well correlated. Indeed, a study using hepatocytes prepared from human donors showed a similar metabolic profile for aceclofenac *in vitro*, as was identified *in vivo* when the same donors were administered with a dose of the same drug (Ponsoda *et al.*, 2001). However, compared to human hepatoma cell lines such as HepG2, freshly human hepatocytes are not as readily available, and unlike isolated animal hepatocytes, are subject to marked inter-individual variability. Cryopreservation and pooling of isolated human hepatocytes has begun to address this problem, however, the use of isolated animal hepatocytes offers an easily controllable model. Fresh animal cells are straightforward to locate and the use of

inbred strains and controlled diets minimises variability. It is also possible to control the model further, by breeding for certain phenotypes, such as slow or fast metabolisers. By using suspensions, as opposed to culture, the enzymatic capability is maintained and allows investigation of both chemistry of metabolism and toxicological end points in the same incubation (O'Brien *et al.*, 2004; O'Brien *et al.*, 2005). This would enable an integrated exploration of structure toxicity relationships.

Despite being readily available and having simpler culture conditions, hepatoma cell lines do not represent a good model for studying hepatic metabolism. This is due to the limited expression of biotransformation enzymes, levels of which are low compared to those in the normal adult liver (Donato *et al.*, 2008). These cell lines however have had a place in investigating the toxicological potential of molecules (Mersch-Sundermann *et al.*, 2004). In order to try and understand the role of metabolism in hepatotoxicity, there has been a drive towards development of transfected enzyme systems in hepatoma derived cell lines. These have included transfecting both human (HepG2) and murine cell lines with both stable and inducible cDNA encoding for both Phase I and Phase II metabolising enzymes and cell defence genes (Aoyama *et al.*, 1990; Goldring *et al.*, 2006; Puga *et al.*, 1990), therefore potentially providing a good model for investigating DILI in a hepatoma cell line. However, the P450 expression in these cells is often transient and of low level when compared to hepatocytes. Recently, a human liver epithelial cell line (THLE-5b), has been stably transfected with cDNA of P450s using simian virus 40 and a cytomegalovirus promoter. Whilst these cells have a hepatocyte-like phenotype, expressing high levels of Phase 2 metabolising enzymes they do not express high levels of P450. When transfected as described above, the THLE-CYP

cell lines express comparable levels of P450 to human hepatocytes (Mace *et al.*, 1997). By engineering several cell lines expressing single human P450s, there is scope for investigating the role of discrete P450 enzymes in the metabolism and cytotoxicity of drugs.

### **1.9 Glutathione Conjugation as a Detoxification Mechanism**

Formation of reactive intermediates leads to an adaptive defence response in the cell, involving nuclear translocation of transcription factors which orchestrate transcription of cellular defence genes (Copple *et al.*, 2010; Kensler *et al.*, 2007). Upregulated enzymes include phase II enzymes, heme oxygenase (Takakusa *et al.*, 2008), and those involved in the formation and conjugation of glutathione (GSH), such as glutamate cysteine ligase, the rate limiting enzyme in GSH synthesis (Randle *et al.*, 2008). Maintaining the pool of the protective tripeptide GSH is critical in preserving the balance of deactivation of reactive metabolites and onset of toxicity (Lu, 2009; Mercer *et al.*, 2009). GSH is found at millimolar concentrations in the liver and is formed in a two step process. The first is catalysed by glutamate cysteine ligase and sees glutamate bound to cysteine, forming  $\gamma$ -glutamylcysteine. The second step is the addition of glycine to the cysteine residue, catalysed by glutathione synthetase. The synthesis and metabolism of GSH takes place as part of the  $\gamma$ -glutamyl cycle (Fig 5.7) and it is the  $\gamma$ -glutamyl linkage and cysteine residue that allow GSH to carry out its various cellular functions, from the detoxification of reactive metabolites (Srivastava *et al.*, 2010) to protecting astrocytes from oxidative stress (Dringen *et al.*, 2003). To prevent covalent binding of electrophilic metabolites in nucleophilic moieties in cellular proteins and halt the resultant onset of toxicity (Anderson *et al.*, 1998), the reactive species can be quenched by reaction with the

thiol group of the cysteine residue of GSH, either by spontaneous Michael addition or by an enzymatic reaction catalysed by glutathione-S-transferases, resulting in a chemically stable metabolite. The resulting glutathione adduct can be excreted in bile, and exceptionally in urine (Johnson *et al.*, 2005), or it can be cleaved by transpeptidase to a cysteinylglycine adduct (Josch *et al.*, 1998), leading to cysteine and mercapturate adducts which can also be excreted via these routes. (Hinchman *et al.*, 1994; Hinchman *et al.*, 1991). Because the linkage and balance of bioactivation and bioinactivation ultimately determines whether or not a reactive metabolite produces a cytotoxic effect, failure to maintain GSH content leads to cellular injury (Reed *et al.*, 1984). Therefore, analysis of GSH depletion is a marker of DILI that can be used to understand mechanisms of injury.

### **1.10 Development of Novel Biomarkers in DILI**

In order to prevent drug attrition and withdrawal of drugs from the market, and in the clinical diagnosis of DILI, biomarkers are an essential tool for assessment of drug safety and clinical injury. Biomarkers consist of proteins or molecules that can be measured in biofluids either quantitatively or qualitatively and give some reflection of the health of the organ or system being assessed. A biomarker can be defined as a measured change in DNA, protein or metabolite levels, reflective of either a normal or disrupted biological process, either inflicted by disease state or pharmacological agent. In terms of DILI, there are a set of classic biomarkers which can be measured in serum, which are used across the board, both in preclinical safety assessment, and also in patient diagnosis. As discussed earlier, these include proteins such as bilirubin and albumin, and a series of hepatic enzymes, whose level in serum is representative of hepatocyte disruption, or organelle damage. Alanine aminotransferase (ALT),

aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) are all enzymes used as markers of cytotoxic hepatocyte injury (Amacher, 2010). However, these tests do not provide information on the mechanism of hepatic injury, and indeed, are not entirely liver specific. For example, measurement of total serum ALT takes into account both ALT1 and ALT2. Whilst ALT1 is expressed highly in human liver tissue (as well as skeletal muscle and kidney), ALT2 is found in heart and skeletal muscle tissue (Lindblom *et al.*, 2007). Enzyme levels can also be elevated in other disease states, such as liver cancer, fatty liver disease and viral hepatitis (Ozer *et al.*, 2008). These classic biomarkers are also only diagnostic of damage, and are unable to detect early signs of injury. Histopathology is often required to fully diagnose the details of injury sustained. There is therefore an effort to find novel biomarkers that improve upon the reflective, predictive and diagnostic value of currently used markers, as well as enhancing organ selectivity. An ideal biomarker would be present at low baseline level in a non-invasive media, and be subject to rapid assay. It would be predictive of the onset of injury and reflective of the type of injury and be transferable across *in vitro*, *in vivo* and clinical situations. When taking into account sampling times following administration of a potential toxin, it is also important to be aware of the kinetics of a biomarker, as this will enable more informed predictions and diagnosis of injury status. Investigations with serum tumour biomarkers have shown that a knowledge of half-life can inform when blood should be sampled, and optimise patient management (Bidart *et al.*, 1999).

Ideally, new biomarkers would also provide more information on the mechanisms behind liver injury spanning *in vitro* and *in vivo* safety testing, and translating into clinical practice. Many current studies are taking advantage of proteomics, metabolomics and mass spectrometry in order to elucidate potential new peptides as

biomarkers, analysing animal biofluids following exposure to toxic xenobiotics (Soga *et al.*, 2006; Yamamoto *et al.*, 2006). Previously, there has also been interest in exploiting biochemical pathways, such as sulphur amino acid metabolism, which may result in a biomarker reflective of the state of free thiol status within the cell, such as urinary taurine levels (Waterfield *et al.*, 1993a; Waterfield *et al.*, 1993b). A similar non-invasive biomarker of hepatic GSH status would clearly be useful in providing preclinical safety information and also potentially in the clinic. Currently, hepatic GSH can only be determined directly via liver tissue and requires biopsy. Recently a metabolomic study revealed a potential candidate for such a role (Soga *et al.*, 2006). Following administration of APAP to mice, one metabolite was consistently elevated in serum, and was determined to be ophthalmic acid (OA). When serum and hepatic OA concentrations from the APAP treated mice were correlated to hepatic GSH, it was found that the expected depletion in hepatic GSH corresponded to an increase in OA concentrations. This suggested to the authors that in cases where cellular cysteine levels are low, 2-aminobutyrate will take its place, leading to the formation of OA as opposed to GSH. They also provided evidence for involvement of glutamate cysteine ligase (the rate limiting enzyme in the synthesis of glutathione) in the formation of OA. When the experiment was repeated with the inhibitor of glutamate cysteine ligase, buthionine sulfoximine, whilst levels of hepatic GSH were depleted, there was no simultaneous increase in the serum or hepatic levels of OA. However, when the experiment was repeated with the direct GSH depletor, diethylmaleate, a similar result was obtained; elevation of serum OA levels, corresponding to hepatic GSH depletion. This suggests that the observed phenomenon is not unique to APAP, and OA may have potential as a biomarker of hepatic GSH depletion. The authors suggest that as well as GSH depletion, OA may



prove useful as a biomarker of general oxidative stress, and be used in the detection and maintenance of several conditions where oxidative stress is indicated, such as Alzheimers, Parkinsons, cardiac infarction and diabetes (Soga *et al.*, 2006).

### **1.11 Aims of this work**

In order to further our understanding of the relationship between metabolism and toxicity, this work aims to employ some of the discussed techniques. In particular, the role of chemical structure in the onset of toxicity will be discussed, using thiophene containing molecules and their bioactivation to *S*-oxides and epoxides as a paradigm. Two cell models will also be investigated, namely freshly isolated rat hepatocytes and single CYP expressing THLE cell lines, to review and reflect on their ability to provide an integrated system for the study of xenobiotic metabolism and toxicity. LC-MS/MS methods will be utilised to identify and define GSH adducts, as biomarkers of thiophene bioactivation in these systems. In vivo studies to assess the suitability of OA as a biomarker of the biochemical impact of GSH conjugation and ensuing depletion will be carried out, including investigations into the kinetics of OA.

The specific questions to be addressed are;

- 1) How mass spectrometry (MS) can be used to provide information on the chemistry of thiophene bioactivation?

In order to investigate the role of MS in identification of reactive metabolites of thiophenes, several thiophene containing molecules will be incubated in in vitro metabolic systems, either rat liver microsomes, isolated rat hepatocyte suspensions or human liver epithelial cell cultures (THLE) stably transfected with single human P450 enzymes. Supernatant from these incubations will be analysed by liquid chromatography linked to various MS machines, including an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, Hertfordshire, UK) and API 2000 and 3000 mass spectrometers (Applied Biosystems, Warrington, Cheshire, UK), and subjected to tandem MS examination and product ion scans. In order to distinguish between *S*-oxides and hydroxyl metabolites of methapyrilene using MS, the above discussed hydrogen deuterium exchange technique will be trialled.

- 2) Are thiophene GSH adducts formed in certain animal and human in vitro models, and if so can they be linked to any observed toxicity?

Use of the above MS techniques should enable identification of any GSH adducts formed in isolated rat hepatocyte suspensions and THLE cell incubations, where toxicological endpoints can also be examined. The importance of bioactivation and detoxification pathways in influencing cell viability will be further investigated by incubation with 1-aminobenzotriazole, a non-specific P450 inhibitor and with diethylmaleate, a known depletor of GSH. Any relationship between the chemistry of thiophene bioactivation and the level of cell death can then be discussed and evaluated, as well as the effects of removing both the bioactivation stage by

incubation with 1-aminobenzotriazole, and removing the detoxification process by using diethylmaleate to reduce the GSH levels within the cell.

- 3) How well do these models serve as predictive tools for DILI due to bioactivation?

The ability of both the isolated rat suspension model and the THLE cell model to provide data on bioactivation and on toxicological potential will be evaluated and discussed with regards to other published reports of metabolism and toxicity of the molecules investigated, as well as the frequency and severity of DILI induced by the thiophenes examined, i.e. MP, 2-PT, TA and TC. Predicting DILI has previously proved to be incredibly complex, but by focusing on the specific aspect of the influence of bioactivation, it may be possible to define a model that enables in depth evaluation of the chemistry of reactive metabolite formation, and the ability to link this to cytotoxicity.

- 4) Can serum OA levels be exploited as a biomarker of hepatic GSH depletion?

In order to improve both diagnostic and predictive tests for DILI both in research and clinical purposes, mechanistic biomarkers would prove to be an invaluable tool for unpicking the biochemical pathways involved in determining the final fate of a cell upon exposure to a potentially toxic xenobiotic. Hepatic GSH is vital in protecting from damage caused by reactive metabolites and any resultant oxidative stress, and from preventing reactive metabolites from covalently modifying cellular proteins by forming GSH adducts. Formation of these adducts can lead to decreased

concentration, leaving the cell unable to cope with any further reactive metabolite. In order to test the suitability of serum OA levels as a reflective and predicative biomarker of hepatic GSH depletion, in vivo investigations will expose animals to GSH depletory agents and subsequent analysis of tissue and biofluids for OA and GSH levels will enable a judgement to be made regarding the use of OA as such a biomarker. Kinetic studies of OA in rat serum, bile and urine will also be carried out to assess which of these media might provide most measurable levels of OA and to investigate how parameters such as half life might affect sampling times.

## References

Abreo K, LaBarre J (1986). Suprofen, acute renal failure, and hematuria. *Ann Intern Med* **105**(5): 799.

Amacher DE (2010). The discovery and development of proteomic safety biomarkers for the detection of drug-induced liver toxicity. *Toxicol Appl Pharmacol* **245**(1): 134-142.

Anderson K, Andrews R, Yin L, McLeod R, MacDonald C, Hayes JD, *et al.* (1998). Cytotoxicity of xenobiotics and expression of glutathione-S-transferases in immortalised rat hepatocyte cell lines. *Hum Exp Toxicol* **17**(3): 131-137.

Aoyama T, Yamano S, Guzelian PS, Gelboin HV, Gonzalez FJ (1990). Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B1. *Proc Natl Acad Sci U S A* **87**(12): 4790-4793.

Baranczewski P, Stanczak A, Sundberg K, Svensson R, Wallin A, Jansson J, *et al.* (2006). Introduction to in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. *Pharmacol Rep* **58**(4): 453-472.

Bates DW, Cullen DJ, Laird N, Petersen LA, Small SD, Servi D, *et al.* (1995). Incidence of adverse drug events and potential adverse drug events. Implications for prevention. ADE Prevention Study Group. *JAMA* **274**(1): 29-34.

Belghazi M, Jean P, Poli S, Schmitter JM, Mansuy D, Dansette PM (2001). Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv Exp Med Biol* **500**: 139-144.

Bidart JM, Thuillier F, Augereau C, Chalas J, Daver A, Jacob N, *et al.* (1999). Kinetics of serum tumor marker concentrations and usefulness in clinical monitoring. *Clin Chem* **45**(10): 1695-1707.

Bredie WL, Mottram DS, Guy RC (2002). Effect of temperature and pH on the generation of flavor volatiles in extrusion cooking of wheat flour. *J Agric Food Chem* **50**(5): 1118-1125.

Broom NJ, Elder JS, Hannan PC, Pons JE, O'Hanlon PJ, Walker G, *et al.* (1995). The chemistry of pseudomonic acid. Part 14. Synthesis and in vivo biological activity of heterocyclyl substituted oxazole derivatives. *J Antibiot (Tokyo)* **48**(11): 1336-1344.

Bryant MS, Korfmacher WA, Wang S, Nardo C, Nomeir AA, Lin CC (1997). Pharmacokinetic screening for the selection of new drug discovery candidates is greatly enhanced through the use of liquid chromatography-atmospheric pressure ionization tandem mass spectrometry. *J Chromatogr A* **777**(1): 61-66.

Castro-Perez J, Plumb R, Liang L, Yang E (2005). A high-throughput liquid chromatography/tandem mass spectrometry method for screening glutathione conjugates using exact mass neutral loss acquisition. *Rapid Commun Mass Spectrom* **19**(6): 798-804.

Classen DC, Pestotnik SL, Evans RS, Lloyd JF, Burke JP (1997). Adverse drug events in hospitalized patients. Excess length of stay, extra costs, and attributable mortality. *JAMA* **277**(4): 301-306.

Copple IM, Goldring CE, Jenkins RE, Chia AJ, Randle LE, Hayes JD, *et al.* (2008). The hepatotoxic metabolite of acetaminophen directly activates the Keap1-Nrf2 cell defense system. *Hepatology* **48**(4): 1292-1301.

Copple IM, Goldring CE, Kitteringham NR, Park BK (2010). The keap1-nrf2 cellular defense pathway: mechanisms of regulation and role in protection against drug-induced toxicity. *Handb Exp Pharmacol*(196): 233-266.

Dansette PM, Bertho G, Mansuy D (2005). First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives. *Biochem Biophys Res Commun* **338**(1): 450-455.

Dansette PM, Libraire J, Bertho G, Mansuy D (2009). Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem Res Toxicol* **22**(2): 369-373.

Desmet VJ (2001). Organisational Principles. In: Arias IM (ed)^(eds). *The Liver. Biology and Pathobiology*, edn: Lippincott Williams & Wilkins. p^pp 3-17.

Donato MT, Lahoz A, Castell JV, Gomez-Lechon MJ (2008). Cell lines: a tool for in vitro drug metabolism studies. *Curr Drug Metab* **9**(1): 1-11.

Dringen R, Hirrlinger J (2003). Glutathione pathways in the brain. *Biol Chem* **384**(4): 505-516.

Einarson TR (1993). Drug-related hospital admissions. *Ann Pharmacother* **27**(7-8): 832-840.

FDA (2000). CDER-PhRMA-AASLD Conference 200: clinical white paper on drug-induced hepatotoxicity, November 2000: FDA Working Group.

Fischer V, Haar JA, Greiner L, Lloyd RV, Mason RP (1991). Possible role of free radical formation in clozapine (clozaril)-induced agranulocytosis. *Mol Pharmacol* **40**(5): 846-853.

Fisher R, Brendel K, Hanzlik RP (1993). Correlation of metabolism, covalent binding and toxicity for a series of bromobenzene derivatives using rat liver slices in vitro. *Chem Biol Interact* **88**(2-3): 191-198.

Freneaux E, Labbe G, Letteron P, The Le D, Degott C, Geneve J, *et al.* (1988). Inhibition of the mitochondrial oxidation of fatty acids by tetracycline in mice and in man: possible role in microvesicular steatosis induced by this antibiotic. *Hepatology* **8**(5): 1056-1062.

Goldring CE, Kitteringham NR, Jenkins R, Lovatt CA, Randle LE, Abdullah A, *et al.* (2006). Development of a transactivator in hepatoma cells that allows expression of phase I, phase II, and chemical defense genes. *Am J Physiol Cell Physiol* **290**(1): C104-115.

Gomez-Lechon MJ, Donato MT, Castell JV, Jover R (2004). Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab* **5**(5): 443-462.

Gomez-Lechon MJ, Donato T, Ponsoda X, Castell JV (2003). Human hepatic cell cultures: in vitro and in vivo drug metabolism. *Altern Lab Anim* **31**(3): 257-265.

Goyal RK, Srivastava D, Lessnau KD (2009). Clopidogrel-induced hepatocellular injury and cholestatic jaundice in an elderly patient: case report and review of the literature. *Pharmacotherapy* **29**(5): 608-612.

Graham E PhD Thesis, University of Liverpool, Liverpool, 2007.

Guengerich FP (2001a). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* **14**(6): 611-650.

Guengerich FP (2001b). Uncommon P450-catalyzed reactions. *Curr Drug Metab* **2**(2): 93-115.

Hagen SE, Domagala J, Gajda C, Lovdahl M, Tait BD, Wise E, *et al.* (2001). 4-Hydroxy-5,6-dihydropyrones as inhibitors of HIV protease: the effect of heterocyclic substituents at C-6 on antiviral potency and pharmacokinetic parameters. *J Med Chem* **44**(14): 2319-2332.

Hinchman CA, Ballatori N (1994). Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J Toxicol Environ Health* **41**(4): 387-409.

Hinchman CA, Matsumoto H, Simmons TW, Ballatori N (1991). Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem* **266**(33): 22179-22185.

Honig PK, Woosley RL, Zamani K, Conner DP, Cantilena LR, Jr. (1992). Changes in the pharmacokinetics and electrocardiographic pharmacodynamics of terfenadine with concomitant administration of erythromycin. *Clin Pharmacol Ther* **52**(3): 231-238.

Huber K, Yasothan U, Hamad B, Kirkpatrick P (2009). Prasugrel. *Nat Rev Drug Discov* **8**(6): 449-450.

Huffman GP, Huggins FE, Shah N, Huggins R, Linak WP, Miller CA, *et al.* (2000). Characterization of fine particulate matter produced by combustion of residual fuel oil. *J Air Waste Manag Assoc* **50**(7): 1106-1114.

Johnson KA, Plumb R (2005). Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *J Pharm Biomed Anal* **39**(3-4): 805-810.

Josch C, Sies H, Akerboom TP (1998). Hepatic mercapturic acid formation: involvement of cytosolic cysteinylglycine S-conjugate dipeptidase activity. *Biochem Pharmacol* **56**(6): 763-771.

Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, *et al.* (2005). A comprehensive listing of bioactivation pathways of organic functional groups. *Curr Drug Metab* **6**(3): 161-225.

Kao J, Carver MP (1990). Cutaneous metabolism of xenobiotics. *Drug Metab Rev* **22**(4): 363-410.

Kensler TW, Wakabayashi N, Biswal S (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* **47**: 89-116.

Kilbourn MR (1989). Thiophenes as phenyl bio-isosteres: application in radiopharmaceutical design--I. Dopamine uptake antagonists. *Int J Rad Appl Instrum B* **16**(7): 681-686.

Lam W, Ramanathan R (2002). In electrospray ionization source hydrogen/deuterium exchange LC-MS and LC-MS/MS for characterization of metabolites. *J Am Soc Mass Spectrom* **13**(4): 345-353.

Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM, Bor DH (2002). Timing of new black box warnings and withdrawals for prescription medications. *JAMA* **287**(17): 2215-2220.

Lavrijsen K, Van Houdt J, Van Dyck D, Hendrickx J, Lauwers W, Hurkmans R, *et al.* (1990). Biotransformation of sufentanil in liver microsomes of rats, dogs, and humans. *Drug Metab Dispos* **18**(5): 704-710.

Lee WM (2003). Drug-induced hepatotoxicity. *N Engl J Med* **349**(5): 474-485.

Liebler DC, Guengerich FP (2005). Elucidating mechanisms of drug-induced toxicity. *Nat Rev Drug Discov* **4**(5): 410-420.



Lim HK, Chen J, Cook K, Sensenhauser C, Silva J, Evans DC (2008). A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom* **22**(8): 1295-1311.

Lindblom P, Rafter I, Copley C, Andersson U, Hedberg JJ, Berg AL, *et al.* (2007). Isoforms of alanine aminotransferases in human tissues and serum--differential tissue expression using novel antibodies. *Arch Biochem Biophys* **466**(1): 66-77.

Liu DQ, Hop CE, Beconi MG, Mao A, Chiu SH (2001). Use of on-line hydrogen/deuterium exchange to facilitate metabolite identification. *Rapid Commun Mass Spectrom* **15**(19): 1832-1839.

Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, *et al.* (1999). Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* **277**(1 Pt 1): G12-21.

Liu ZX, Kaplowitz N (2002). Immune-mediated drug-induced liver disease. *Clin Liver Dis* **6**(3): 755-774.

Lopez-Garcia MP, Dansette PM, Mansuy D (1994). Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry* **33**(1): 166-175.

Lu SC (2009). Regulation of glutathione synthesis. *Mol Aspects Med* **30**(1-2): 42-59.

Mace K, Aguilar F, Wang JS, Vautravers P, Gomez-Lechon M, Gonzalez FJ, *et al.* (1997). Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* **18**(7): 1291-1297.

Mason RP, Fischer V (1992). Possible role of free radical formation in drug-induced agranulocytosis. *Drug Saf* **7 Suppl 1**: 45-50.

Masubuchi N, Makino C, Murayama N (2007). Prediction of in vivo potential for metabolic activation of drugs into chemically reactive intermediate: correlation of in vitro and in vivo generation of reactive intermediates and in vitro glutathione conjugate formation in rats and humans. *Chem Res Toxicol* **20**(3): 455-464.

Meneses-Lorente G, Sakatis MZ, Schulz-Utermoehl T, De Nardi C, Watt AP (2006). A quantitative high-throughput trapping assay as a measurement of potential for bioactivation. *Anal Biochem* **351**(2): 266-272.

Mercer AE, Regan SL, Hirst CM, Graham EE, Antoine DJ, Benson CA, *et al.* (2009). Functional and toxicological consequences of metabolic bioactivation of methapyrilene via thiophene S-oxidation: Induction of cell defence, apoptosis and hepatic necrosis. *Toxicol Appl Pharmacol* **239**(3): 297-305.

Mersch-Sundermann V, Knasmüller S, Wu XJ, Darroudi F, Kassie F (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology* **198**(1-3): 329-340.

Methven L, Tsoukka M, Oruna-Concha MJ, Parker JK, Mottram DS (2007). Influence of sulfur amino acids on the volatile and nonvolatile components of cooked salmon (*Salmo salar*). *J Agric Food Chem* **55**(4): 1427-1436.

Nassar AE (2003). Online hydrogen-deuterium exchange and a tandem-quadrupole time-of-flight mass spectrometer coupled with liquid chromatography for metabolite identification in drug metabolism. *J Chromatogr Sci* **41**(8): 398-404.

Navarro VJ, Senior JR (2006). Drug-related hepatotoxicity. *N Engl J Med* **354**(7): 731-739.

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, *et al.* (1993). The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**(1): 1-51.

Nelson SD, Mitchell JR, Timbrell JA, Snodgrass WR, Corcoran GB, 3rd (1976). Isoniazid and iproniazid: activation of metabolites to toxic intermediates in man and rat. *Science* **193**(4256): 901-903.

Ng VL (2009). Anticoagulation monitoring. *Clin Lab Med* **29**(2): 283-304.

Nishiya T, Kato M, Suzuki T, Maru C, Kataoka H, Hattori C, *et al.* (2008). Involvement of cytochrome P450-mediated metabolism in tienilic acid hepatotoxicity in rats. *Toxicol Lett* **183**(1-3): 81-89.

Nishiya Y, Hagihara K, Ito T, Tajima M, Miura S, Kurihara A, *et al.* (2009a). Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab Dispos* **37**(3): 589-593.

Nishiya Y, Hagihara K, Kurihara A, Okudaira N, Farid NA, Okazaki O, *et al.* (2009b). Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**(11): 836-843.

O'Brien PJ, Chan K, Silber PM (2004). Human and animal hepatocytes in vitro with extrapolation in vivo. *Chem Biol Interact* **150**(1): 97-114.

O'Brien PJ, Siraki AG (2005). Accelerated cytotoxicity mechanism screening using drug metabolising enzyme modulators. *Curr Drug Metab* **6**(2): 101-109.

O'Donnell JP, Dalvie DK, Kalgutkar AS, Obach RS (2003). Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31**(11): 1369-1377.

Obach RS, Kalgutkar AS, Soglia JR, Zhao SX (2008). Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem Res Toxicol* **21**(9): 1814-1822.

Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, *et al.* (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med* **137**(12): 947-954.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**(3): 194-205.

Park BK, Kitteringham NR, Maggs JL, Pirmohamed M, Williams DP (2005). The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* **45**: 177-202.

Park BK, Pirmohamed M, Kitteringham NR (1998). Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. *Chem Res Toxicol* **11**(9): 969-988.

Pascoe GA, Calleman CJ, Baille TA (1988). Identification of S-(2,5-dihydroxyphenyl)-cysteine and S-(2,5-dihydroxyphenyl)-N-acetyl-cysteine as urinary metabolites of acetaminophen in the mouse. Evidence for p-benzoquinone as a reactive intermediate in acetaminophen metabolism. *Chem Biol Interact* **68**(1-2): 85-98.

Pelkonen O, Raunio H (1997). Metabolic activation of toxins: tissue-specific expression and metabolism in target organs. *Environ Health Perspect* **105 Suppl 4**: 767-774.

Pirmohamed M, Naisbitt DJ, Gordon F, Park BK (2002). The danger hypothesis--potential role in idiosyncratic drug reactions. *Toxicology* **181-182**: 55-63.

Pizarro AE, Andrade RJ, Garcia-Cortes M, Lucena MI, Perez-Moreno JM, Puertas M, *et al.* (2001). [Acute hepatitis due to ticlopidine. A report of 12 cases and review of the literature]. *Rev Neurol* **33**(11): 1014-1020.

Pons C, Dansette PM, Amar C, Jaouen M, Wolf CR, Gregeois J, *et al.* (1991). Detection of human hepatitis anti-liver kidney microsomes (LKM2) autoantibodies on rat liver sections is predominantly due to reactivity with rat liver P-450 IIC11. *J Pharmacol Exp Ther* **259**(3): 1328-1334.

Ponsoda X, Pareja E, Gomez-Lechon MJ, Fabra R, Carrasco E, Trullenque R, *et al.* (2001). Drug biotransformation by human hepatocytes. In vitro/in vivo metabolism by cells from the same donor. *J Hepatol* **34**(1): 19-25.

Press JB, McNally JJ, Sanfilippo PJ, Addo MF, Loughney D, Giardino E, *et al.* (1993). Novel thieno[2,3-b]- and [3,4-b]pyrans as potassium channel openers. Thiophene systems--XVII. *Bioorg Med Chem* **1**(6): 423-435.

Puga A, Raychaudhuri B, Salata K, Zhang YH, Nebert DW (1990). Stable expression of mouse Cyp1a1 and human CYP1A2 cDNAs transfected into mouse hepatoma cells lacking detectable P450 enzyme activity. *DNA Cell Biol* **9**(6): 425-436.

Randle LE, Goldring CE, Benson CA, Metcalfe PN, Kitteringham NR, Park BK, *et al.* (2008). Investigation of the effect of a panel of model hepatotoxins on the Nrf2-Keap1 defence response pathway in CD-1 mice. *Toxicology* **243**(3): 249-260.

Ratra GS, Cottrell S, Powell CJ (1998). Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* **46**(1): 185-196.

Raucy JL, Lasker JM, Lieber CS, Black M (1989). Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. *Arch Biochem Biophys* **271**(2): 270-283.

Reed DJ, Fariss MW (1984). Glutathione depletion and susceptibility. *Pharmacol Rev* **36**(2 Suppl): 25S-33S.

Rodenhuis N, Timmerman W, Wikstrom HV, Dijkstra D (2000). Thiophene analogs of naphthoxazines and 2-aminotetralins: bioisosteres with improved relative oral bioavailability, as compared to 5-OH-DPAT. *Eur J Pharmacol* **394**(2-3): 255-263.

Rosenthal T, Boichis H (1971). Nephrotoxicity of cephaloridine. *Br Med J* **4**(5779): 115.

Ross D, Siegel D, Schattenberg DG, Sun XM, Moran JL (1996). Cell-specific activation and detoxification of benzene metabolites in mouse and human bone marrow: identification of target cells and a potential role for modulation of apoptosis in benzene toxicity. *Environ Health Perspect* **104 Suppl 6**: 1177-1182.

Sanderson JP, Naisbitt DJ, Park BK (2006). Role of bioactivation in drug-induced hypersensitivity reactions. *AAPS J* **8**(1): E55-64.

Shelley WB, Goldwein MI (1976). High dose dapsone toxicity. *Br J Dermatol* **95**(1): 79-82.

Shimizu S, Atsumi R, Nakazawa T, Fujimaki Y, Sudo K, Okazaki O (2009). Metabolism of ticlopidine in rats: identification of the main biliary metabolite as a glutathione conjugate of ticlopidine S-oxide. *Drug Metab Dispos* **37**(9): 1904-1915.

Smith MT, Yager JW, Steinmetz KL, Eastmond DA (1989). Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environ Health Perspect* **82**: 23-29.

Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T, *et al.* (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem* **281**(24): 16768-16776.

Srivastava A, Maggs JL, Antoine DJ, Williams DP, Smith DA, Park BK (2010). Role of reactive metabolites in drug-induced hepatotoxicity. *Handb Exp Pharmacol*(196): 165-194.

Takakusa H, Masumoto H, Mitsuru A, Okazaki O, Sudo K (2008). Markers of electrophilic stress caused by chemically reactive metabolites in human hepatocytes. *Drug Metab Dispos* **36**(5): 816-823.

Temple RJ, Himmel MH (2002). Safety of newly approved drugs: implications for prescribing. *JAMA* **287**(17): 2273-2275.

Thevis M, Geyer H, Bahr D, Schanzer W (2005). Identification of fentanyl, alfentanil, sufentanil, remifentanil and their major metabolites in human urine by liquid chromatography/tandem mass spectrometry for doping control purposes. *Eur J Mass Spectrom (Chichester, Eng)* **11**(4): 419-427.

Tirmenstein MA, Hu CX, Gales TL, Maleeff BE, Narayanan PK, Kurali E, *et al.* (2002). Effects of troglitazone on HepG2 viability and mitochondrial function. *Toxicol Sci* **69**(1): 131-138.

Trontelj J, Bogataj M, Marc J, Mrhar A (2007). Development and validation of a liquid chromatography-tandem mass spectrometry assay for determination of raloxifene and its metabolites in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **855**(2): 220-227.

Utrecht JP (1992). The role of leukocyte-generated reactive metabolites in the pathogenesis of idiosyncratic drug reactions. *Drug Metab Rev* **24**(3): 299-366.

Velayudham LS, Farrell GC (2003). Drug-induced cholestasis. *Expert Opin Drug Saf* **2**(3): 287-304.

Waterfield CJ, Turton JA, Scales MD, Timbrell JA (1993a). Effect of various non-hepatotoxic compounds on urinary and liver taurine levels in rats. *Arch Toxicol* **67**(8): 538-546.

Waterfield CJ, Turton JA, Scales MD, Timbrell JA (1993b). Investigations into the effects of various hepatotoxic compounds on urinary and liver taurine levels in rats. *Arch Toxicol* **67**(4): 244-254.

Williams DP, Antoine DJ, Butler PJ, Jones R, Randle L, Payne A, *et al.* (2007). The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: a chemical and biochemical definition of the toxicophore. *J Pharmacol Exp Ther* **322**(3): 1208-1220.

Yamamoto T, Kikkawa R, Yamada H, Horii I (2006). Investigation of proteomic biomarkers in in vivo hepatotoxicity study of rat liver: toxicity differentiation in hepatotoxicants. *J Toxicol Sci* **31**(1): 49-60.

Yeh SP, Hsueh EJ, Wu H, Wang YC (1998). Ticlopidine-associated aplastic anemia. A case report and review of literature. *Ann Hematol* **76**(2): 87-90.

Yukinaga H, Takami T, Shioyama SH, Tozuka Z, Masumoto H, Okazaki O, *et al.* (2007). Identification of cytochrome P450 3A4 modification site with reactive metabolite using linear ion trap-Fourier transform mass spectrometry. *Chem Res Toxicol* **20**(10): 1373-1378.

Zhang Z, Zhu M, Tang W (2009). Metabolite identification and profiling in drug design: current practice and future directions. *Curr Pharm Des* **15**(19): 2220-2235.

Zimmerman HJ (1999). *Hepatotoxicity*. 2nd edn. Lippincott Williams & Wilkins.

CHEMICAL AND BIOCHEMICAL ASPECTS OF DRUG- INDUCED LIVER INJURY

## CHAPTER TWO

---

# USE OF MASS SPECTROMETRY TO DEFINE THE REACTIVE METABOLITE OF METHAPYRILENE

## Contents

2.1 Introduction.....	49
2.2 Materials and Methods.....	56
2.2.1 Materials.....	56
2.2.2 Animals.....	56
2.2.3 Preparation and Incubation of Rat Hepatic Microsomes.....	56
2.2.4 LC-MS/MS/MS Analysis of Microsomal Incubations.....	57
2.2.5 Characterization of glutathione adduct by deuterium exchange.....	57
2.3 Results.....	59
2.3.1 Determination of metabolism by MS/MS/MS.....	59
2.3.2 Characterization of glutathione adduct by deuterium exchange.....	60
2.4 Discussion.....	71

## Abbreviations

CYP450, cytochrome P450 mixed function oxidase; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography tandem mass spectrometry; MeOH, methanol; MeOD, monodeuteromethanol; MP, methapyrilene; NADPH,  $\beta$ -nicotinamide adenine dinucleotide phosphate (reduced); RLM, rat hepatic microsomes.

**NB.** The work described in this chapter has previously been published as detailed below.

Graham, EE, Walsh, RJ, Hirst, CM, Maggs, JL, Martin, S, Wild, MJ, Wilson, ID, Harding, JR, Kenna, JG, Peter, RM, Williams, DP, Park, BK (2008) Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* 326(2): 657-671.



## 2.1 Introduction

Thiophene derivatives are commonly found in nature. Exposure as a consequence of human activity occurs when thiophenes are released into the environment through fossil fuel combustion (Huffman *et al.*, 2000) and generated in cooking processes (Bredie *et al.*, 2002; Methven *et al.*, 2007). Thiophene rings, acting as bioisosteres for benzo, phenyl, phenol and heterocyclic moieties (Broom *et al.*, 1995; Kilbourn, 1989; Press *et al.*, 1993; Rodenhuis *et al.*, 2000) are also present in many drugs, incorporated to increase potency and efficacy (Broom *et al.*, 1995; Hagen *et al.*, 2001; Press *et al.*, 1993) and additionally to enhance pharmacokinetic properties (Broom *et al.*, 1995; Hagen *et al.*, 2001; Rodenhuis *et al.*, 2000). Notwithstanding their occasional association with toxicity, thiophene drugs can display both excellent potency and low cytotoxicity in vitro (Hagen *et al.*, 2001). However, some thiophene containing drugs have been withdrawn due to adverse effects, such as the diuretic, tienilic acid, withdrawn due to cases of autoimmune hepatitis (Lecoeur *et al.*, 1996), and the H<sub>1</sub> receptor antagonist, methapyrilene (MP), withdrawn following reports of hepatic carcinomas in the rat (Lijinsky *et al.*, 1980). Metabolism to reactive moieties may be in part to blame for these adverse reactions but it is also important to note that a number of drugs rely on metabolic activation and covalent binding to proteins for their efficacy, and thus prevention of adverse covalent binding through chemical modification of the compound may inadvertently lead to loss of efficacy. For example, oxidative metabolic activation of the (fused) thiophene ring is a generic pharmacophoric characteristic of thienopyridine antiplatelet agents (Hagihara *et al.*, 2009).

While C-oxidation of the thiophene ring of tienilic acid yields a stable C-5 hydroxyl derivative (Mansuy *et al.*, 1984), metabolic investigations with thiophene derivatives have provided evidence for the formation of both an *S*-oxide and epoxide of the thiophene ring (Dansette *et al.*, 2005; Shimizu *et al.*, 2009). Oxidation of thiophenes to thiolacetone (Hagihara *et al.*, 2009; Shimizu *et al.*, 2009), thiol (Hagihara *et al.*, 2009), sulphenic acid (Dansette *et al.*, 2009) and  $\gamma$ -thio keto- $\alpha,\beta$ -unsaturated aldehyde (O'Donnell *et al.*, 2003) intermediates is also known. The sulphenic acid and aldehyde intermediates are highly reactive species. *S*-oxidation seems to occur as the major *in vivo* pathway of thiophene-ring bioactivation in the rat; the main metabolites, as detected in bile and urine, being variously the sulphoxide dimer formed via the Diels-Alder reaction and the GSH adduct, or its mercapturate catabolite (Dansette *et al.*, 1992; Shimizu *et al.*, 2009; Valadon *et al.*, 1996) formed from Michael addition of GSH to the *S*-oxide (Dansette *et al.*, 1992; Mansuy *et al.*, 1984; Nishiya *et al.*, 2008a; Nishiya *et al.*, 2008b; Shimizu *et al.*, 2009; Valadon *et al.*, 1996).

*In vitro* studies of the metabolic activation of drugs are usually carried out by using isolated hepatocytes or microsomal incubations supplemented with NADPH. In the case of the latter, the lack of co-factors restricts Phase II metabolism, and presence of reactive metabolites can be inferred from assessing irreversible binding of radiolabelled substrate to the microsomes, or by addition of a trapping agent (Masubuchi *et al.*, 2007). The trapping technique can be used to identify formation of hard electrophiles using cyanide, and for soft electrophiles using thiols such as glutathione (GSH), N-acetyl cysteine (NAC) and glutathione ethyl ester (Rousu *et al.*, 2009).

Both in vitro and in vivo investigations rely on mass spectrometry as the main technique for elucidating which metabolites are formed (Ma *et al.*, 2009), but often turn to nuclear magnetic resonance spectroscopy in order to fully determine chemical structure, by defining the number, location and interactions of hydrogen atoms in the molecule (Dear *et al.*, 2000). Although powerful NMR spectrometers are increasingly available for metabolite analysis (Dansette *et al.*, 2005; Shimizu *et al.*, 2009), extended isolation procedures are still required for minor metabolites (Srivastava *et al.*, 2009). However, over the past ten years, hydrogen/deuterium exchange has become recognised as a valuable tool for studying fragmentation in mass spectrometry and thereby in differentiating alternate metabolite structures, by determining the number and (approximate) location of exchanged-in deuterium atoms within a metabolite (Chen *et al.*, 2009; Lam *et al.*, 2002; Liu *et al.*, 2001; Medower *et al.*, 2008; Nassar, 2003; Ohashi *et al.*, 1998).

MP was introduced in the 1950s and used commonly as a sleeping aid for 25 years until being withdrawn due to hepatocarcinogenicity in chronically dosed rats (Lijinsky *et al.*, 1980). No carcinomas or hepatotoxic events were reported in humans. Giving a negative result in the Ames test, MP is a non mutagenic carcinogen, and appears to be species selective, with no carcinomas reported in hamsters, guinea pigs and mice (Brennan *et al.*, 1982; Reznik-Schuller *et al.*, 1981). Dose dependent hepatotoxicity in rats has also been reported following both acute (Mercer *et al.*, 2009; Ratra *et al.*, 2000) and chronic (Cunningham *et al.*, 1995) dosing and MP produces concentration dependent toxicity in isolated rat hepatocytes (Graham *et al.*, 2008; Ratra *et al.*, 1998b). Chronic dosing regimens of between 100mg/kg/day to 300mg/kg/day for three days produce mild to moderate injury,

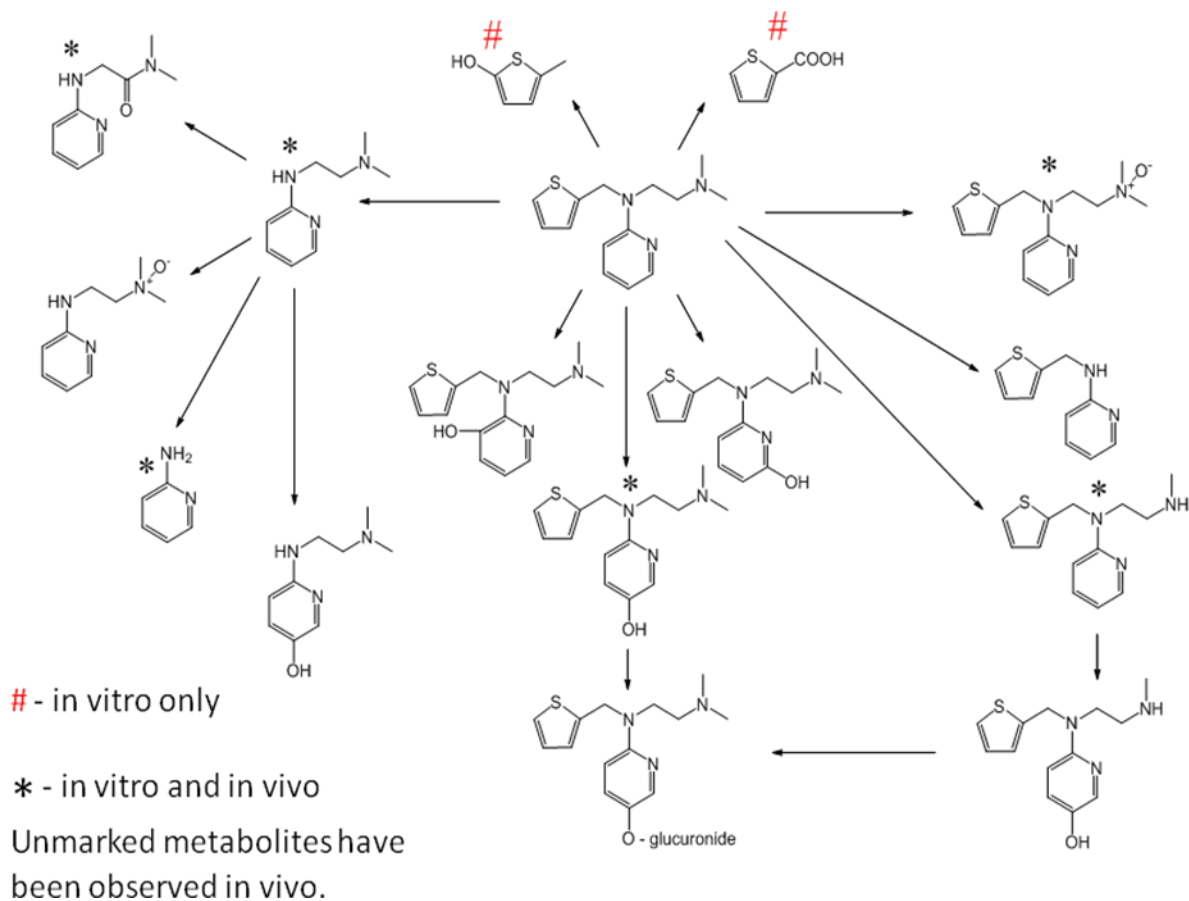
consisting of necrosis, inflammatory cell infiltration and bile duct proliferation. Structure toxicity studies with rat hepatocytes and MP analogues have identified the thiophene moiety as the toxicophore of MP (Graham *et al.*, 2008). The mechanism of such toxicity has been investigated in some detail (Beekman *et al.*, 2006; Craig *et al.*, 2006; Methven *et al.*, 2007; Ratra *et al.*, 1998a; Ratra *et al.*, 1998b; Ratra *et al.*, 2000) but the chemistry of any reactive metabolite formed was hitherto defined uncertainly (Kammerer *et al.*, 1986; Kammerer *et al.*, 1988; Kelly *et al.*, 1992; Lampe *et al.*, 1990; Singer *et al.*, 1987; Ziegler *et al.*, 1981)

Metabolism of MP in the rat has been investigated previously, and several metabolites identified *in vivo* (Fig. 2.1) (Kammerer *et al.*, 1988; Kelly *et al.*, 1990; Ratra *et al.*, 2000; Singer *et al.*, 1987). MP has several structural alerts for bioactivation to reactive intermediates (Kalgutkar *et al.*, 2005), via N-oxidation to iminium ions, S-oxidation and arene oxidation. Irreversible binding has been detected with radiolabelled MP *in vivo* and *in vitro*, providing evidence for bioactivation to an electrophilic metabolite able to form protein adducts (Lampe *et al.*, 1990; Singer *et al.*, 1987). The *in vitro* metabolism of MP has also been investigated using rat liver microsomes (RLM) and rat hepatocytes (Graham, 2007; Graham *et al.*, 2008). Several metabolites have been identified as detailed in Figure 2.1. A reactive metabolite has not yet been identified in rat systems, but an iminium ion was trapped using cyanide in rabbit liver microsomes (Ziegler *et al.*, 1981).

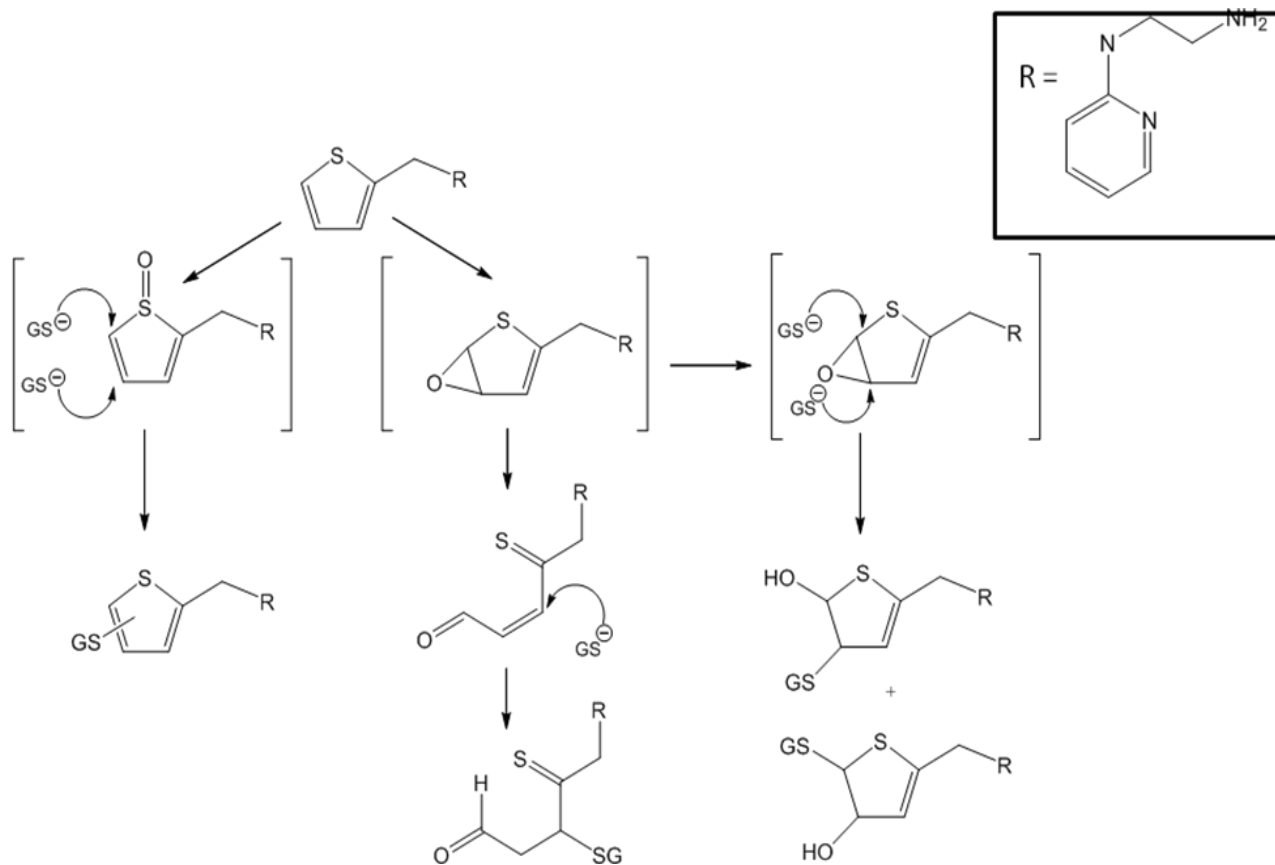
Recently, Graham (2007) investigated metabolism of MP in the rat using radiolabelled drug. The tritiated form of MP was administered to anaesthetised male Wistar rats and bile and urine were collected at various time points and then analysed by radiometric HPLC and LC-MS. Incubations were also carried out with hepatocytes and hepatic microsomes isolated from the same strain of rat. The major

metabolite in bile and hepatocytes, and in microsomal incubations supplemented with GSH, was defined as a mono-oxygenated (M+O+GSH) glutathione adduct. Fragmentation revealed by a process of elimination that the site of glutathione conjugation was the thiophene ring (Graham *et al.*, 2008). However, whether the adduct was formed from an *S*-oxide or an epoxide (Dansette *et al.*, 2005; Shimizu *et al.*, 2009), or a  $\gamma$ -thioketo- $\alpha$ ,  $\beta$ -unsaturated aldehyde (O'Donnell *et al.*, 2003) of the heterocycle remained elusive.

The investigations described in this chapter were intended to define the reactive metabolite of methapyrilene produced in rats by employing rat hepatic microsomes as the source of metabolic enzymes, and using glutathione as a trapping agent. In order to achieve definition of the chemistry of metabolites detected, LC-MS/MS/MS will be used to determine absolute mass and to observe fragmentation of metabolites, focusing on formation of glutathione adducts. Approximate position and number of labile hydrogen atoms can then be determined by H/D exchange followed by LC-MS/MS.



**Figure 2.1.** Schematic representation of the metabolism of methapyrilene (MP) in the rat, both in vivo and in vitro. (Kammerer *et al.*, 1986; Kammerer *et al.*, 1988; Kelly *et al.*, 1990; Lampe *et al.*, 1990; Ratra *et al.*, 2000; Singer *et al.*, 1987)



**Figure 2.2** Alternative hypothetical pathways of bioactivation of MP to the intermediate trapped as a glutathione conjugate: to MP *S*-oxide, an epoxide that reacts with GSH without opening of the thiophene ring, and an epoxide that undergoes concerted opening of the epoxide and thiophene rings to produce a  $\gamma$ -thioketo- $\alpha, \beta$ -unsaturated aldehyde.

## **2.2 Materials and Methods**

### **2.2.1 Materials.**

MP and monodeuteromethanol (MeOD; 99.5 atom % D) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Non-deuterated methanol was HPLC grade from Fisher Scientific UK (Loughborough, Leicestershire, UK). Unless otherwise stated all other reagents were obtained from Sigma-Aldrich. Organic solvents were products of VWR (Lutterworth, Leicestershire, UK) and were of chromatographic, analytical, or cell-culture grade.

### **2.2.2 Animals.**

Adult male Wistar rats were obtained from Charles River Laboratories (Margate, Kent, UK). All experiments with live animals were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the Animal Ethics Committee of the University of Liverpool.

### **2.2.3 Preparation and Incubation of Rat Hepatic Microsomes.**

RLM were prepared from tissue minced in ice-cold 67 mM phosphate buffer, pH 7.5, containing KCl (1.15%, w/v). Homogenates were produced with a motor-driven homogenizer, and centrifuged (10,000g; 20 min; 4°C) to obtain supernatant from which microsomes were sedimented (105,000g; 60 min; 4°C). The microsomes were resuspended in KCl phosphate buffer, centrifuged (105,000g; 60 min; 4°C) and reconstituted in 67mM phosphate buffer, pH 7.5. Protein content measured by the Bradford assay (Bradford, 1976). Incubations were carried out in a final volume of 1ml 0.2 M Tris-HCl buffer, pH 7.4, containing 1 g/ml microsomal protein, various



concentrations of MP (10-1000 $\mu$ M) and a NADPH regenerating system (0.4mM NADP, 7.5 mM glucose-6-phosphate, 1U/ml glucose-6-phosphate dehydrogenase, 5.0mM MgCl<sub>2</sub>), supplemented with 1mM GSH. The regenerating system was omitted from control incubations. After 60-min incubation at 37°C, the reaction was terminated by the addition of an equal volume of ice-cold acetonitrile, centrifuged (18,400g, 5 min), and the final supernatant analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS/MS).

#### **2.2.4 LC-MS/MS/MS Analysis of Microsomal Incubations**

Supernatant (10 $\mu$ l) from microsomal incubations was injected onto a Gemini 250 x 2 mm, 5 $\mu$ M column (Phenomenex, UK) and eluted with a gradient of MeOH (containing 10mM ammonium acetate) in 10mM aqueous ammonium acetate at a flow rate of 300 $\mu$ l/min. The gradient was as follows, 0% B for 3min, 100% B at 13min to 15min, and 0% B from 15.1min to 20min. The column eluent was analysed by an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, Hertfordshire, UK). The capillary and spray voltages were 4 V and 3.8 kV, respectively, and the capillary temperature was 275°C. Full-scanning (m/z 100-900) and selected MS/MS/MS acquisitions in positive-ion mode were performed at 100,000 resolution through Xcaliber 2.0 SR2. The instrument was calibrated for accurate mass measurements on the day of analysis.

#### **2.2.5 Characterization of glutathione adduct by deuterium exchange.**

MP (100 $\mu$ M) was incubated at 37°C with RLM (protein concentration, 1mg/ml) in a final volume of 1ml 0.2M Tris-HCl buffer, pH 7.4, containing NADPH regenerating system and GSH (1mM). After 60 min, an equal volume of ice-cold acetonitrile was added to the single incubation and the mixture was stored at -20°C until further use. Following centrifugation at 870g for 10min, the supernatant was evaporated to dryness under nitrogen and the residue was reconstituted immediately in either MeOH or MeOD (200 $\mu$ l) at room temperature. The solution was clarified by centrifugation at 10,000g for 5 min, and immediately thereafter infused (20 $\mu$ l/min) directly into the electrospray ionisation source of an LTQ Orbitrap mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, Hertfordshire, UK). The capillary and spray voltages were 4 V and 3.8 kV, respectively, and the capillary temperature was 275°C. Full-scanning (m/z 100-900) and selected MS/MS acquisitions (MS<sup>2</sup> and MS<sup>3</sup> product ion scans) in positive-ion mode were performed at 100,000 resolution through Xcaliber 2.0 SR2. The instrument was calibrated for accurate mass measurements on the day of analysis.

## 2.3 Results

### 2.3.1 Determination of metabolism by MS/MS/MS

Previous work had indicated that RLM supplemented with 1mM GSH produce hydroxymethapyrilene, methapyrilene N-oxide, desmethylmethapyrilene and a glutathione adduct. The presence of these metabolites was confirmed by LC-MS/MS/MS. Figure 2.3 shows extracted ion chromatograms for the following m/z values of  $[M+1]^+$  species; 262 (parent drug), 278 (monooxygenated MP, MP[O]), 248 (desmethyl MP) and 585 (GSH adduct of monooxygenated MP, MP[O]SG). A single peak was detected for m/z 262. MS/MS yielded a major fragment at m/z 217, corresponding to neutral loss of the dialkylamine group ( $\text{HN}(\text{CH}_3)_2$ ). MS/MS/MS, i.e. fragmentation of m/z 217, yielded peaks at m/z 119 and 97, representing the pyridine and thiophene rings, respectively (Fig 2.4).

Two peaks were detected in the chromatogram for m/z 278, at retention times 11.75 min and 13.19 min. The peak at 11.75 min was attributed to a hydroxylated metabolite. Fragmentation revealed the pyridine ring as the location of hydroxylation, due to the presence of m/z 135 in the MS/MS/MS scan (Fig. 2.5). The peak at retention time 13.19min, eluting characteristically after MP, yielded a diagnostic  $[M+1-O]^+$  fragment in the full scan spectrum (Ramanathan *et al.*, 2000), and was therefore defined as MP N-oxide (Fig. 2.6). Desmethylmethapyrilene exhibited a similar fragmentation pattern to the parent drug (Fig. 2.7). The peak at

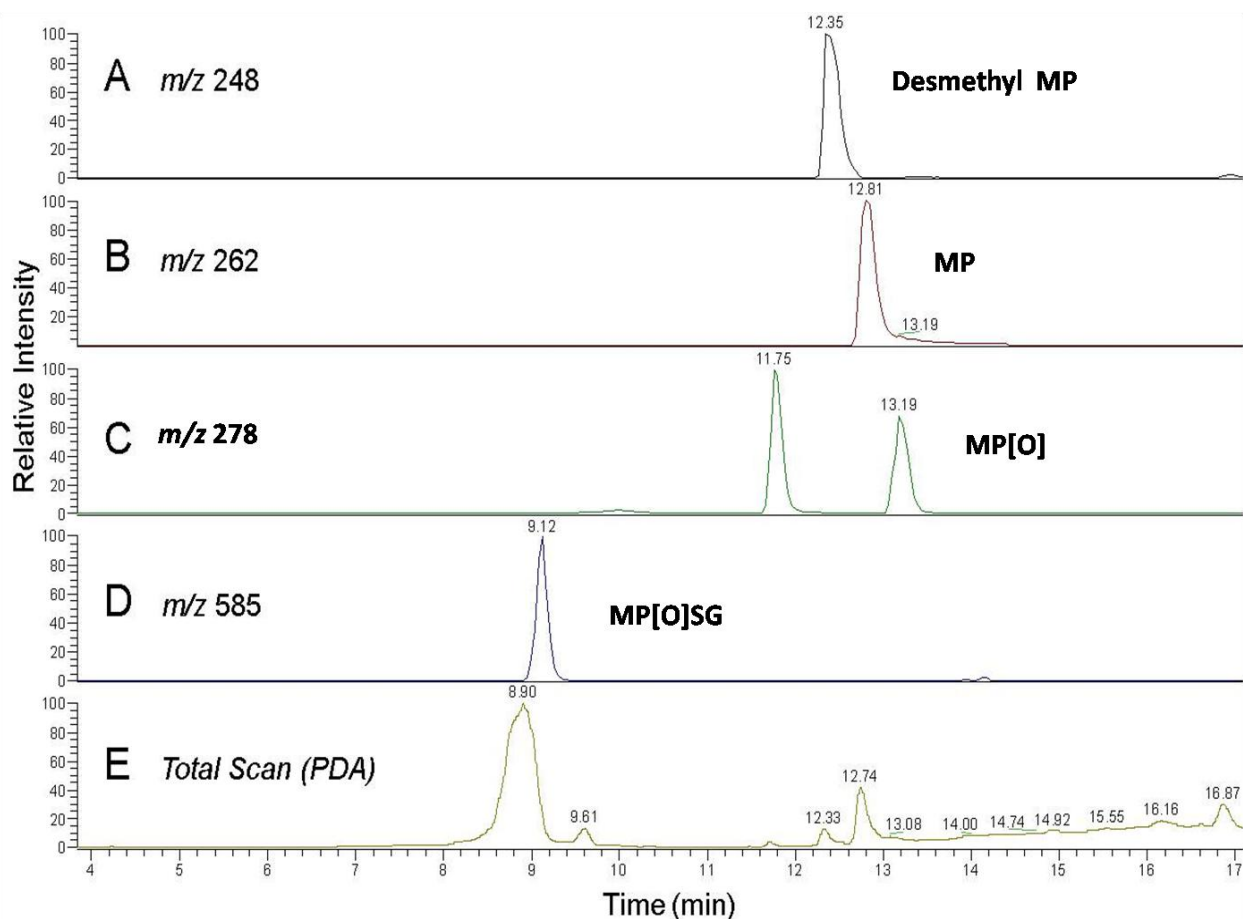
$m/z$  585 was defined as an oxygenated glutathione adduct. MS/MS analysis revealed fragments at  $m/z$  540  $[585\text{-HN}(\text{CH}_3)_2]^+$ ,  $m/z$  278  $[585\text{-GSH}]^+$  (retro-Michael elimination of GSH) and  $m/z$  456  $[585\text{-129}]^+$  (neutral loss of pyroglutamate from the GSH residue) In addition,  $m/z$  522  $([540\text{-H}_2\text{O}]^+)$ , 411  $([540\text{-129}]^+)$ , 393  $([411\text{-H}_2\text{O}]^+)$ , 267  $([233\text{+S+2H}]^+)$  and 233  $([540\text{-GSH}]^+)$  were obtained as fragments of  $m/z$  540 in an MS/MS/MS product-ion scan (Fig. 2.8).

### 2.3.2 Characterization of glutathione adduct by deuterium exchange.

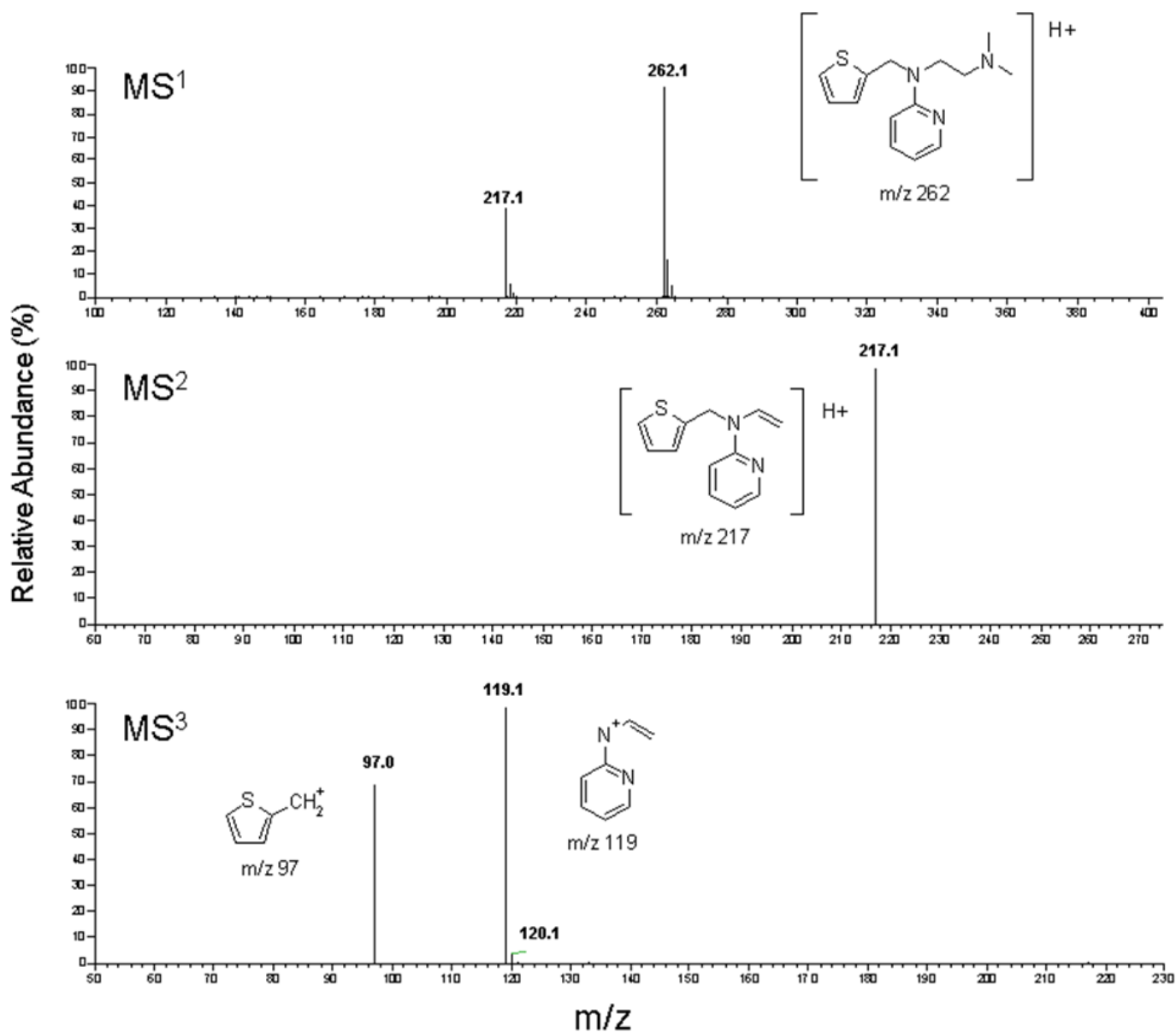
The mass spectral identification of the glutathione adduct ( $m/z$  585) having been confirmed using the LTQ Orbitrap, it was next established that GSH dissolved in MeOD exchanged all seven of its labile amine, amide, carboxyl and sulphydryl protons, with a consequential increase in the mass of the protonated molecule from 308.0917  $([\text{M+H}]^+; \Delta +0.1 \text{ ppm})$  to 316.1419  $([\text{M}_\text{D}+\text{D}]^+; \Delta +0.1 \text{ ppm})$ ; where  $\text{M}_\text{D}$  represents the weight of the fully deuterated analyte and  $\Delta \pm \text{ ppm}$  represents the deviation of the measured  $m/z$  from the calculated  $m/z$ . . The mass of the protonated microsomal metabolite increased from 585.2173 ( $\text{C}_{24}\text{H}_{37}\text{N}_6\text{O}_7\text{S}_2$ ;  $\Delta +0.8 \text{ ppm}$ ) to 592.2618 ( $\text{C}_{24}\text{H}_{30}\text{D}_7\text{N}_6\text{O}_7\text{S}_2$ ;  $([\text{M}_\text{D}+\text{D}]^+; \Delta +1.4 \text{ ppm})$ ) when the compound was deuterated under the same conditions (Fig. 2.9 B and C). This was interpreted as representing exchange of the six amine, amide and carboxyl protons on the glutathionyl residue of an *S*-oxide adduct (Fig. 2.10). The ion at  $m/z$  593.2648 was identified as  $([\text{M}_\text{D}+\text{D}]^+)$  for the mono- $^{13}\text{C}$  isotopomer of hexadeutero-MP[O]SG by comparison of the measured mass with the calculated mass: 593.2648 (Fig. 2.11). The ions at  $m/z$  590.2491 and 591.2553 were due to incomplete deuteration of MP[O]SG. An isomeric dihydroxythiophenyl-*S*-glutathionyl adduct derived

from a thiophene epoxide of MP would be expected to exchange seven protons, including an hydroxyl proton on the thiophene ring, and yield a monoisotopic  $[M_D+D]^+$  ion at  $m/z$  593.2662 (calculated) (Fig. 2.11).

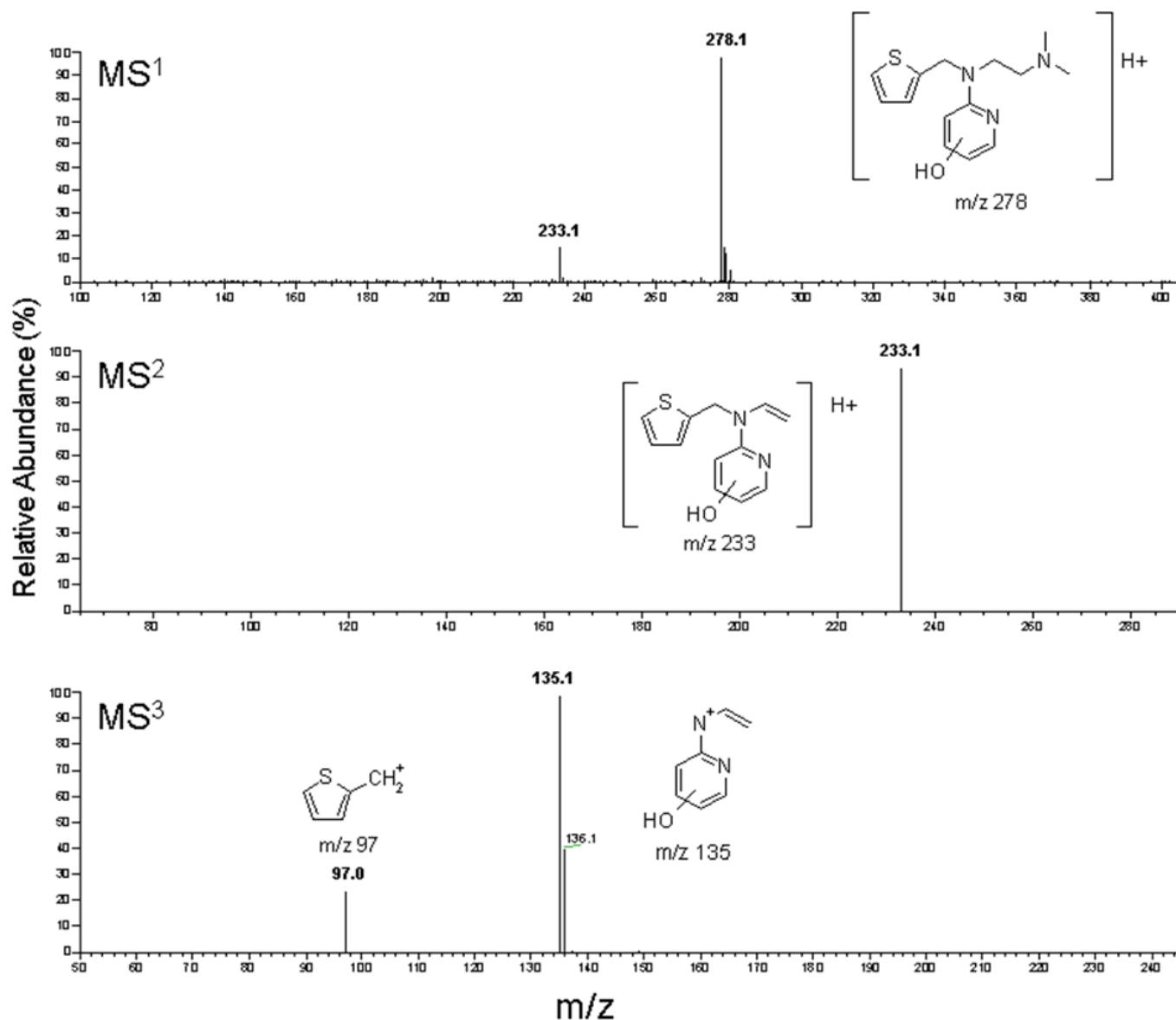
The abundant MS/MS product ions of MP[O]SG, namely  $[M+1-307]^+$  and  $[M+1-HN(CH_3)_2]^+$ , underwent mass shifts upon deuteration of the metabolite that were consistent with the six proton exchanges being confined to the glutathionyl residue (Fig. 2.10 A, B and C).



**Figure 2.3.** LC/MS chromatograms showing metabolites of MP in rat hepatic microsomal incubations as analysed on a LTQ-orbitrap mass spectrometer (ThermoFischer, UK). **A)** Extracted chromatogram of  $m/z$  248 showing single peak representing desmethylMP. **B)** Extracted chromatogram of  $m/z$  262. Single peak represents parent drug. **C)** Chromatogram showing  $m/z$  248. Peak at retention time 11.75min corresponds to hydroxyMP and peak at retention time 13.19min corresponds to MP N-oxide. **D)** Extracted chromatogram showing mono-oxygenated GSH adduct of MP (MP[O]SG) at  $m/z$  585. **E)** Total diode array scan of incubation.

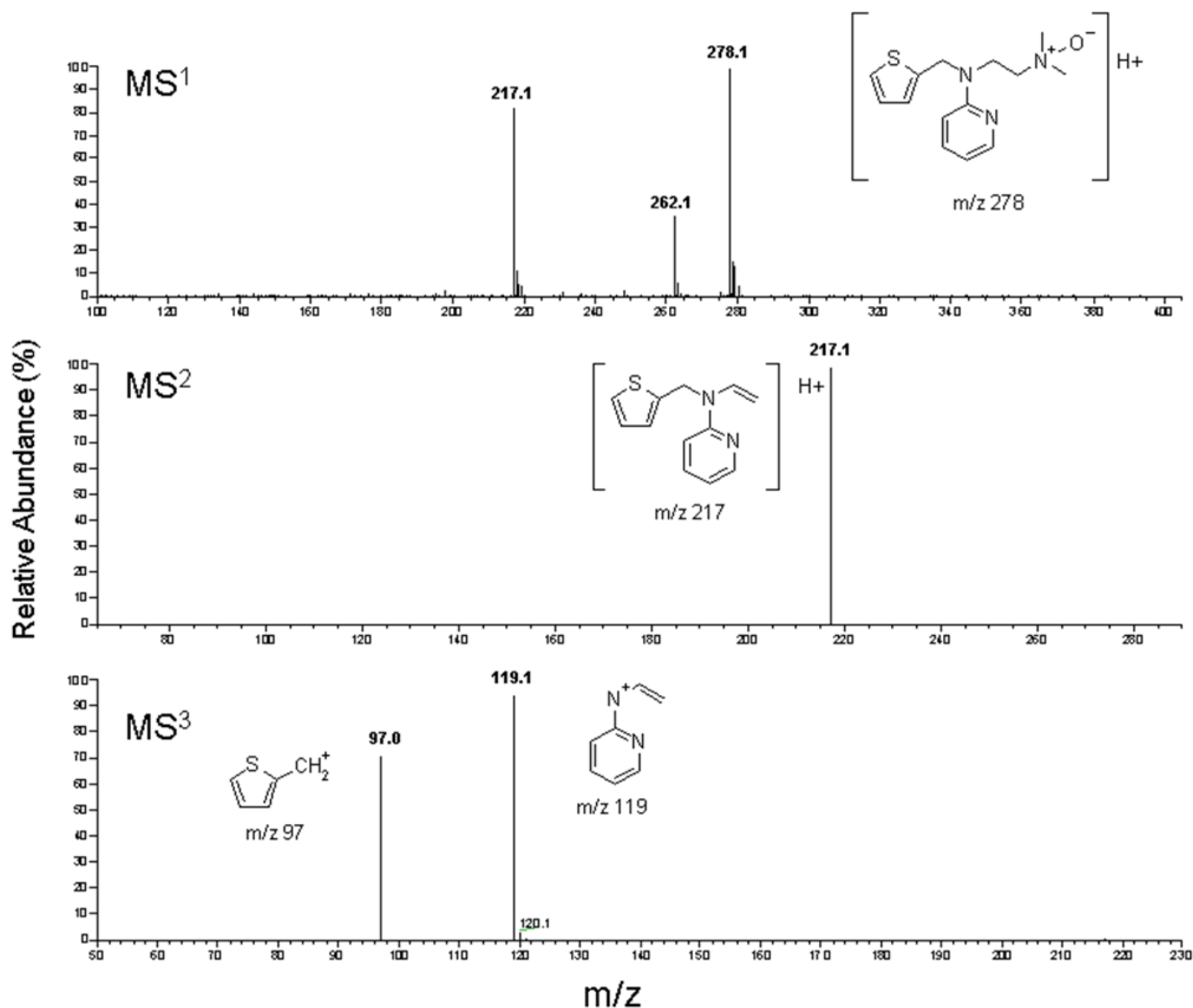


**Figure 2.4.** MS<sup>1</sup>, MS<sup>2</sup> and MS<sup>3</sup> spectra representative of parent drug as recovered from incubation of RLM supplemented with 100 $\mu$ M MP, 1mM GSH and NADPH regenerating system. Analysis was carried out on a LTQ-Orbitrap mass spectrometer. MS<sup>2</sup> and MS<sup>3</sup> spectra are product ion spectra of the base peak in the preceding spectrum.

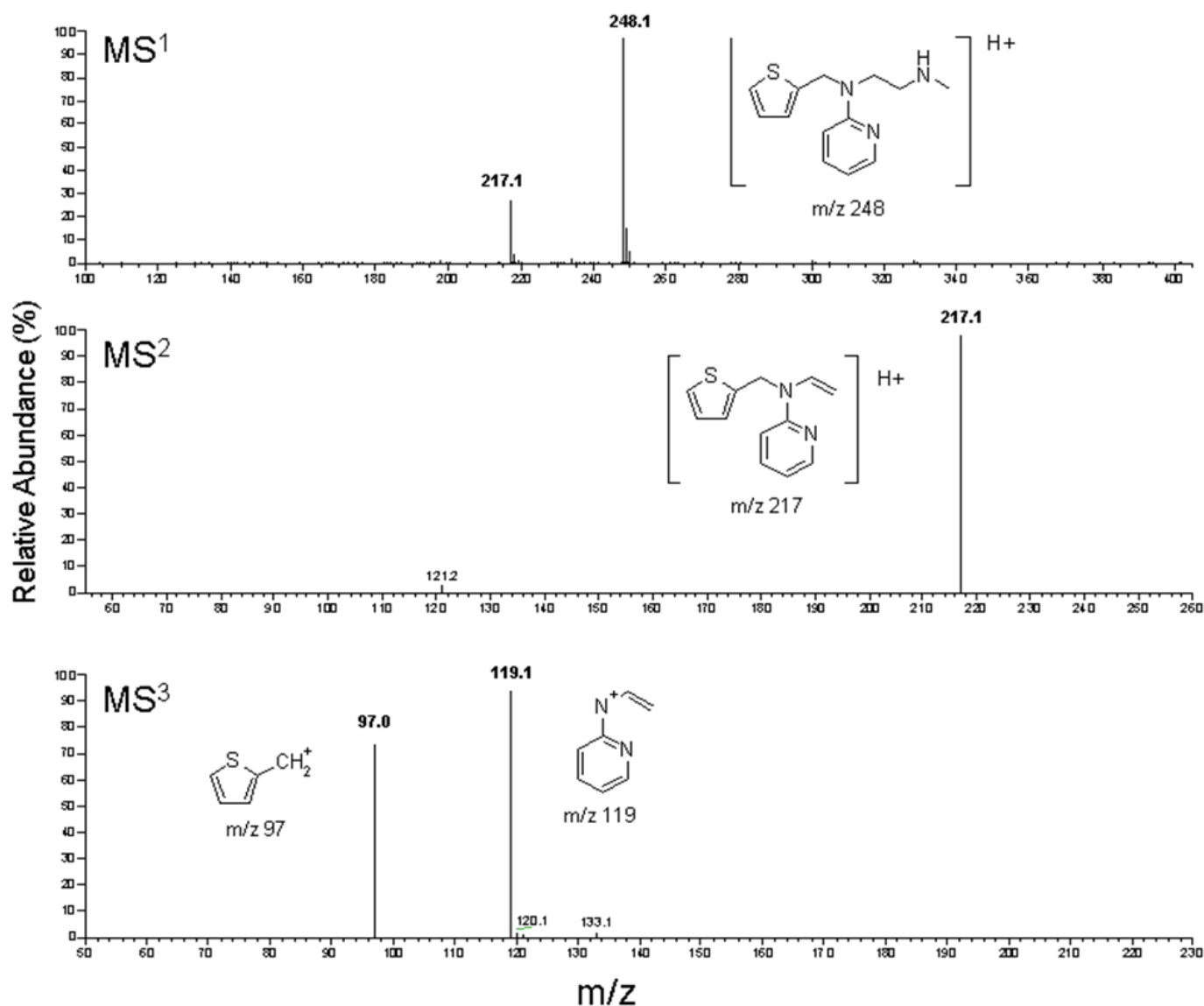


**Figure 2.5.** MS<sup>1</sup>, MS<sup>2</sup> and MS<sup>3</sup> spectra representative of metabolite with retention time 11.75min as detected in extracted chromatogram of for *m/z* 278. Metabolites were generated by RLM supplemented with 100 $\mu$ M MP, 1mM GSH and NADPH regenerating system. Analysis was carried out on a LTQ-Orbitrap mass spectrometer. Fragmentation suggests that site of hydroxylation is the pyridine ring, although exact position of the hydroxyl group has not been determined. MS<sup>2</sup> and MS<sup>3</sup> spectra are product ion spectra of the base peak in the preceding spectrum.

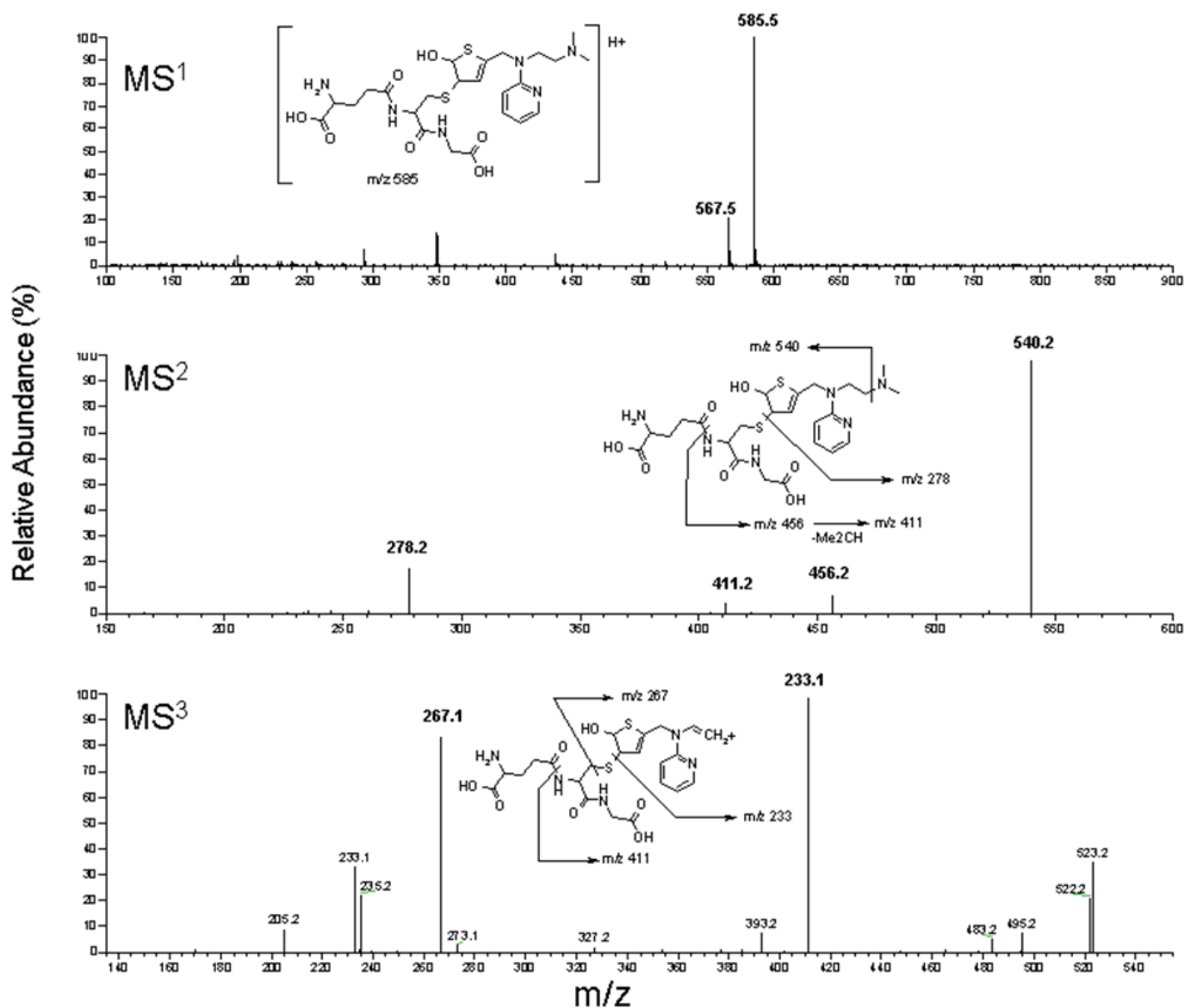




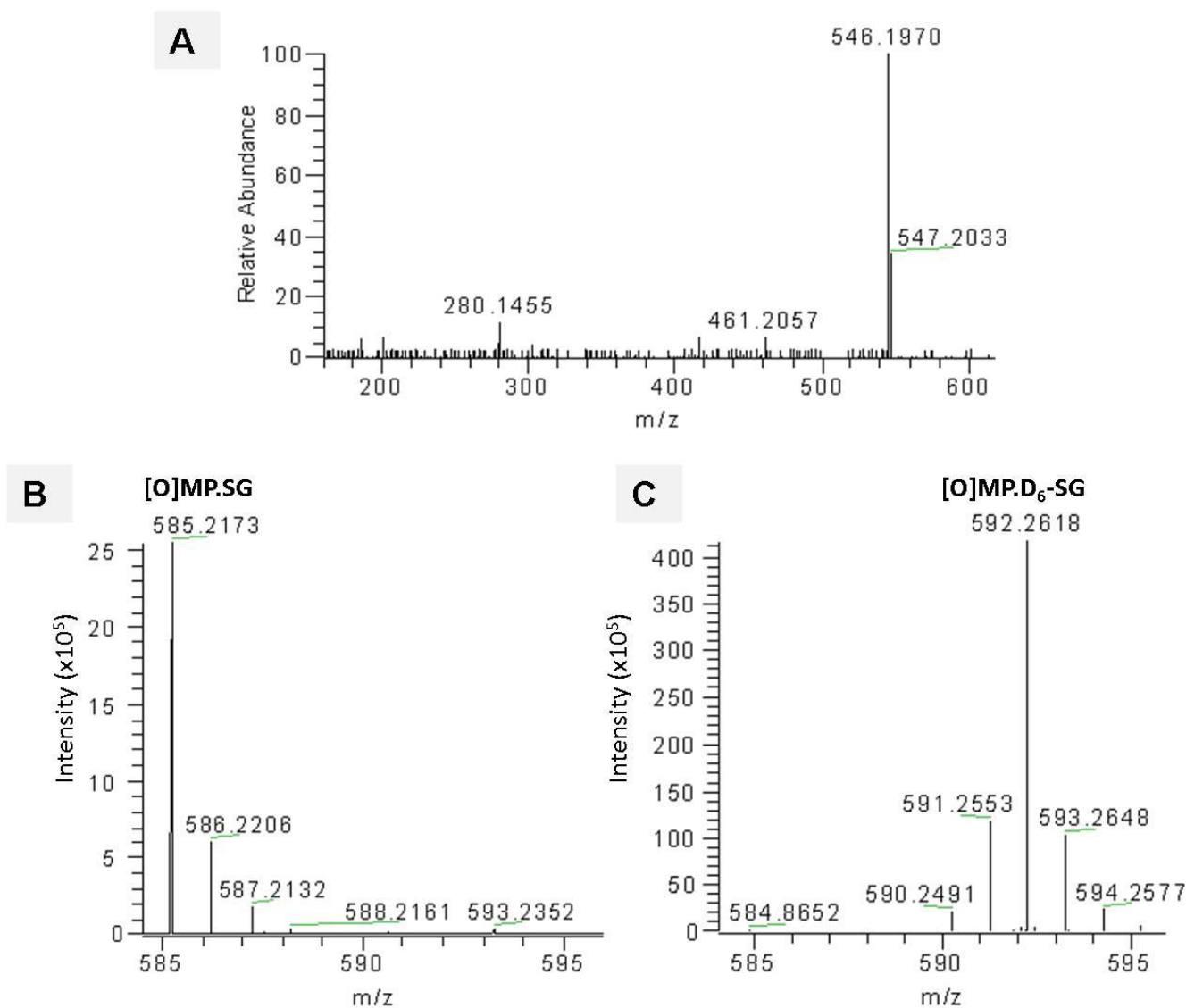
**Figure 2.6.** MS<sup>1</sup>, MS<sup>2</sup> and MS<sup>3</sup> spectra representative of metabolite with retention time 13.19 as detected in extracted chromatogram of *m/z* 278. Metabolites were generated by RLM supplemented with 100 $\mu$ M MP, 1mM GSH and NADPH regenerating system. Analysis was carried out on a LTQ-Orbitrap mass spectrometer. Fragmentation is consistent with MP N-oxide, due to lack of hydroxylation of either the thiophene or pyridine ring. MS<sup>2</sup> and MS<sup>3</sup> spectra are product ion spectra of the base peak in the preceding spectrum.



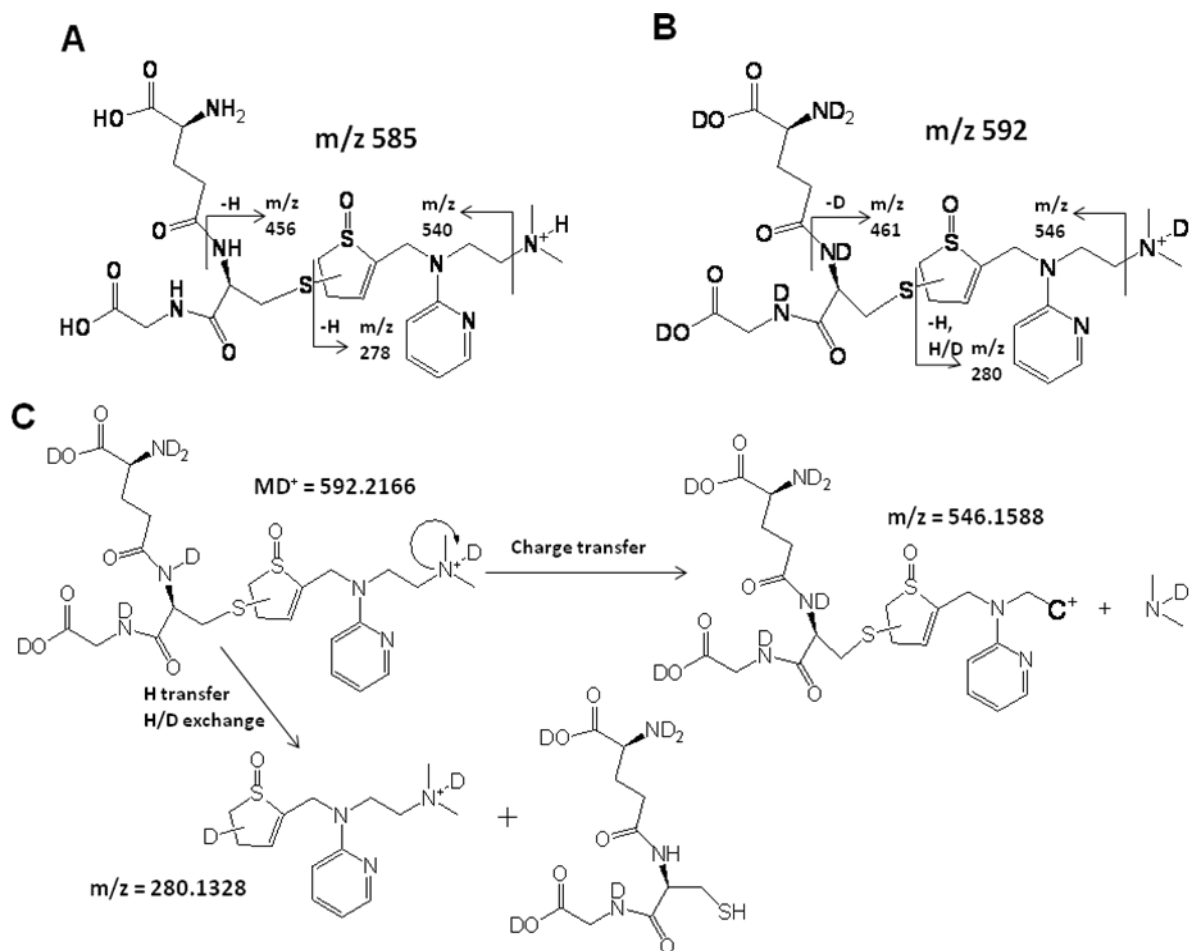
**Figure 2.7.** MS<sup>1</sup>, MS<sup>2</sup> and MS<sup>3</sup> spectra representative of metabolite with retention time 12.35 as detected with selective monitoring for  $m/z$  248. Metabolites were generated by RLM supplemented with 100 $\mu$ M MP, 1mM GSH and NADPH regenerating system. Analysis was carried out on a LTQ-Orbitrap mass spectrometer. Parent ion mass and fragmentation are indicative of desmethyl methapyrilene. Fragmentation confirms no alteration to thiophene or pyridine rings. MS<sup>2</sup> and MS<sup>3</sup> spectra are product ion spectra of the base peak in the preceding spectrum.



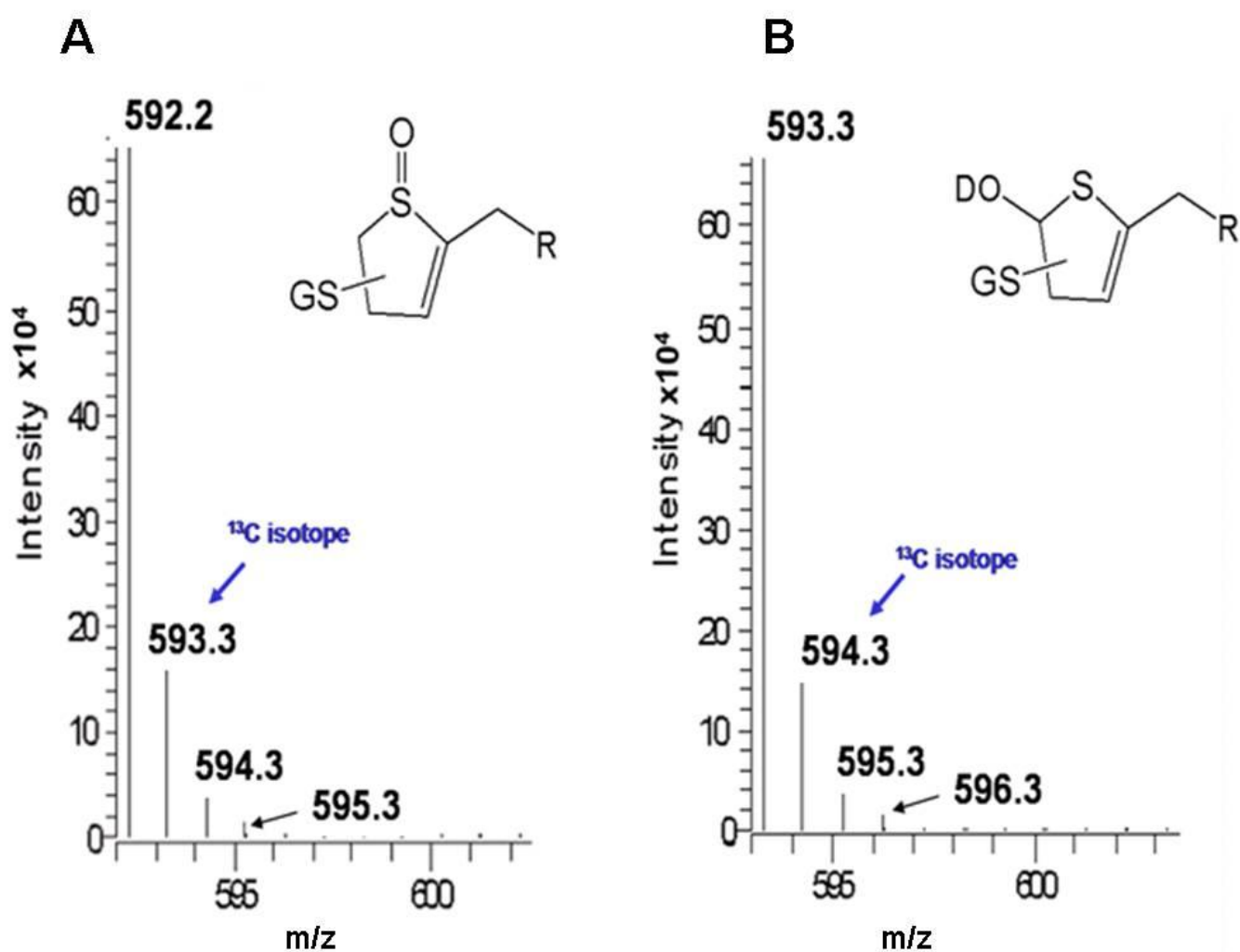
**Figure 2.8.** MS<sup>1</sup>, MS<sup>2</sup> and MS<sup>3</sup> spectra representative of metabolite with retention time 9.12 as detected with selective monitoring for  $m/z$  585. Metabolites were generated by RLM supplemented with 100 $\mu$ M MP, 1mM GSH and NADPH regenerating system. Analysis was carried out on a LTQ-Orbitrap mass spectrometer. MS<sup>1</sup> mass is indicative of a glutathione adduct [262 +307]<sup>+</sup>. This is confirmed presence of fragments  $m/z$  456 [M+1-129]<sup>+</sup> representing loss of pyroglutamate and  $m/z$  278 [M+1-308]<sup>+</sup> representing loss of GSH in the MS<sup>2</sup> spectra.



**Figure 2.9.** Full-scan and partial electrospray mass spectra of glutathione adduct (MP[O]SG) produced by an incubation of MP with RLM and GSH. (A) Full-scan spectrum of MP[O]SG dissolved in MeOD, showing at  $m/z$  592 ( $[M_D+D]^+$ ) the deuterated hexadeuterated derivative ([O]MP.D<sub>6</sub>-SG) attributed to exchange of the six labile protons on the glutathionyl residue. (B) Parent-ion region of the spectrum of MP[O]SG ([O]MP.SG) dissolved in methanol showing  $[M+H]^+$  at  $m/z$  585.2173. (C) Parent-ion region of spectrum of (A) showing  $[M_D+D]^+$  at  $m/z$  592.2618 and the mono-<sup>13</sup>C isotopomer of hexadeutero-MP[O]SG at  $m/z$  593.2648.



**Figure 2.10.** Interpretations of mass spectral fragmentation of (A) MP[O]SG ( $[M+H]^+$  at  $m/z$  585) recovered from an incubation of RLM with MP and GSH, represented as the glutathione adduct of MP *S*-oxide (position of glutathionylation not determined), and (B) and (C) hexadeuterated MP[O]SG ( $[M_D+D]^+$  at  $m/z$  592) obtained by H/D exchange of the metabolite in MeOD. The mass shifts of fragments  $[M+1-HN(CH_3)_2]^+$  ( $m/z$  540 to  $m/z$  546),  $[M+1-pyroglutamate]^+$  ( $m/z$  456 to  $m/z$  461) and  $[M+1-GSH]^+$  ( $m/z$  278 to  $m/z$  280) produced by deuteration are consistent with the six H/D exchanges being confined to the glutathionyl residue and thereby with the proposed *S*-oxide structure of MP[O]SG. The mass shift of  $[M+1-GSH]^+$  from  $m/z$  278 to  $m/z$  280 rather than to  $m/z$  279 implies H/D exchange between the thiophene ring and a hexadeuterated glutathionyl residue (mass, 312) during fragmentation.



**Figure 2.11.** Calculated MS spectra (parent ion region) obtained in Xcaliber 2.0 SR2 representing spectra expected following H/D exchange of (A) a glutathione adduct formed from an S-oxide of MP and (B) a glutathione adduct formed via an epoxide of MP resulting in a hydroxyl glutathione adduct.

## 2.4 Discussion.

The purpose of the work described in this chapter was to define the chemistry of the toxicophoric reactive metabolite of MP formed in the rat. In order to do this, incubations were carried out using RLM supplemented with a 10-fold molar excess of GSH (1mM) in order to trap any electrophilic metabolite formed. It has been shown that GSH does not inhibit any major CYP450 isoforms at concentrations up to 10mM (Zhang *et al.*, 2009). Incubations were firstly analysed by LC-MS/MS/MS in order to confirm turnover to a glutathione adduct had been achieved and to investigate which other metabolites were identifiable. A mono-oxygenated glutathione adduct (MP[O]SG) of the M+O+GSH type was found, and its fragmentation corresponded with that previously reported (Graham, 2007). An incubation of liver microsomes and GSH or a GSH derivative is now the standard preparation used in screens for reactive drug metabolites that are soft electrophiles (Gan *et al.*, 2009). However, it can yield more (Bu *et al.*, 2007; Rousu *et al.*, 2009) or fewer (Srivastava *et al.*, 2009) conjugates than can be accounted for in vitro, thereby giving a false impression of the bioactivation pathways that operate in vivo. This was not the case with MP, only one GSH adduct being found in hepatic microsomal incubations, rat hepatocytes and rats (Graham *et al.*, 2008). Also detected in the incubations were other previously reported metabolites, namely desmethyl MP, MP N-oxide and mono-oxygenated MP (Graham *et al.*, 2008). Whilst the pyridine ring is suggested as the site of hydroxylation, the exact position of the hydroxyl group was not determined. Therefore it was confirmed that

microsomal bioactivation occurred in the context of the established pathways of MP's transformation to stable metabolites.

Passive hydrogen/deuterium exchange (H/D exchange) was then used to define the structure of the GSH adduct and thence to derive the chemistry of the reactive intermediate, be it an *S*-oxide or epoxide of the thiophene ring, or a ring opened  $\alpha,\beta$ -unsaturated aldehyde. H/D exchange has previously been found to be effective in distinguishing between *S*-oxides or N-oxides and isomeric hydroxyl metabolites due to the difference between the number of labile hydrogen atoms, hydroxyl metabolites having an extra hydrogen atom available to exchange (Chen *et al.*, 2009; Liu *et al.*, 2001). Liu *et al.* (2001) used online H/D exchange to identify an *S*-oxide metabolite of an investigational compound. Having characterised a mono-oxygenated metabolite due to a mass increase of 16Da, and having shown that the parent compound had two exchangeable hydrogen atoms, they used a D<sub>2</sub>O/acetonitrile LC gradient to effect online H/D exchange that revealed a mass increase of 2Da on the mono-oxygenated metabolite, therefore ruling out the hydroxyl metabolite and supporting formation of an *S*-oxide (Chen *et al.*, 2009; Liu *et al.*, 2001). Other groups used this technique successfully to aid definition of metabolic structures (Gianelli *et al.*, 2000; Miao *et al.*, 2005; Ohashi *et al.*, 1998). It would appear from our findings and those of other groups, that using H/D exchange is an effective way of deducing the structures of reactive intermediates of thiophene rings.

Following the H/D exchange experiment described in this chapter, it is proposed that the toxicophore of MP is the *S*-oxide of the thiophene ring. As mentioned previously, *S*-oxidation appears to be the major route of bioactivation of thiophene molecules in the rat (Dansette *et al.*, 1992; Shimizu *et al.*, 2009), and has also been detected



previously in *in vitro* investigations (Belghazi *et al.*, 2001; Dansette *et al.*, 2005; Joshi *et al.*, 2004). However, it should be noted that the metabolic activation of thiophene compounds with more than one bioactivatable moiety is not necessarily restricted to the thiophene ring (Lim *et al.*, 2008). Isomeric thioether adducts formed by addition of GSH to MP *S*-oxide and the  $\alpha,\beta$ -unsaturated aldehyde intermediate (Fig. 2.2) would both have structures consistent with the observed six H/D exchanges in MeOD: H/D exchange on the MP-derived moiety would not be expected in either case. Nevertheless, there are several factors suggesting that a thioketo aldehyde is an improbable precursor of MP[O]SG. Firstly, in microsomal incubations of suprofen, a 2-arylthiophene, the putative 4,5-epoxide precursor of the  $\gamma$ -thioketo- $\alpha,\beta$ -unsaturated aldehyde intermediate, and not the aldehyde, was trapped with GSH as a thioether adduct. The aldehyde itself was stabilized separately, as a pyridazine derivative, with semicarbazide (O'Donnell *et al.*, 2003). Secondly, investigations with methapyrilene and semicarbazide found neither inhibition of the irreversible binding of [ $^3$ H]MP to liver microsomes nor yielded an additional metabolite, and specifically no pyridazine derivative (Graham *et al.*, 2008). Finally, furans and pyrroles are also metabolized to GSH adducts without apparent opening of the heterocycle (Kalgutkar *et al.*, 2005; Williams *et al.*, 2007), and, in general, it seems that  $\alpha,\beta$ -unsaturated aldehydes derived from heteroarene epoxides by ring opening are not trapped in ring-opened forms by GSH. Recyclization requires dehydration, yielding a M-2+GSH type adduct. The glutathione adduct of a  $\gamma$ -thioketo- $\alpha,\beta$ -unsaturated aldehyde, as implied by Medower *et al.* (2008), might also be formed from a thiophene epoxide by concerted addition of GSH and ring opening, but no published precedent for such a reaction has been

found. Therefore, the available data are most readily understood in terms of MP[O]SG being a glutathione adduct of MP *S*-oxide.

These findings represent the first detailed structural definition of an electrophilic metabolite of MP modified in the toxicophore moiety. Whilst iminium ions have previously been trapped using cyanide (Ziegler *et al.*, 1981), this was in rabbit liver microsomes, so it is not apparent how this relates to the *in vivo* hepatotoxic rat model. The metabolic activation of MP appears to play a role in the toxicity of MP. Irreversible binding of tritiated MP in both RLM and isolated rat hepatocytes has previously been investigated parallel to cytotoxicity (Graham, 2007). Irreversible binding in hepatocytes was 4.56nmol/mg protein at 200 $\mu$ M MP, but was significantly reduced following preincubations with non specific P450 inhibitors aminobenzotriazole and SKF-525A (Graham, 2007; Graham *et al.*, 2008). These inhibitors also attenuated the cytotoxicity caused by 200 $\mu$ M MP. Incubations with RLM exhibited 0.7nmol/mg protein irreversible binding, but following supplementation with glutathione, this decreased to 0.1nmol/mg and a glutathione adduct was formed. It can therefore be asserted that the *S*-oxide metabolite described in this chapter is responsible for the hepatotoxic effects of MP in the rat.

This chapter adds to the convincing and substantial evidence that metabolic activation can be a mechanism involved in drug induced liver injury (Park *et al.*, 2005). There is therefore a serious need for preclinical systems in which to investigate the potential of compounds to undergo metabolism to reactive metabolic and to investigate structure metabolism and toxicity relationships (Groneberg *et al.*, 2002).

## References

- Beekman, JM, Boess, F, Hildebrand, H, Kalkuhl, A, Suter, L (2006) Gene expression analysis of the hepatotoxicant methapyrilene in primary rat hepatocytes: an interlaboratory study. *Environ Health Perspect* **114**(1): 92-99.
- Belghazi, M, Jean, P, Poli, S, Schmitter, JM, Mansuy, D, Dansette, PM (2001) Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv Exp Med Biol* **500**: 139-144.
- Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.
- Bredie, WL, Mottram, DS, Guy, RC (2002) Effect of temperature and pH on the generation of flavor volatiles in extrusion cooking of wheat flour. *J Agric Food Chem* **50**(5): 1118-1125.
- Brennan, LM, Creasia, DA (1982) The effects of methapyrilene hydrochloride on hepatocarcinogenicity and pentobarbital-induced sleeping time in rats and mice. *Toxicology and applied pharmacology* **66**(2): 252-258.
- Broom, NJ, Elder, JS, Hannan, PC, Pons, JE, O'Hanlon, PJ, Walker, G, Wilson, J, Woodall, P (1995) The chemistry of pseudomonic acid. Part 14. Synthesis and in vivo biological activity of heterocyclyl substituted oxazole derivatives. *J Antibiot (Tokyo)* **48**(11): 1336-1344.
- Bu, HZ, Zhao, P, Dalvie, DK, Pool, WF (2007) Identification of primary and sequential bioactivation pathways of carbamazepine in human liver microsomes using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **21**(20): 3317-3322.
- Chen, G, Daaro, I, Pramanik, BN, Piwinski, JJ (2009) Structural characterization of in vitro rat liver microsomal metabolites of antihistamine desloratadine using LTQ-Orbitrap hybrid mass spectrometer in combination with online hydrogen/deuterium exchange HR-LC/MS. *J Mass Spectrom* **44**(2): 203-213.
- Craig, A, Sidaway, J, Holmes, E, Orton, T, Jackson, D, Rowlinson, R, Nickson, J, Tonge, R, Wilson, I, Nicholson, J (2006) Systems toxicology: integrated genomic, proteomic and metabolomic analysis of methapyrilene induced hepatotoxicity in the rat. *J Proteome Res* **5**(7): 1586-1601.
- Cunningham, ML, Pippin, LL, Anderson, NL, Wenk, ML (1995) The hepatocarcinogen methapyrilene but not the analog pyrilamine induces sustained hepatocellular replication and protein alterations in F344 rats in a 13-week feed study. *Toxicol Appl Pharmacol* **131**(2): 216-223.
- Dansette, PM, Bertho, G, Mansuy, D (2005) First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives. *Biochem Biophys Res Commun* **338**(1): 450-455.
- Dansette, PM, Libraire, J, Bertho, G, Mansuy, D (2009) Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem Res Toxicol* **22**(2): 369-373.

Dansette, PM, Thang, DC, el Amri, H, Mansuy, D (1992) Evidence for thiophene-S-oxide as a primary reactive metabolite of thiophene in vivo: formation of a dihydrothiophene sulfoxide mercapturic acid. *Biochem Biophys Res Commun* **186**(3): 1624-1630.

Dear, GJ, Plumb, RS, Sweatman, BC, Parry, PS, Roberts, AD, Lindon, JC, Nicholson, JK, Ismail, IM (2000) Use of directly coupled ion-exchange liquid chromatography-mass spectrometry and liquid chromatography-nuclear magnetic resonance spectroscopy as a strategy for polar metabolite identification. *J Chromatogr B Biomed Sci Appl* **748**(1): 295-309.

Gan, J, Ruan, Q, He, B, Zhu, M, Shyu, WC, Humphreys, WG (2009) In vitro screening of 50 highly prescribed drugs for thiol adduct formation--comparison of potential for drug-induced toxicity and extent of adduct formation. *Chem Res Toxicol* **22**(4): 690-698.

Gianelli, L, Mellerio, GG, Siviero, E, Rossi, A, Cabri, W, Sogli, L (2000) Mass spectrometry of avermectins: structural determination of two new derivatives of Ivermectin B(1a). *Rapid Commun Mass Spectrom* **14**(14): 1260-1265.

Graham, E PhD Thesis, University of Liverpool, Liverpool, 2007.

Graham, EE, Walsh, RJ, Hirst, CM, Maggs, JL, Martin, S, Wild, MJ, Wilson, ID, Harding, JR, Kenna, JG, Peter, RM, Williams, DP, Park, BK (2008) Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* **326**(2): 657-671.

Groneberg, DA, Grosse-Siestrup, C, Fischer, A (2002) In vitro models to study hepatotoxicity. *Toxicol Pathol* **30**(3): 394-399.

Hagen, SE, Domagala, J, Gajda, C, Lovdahl, M, Tait, BD, Wise, E, Holler, T, Hupe, D, Nouhan, C, Urumov, A, Zeikus, G, Zeikus, E, Lunney, EA, Pavlovsky, A, Gracheck, SJ, Saunders, J, VanderRoest, S, Brodfuehrer, J (2001) 4-Hydroxy-5,6-dihydropyrones as inhibitors of HIV protease: the effect of heterocyclic substituents at C-6 on antiviral potency and pharmacokinetic parameters. *J Med Chem* **44**(14): 2319-2332.

Hagihara, K, Kazui, M, Kurihara, A, Yoshiike, M, Honda, K, Okazaki, O, Farid, NA, Ikeda, T (2009) A possible mechanism for the differences in efficiency and variability of active metabolite formation from thienopyridine antiplatelet agents, prasugrel and clopidogrel. *Drug Metab Dispos* **37**(11): 2145-2152.

Huffman, GP, Huggins, FE, Shah, N, Huggins, R, Linak, WP, Miller, CA, Pugmire, RJ, Meuzelaar, HL, Seehra, MS, Manivannan, A (2000) Characterization of fine particulate matter produced by combustion of residual fuel oil. *J Air Waste Manag Assoc* **50**(7): 1106-1114.

Joshi, EM, Heasley, BH, Chordia, MD, Macdonald, TL (2004) In vitro metabolism of 2-acetylbenzothiophene: relevance to zileuton hepatotoxicity. *Chem Res Toxicol* **17**(2): 137-143.

Kalgutkar, AS, Soglia, JR (2005) Minimising the potential for metabolic activation in drug discovery. *Expert Opin Drug Metab Toxicol* **1**(1): 91-142.

Kammerer, RC, Schmitz, DA (1986) Metabolism of methapyrilene by rat-liver homogenate. *Xenobiotica* **16**(7): 671-680.

Kammerer, RC, Schmitz, DA, Lampe, MA, Kloc, K (1988) The in vivo metabolism of methapyrilene, a hepatocarcinogen, in the rat. *Xenobiotica* **18**(7): 869-881.

Kelly, DW, Holder, CL, Korfmacher, WA, Getek, TA, Lay, JO, Jr., Casciano, DA, Shaddock, JG, Duhart, HM, Slikker, W, Jr. (1992) Metabolism of methapyrilene by Fischer-344 rat and B6C3F1 mouse hepatocytes. *Xenobiotica* **22**(12): 1367-1381.

Kelly, DW, Holder, CL, Korfmacher, WA, Slikker, W, Jr. (1990) Plasma elimination and urinary excretion of methapyrilene in the rat. *Drug metabolism and disposition: the biological fate of chemicals* **18**(6): 1018-1024.

Kilbourn, MR (1989) Thiophenes as phenyl bio-isosteres: application in radiopharmaceutical design--I. Dopamine uptake antagonists. *Int J Rad Appl Instrum B* **16**(7): 681-686.

Lam, W, Ramanathan, R (2002) In electrospray ionization source hydrogen/deuterium exchange LC-MS and LC-MS/MS for characterization of metabolites. *J Am Soc Mass Spectrom* **13**(4): 345-353.

Lampe, MA, Kammerer, RC (1990) Species differences in the metabolism and macromolecular binding of methapyrilene: a comparison of rat, mouse and hamster. *Xenobiotica; the fate of foreign compounds in biological systems* **20**(12): 1269-1280.

Lecoeur, S, Andre, C, Beaune, PH (1996) Tienilic acid-induced autoimmune hepatitis: anti-liver and-kidney microsomal type 2 autoantibodies recognize a three-site conformational epitope on cytochrome P4502C9. *Mol Pharmacol* **50**(2): 326-333.

Lijinsky, W, Reuber, MD, Blackwell, BN (1980) Liver tumors induced in rats by oral administration of the antihistaminic methapyrilene hydrochloride. *Science* **209**(4458): 817-819.

Lim, HK, Chen, J, Cook, K, Sensenhaus, C, Silva, J, Evans, DC (2008) A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom* **22**(8): 1295-1311.

Liu, DQ, Hop, CE, Beconi, MG, Mao, A, Chiu, SH (2001) Use of on-line hydrogen/deuterium exchange to facilitate metabolite identification. *Rapid Commun Mass Spectrom* **15**(19): 1832-1839.

Ma, S, Zhu, M (2009) Recent advances in applications of liquid chromatography-tandem mass spectrometry to the analysis of reactive drug metabolites. *Chem Biol Interact* **179**(1): 25-37.

Mansuy, D, Dansette, PM, Foures, C, Jaouen, M, Moinet, G, Bayer, N (1984) Metabolic hydroxylation of the thiophene ring: isolation of 5-hydroxy-tienilic acid as the major urinary metabolite of tienilic acid in man and rat. *Biochem Pharmacol* **33**(9): 1429-1435.

Masubuchi, N, Makino, C, Murayama, N (2007) Prediction of in vivo potential for metabolic activation of drugs into chemically reactive intermediate: correlation of in vitro and in vivo generation of reactive intermediates and in vitro glutathione conjugate formation in rats and humans. *Chem Res Toxicol* **20**(3): 455-464.

Medower, C, Wen, L, Johnson, WW (2008) Cytochrome P450 oxidation of the thiophene-containing anticancer drug 3-[(quinolin-4-ylmethyl)-amino]-thiophene-2-carboxylic acid (4-

trifluoromethoxy-phenyl)-amide to an electrophilic intermediate. *Chem Res Toxicol* **21**(8): 1570-1577.

Mercer, AE, Regan, SL, Hirst, CM, Graham, EE, Antoine, DJ, Benson, CA, Williams, DP, Foster, J, Kenna, JG, Park, BK (2009) Functional and toxicological consequences of metabolic bioactivation of methapyrilene via thiophene S-oxidation: Induction of cell defence, apoptosis and hepatic necrosis. *Toxicol Appl Pharmacol* **239**(3): 297-305.

Methven, L, Tsoukka, M, Oruna-Concha, MJ, Parker, JK, Mottram, DS (2007) Influence of sulfur amino acids on the volatile and nonvolatile components of cooked salmon (*Salmo salar*). *J Agric Food Chem* **55**(4): 1427-1436.

Miao, Z, Kamel, A, Prakash, C (2005) Characterization of a novel metabolite intermediate of ziprasidone in hepatic cytosolic fractions of rat, dog, and human by ESI-MS/MS, hydrogen/deuterium exchange, and chemical derivatization. *Drug Metab Dispos* **33**(7): 879-883.

Nassar, AE (2003) Online hydrogen-deuterium exchange and a tandem-quadrupole time-of-flight mass spectrometer coupled with liquid chromatography for metabolite identification in drug metabolism. *J Chromatogr Sci* **41**(8): 398-404.

Nishiya, T, Kato, M, Suzuki, T, Maru, C, Kataoka, H, Hattori, C, Mori, K, Jindo, T, Tanaka, Y, Manabe, S (2008a) Involvement of cytochrome P450-mediated metabolism in tienilic acid hepatotoxicity in rats. *Toxicol Lett* **183**(1-3): 81-89.

Nishiya, T, Mori, K, Hattori, C, Kai, K, Kataoka, H, Masubuchi, N, Jindo, T, Manabe, S (2008b) The crucial protective role of glutathione against tienilic acid hepatotoxicity in rats. *Toxicol Appl Pharmacol* **232**(2): 280-291.

O'Donnell, JP, Dalvie, DK, Kalgutkar, AS, Obach, RS (2003) Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31**(11): 1369-1377.

Ohashi, N, Furuuchi, S, Yoshikawa, M (1998) Usefulness of the hydrogen--deuterium exchange method in the study of drug metabolism using liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* **18**(3): 325-334.

Park, BK, Kitteringham, NR, Maggs, JL, Pirmohamed, M, Williams, DP (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* **45**: 177-202.

Press, JB, McNally, JJ, Sanfilippo, PJ, Addo, MF, Loughney, D, Giardino, E, Katz, LB, Falotico, R, Haertlein, BJ (1993) Novel thieno[2,3-b]- and [3,4-b]pyrans as potassium channel openers. Thiophene systems--XVII. *Bioorg Med Chem* **1**(6): 423-435.

Ramanathan, R, Su, AD, Alvarez, N, Blumenkrantz, N, Chowdhury, SK, Alton, K, Patrick, J (2000) Liquid chromatography/mass spectrometry methods for distinguishing N-oxides from hydroxylated compounds. *Anal Chem* **72**(6): 1352-1359.

Ratra, GS, Cottrell, S, Powell, CJ (1998a) Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* **46**(1): 185-196.

Ratra, GS, Morgan, WA, Mullervy, J, Powell, CJ, Wright, MC (1998b) Methapyrilene hepatotoxicity is associated with oxidative stress, mitochondrial dysfunction and is prevented by the Ca<sup>2+</sup> channel blocker verapamil. *Toxicology* **130**(2-3): 79-93.

Ratra, GS, Powell, CJ, Park, BK, Maggs, JL, Cottrell, S (2000) Methapyrilene hepatotoxicity is associated with increased hepatic glutathione, the formation of glucuronide conjugates, and enterohepatic recirculation. *Chem Biol Interact* **129**(3): 279-295.

Reznik-Schuller, HM, Lijinsky, W (1981) Morphology of early changes in liver carcinogenesis induced by methapyrilene. *Archives of toxicology* **49**(1): 79-83.

Rodenhuis, N, Timmerman, W, Wikstrom, HV, Dijkstra, D (2000) Thiophene analogs of naphthoxazines and 2-aminotetralins: bioisosteres with improved relative oral bioavailability, as compared to 5-OH-DPAT. *Eur J Pharmacol* **394**(2-3): 255-263.

Rousu, T, Pelkonen, O, Tolonen, A (2009) Rapid detection and characterization of reactive drug metabolites in vitro using several isotope-labeled trapping agents and ultra-performance liquid chromatography/time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* **23**(6): 843-855.

Shimizu, S, Atsumi, R, Nakazawa, T, Fujimaki, Y, Sudo, K, Okazaki, O (2009) Metabolism of ticlopidine in rats: identification of the main biliary metabolite as a glutathione conjugate of ticlopidine S-oxide. *Drug Metab Dispos* **37**(9): 1904-1915.

Singer, SS, Lijinsky, W, Kratz, LE, Castagnoli, N, Jr., Rose, JE (1987) A comparison of in vivo and in vitro metabolites of the H1-antagonist N,N-dimethyl-N'-2-pyridyl-N'-(2-thienylmethyl)-1,2-ethanediamine (methapyrilene) in the rat. *Xenobiotica; the fate of foreign compounds in biological systems* **17**(11): 1279-1291.

Srivastava, A, Lian, LY, Maggs, JL, Chaponda, M, Pirmohamed, M, Williams, DP, Park, BK (2009) Quantifying the Metabolic Activation of Nevirapine in Patients by Integrated Applications of NMR and Mass Spectrometries. *Drug Metab Dispos*.

Valadon, P, Dansette, PM, Girault, JP, Amar, C, Mansuy, D (1996) Thiophene sulfoxides as reactive metabolites: formation upon microsomal oxidation of a 3-arylthiophene and fate in the presence of nucleophiles in vitro and in vivo. *Chemical research in toxicology* **9**(8): 1403-1413.

Williams, DP, Antoine, DJ, Butler, PJ, Jones, R, Randle, L, Payne, A, Howard, M, Gardner, I, Blagg, J, Park, BK (2007) The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: a chemical and biochemical definition of the toxicophore. *J Pharmacol Exp Ther* **322**(3): 1208-1220.

Zhang, C, Wong, S, Delarosa, EM, Kenny, JR, Halladay, JS, Hop, CE, Khojasteh-Bakht, SC (2009) Inhibitory properties of trapping agents: glutathione, potassium cyanide, and methoxylamine, against major human cytochrome p450 isoforms. *Drug Metab Lett* **3**(2): 125-129.

Ziegler, R, Ho, B, Castagnoli, N, Jr. (1981) Trapping of metabolically generated electrophilic species with cyanide ion: metabolism of methapyrilene. *J Med Chem* **24**(10): 1133-1138.





## CHAPTER THREE

---

AN INVESTIGATION OF THE  
BIOACTIVATION AND TOXICITY OF  
THIOPHENES IN ISOLATED RAT  
HEPATOCTES

## Contents

3.1 Introduction .....	83
Materials and Methods.....	87
3.2.1 Materials .....	87
3.2.2 Animals .....	87
3.2.3 Hepatocyte isolation procedure .....	87
3.2.4 Hepatocyte incubations for assessment of cytotoxicity and metabolism of thiophene compounds.....	88
3.2.5 Determination of thiophene cytotoxicity.....	89
3.2.6 Preparation of samples for assessment of metabolism by LC-MS.....	89
3.2.7 Assessment of metabolism of thiophene compounds by LC-MS/MS analysis.....	89
3.2.8. Statistics .....	91
3.3. Results .....	92
3.3.1 Cytotoxicity of thiophene-containing compounds in rat hepatocytes suspensions .....	92
3.3.2 Formation of Thiophene Glutathione Adducts in Rat Hepatocyte Suspensions .....	97
3.4 Discussion .....	104

## Abbreviations

2-PT, 2-Phenylthiophene; ABT, 1-aminobenzotriazole; ACN, acetonitrile; CYP450, cytochrome P450 mixed function oxidase; DEM, diethylmaleate; DILI, drug-induced liver injury; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography tandem mass spectrometry; MeOH, methanol; MP, methapyrilene; rt, retention time; TA, tienilic acid; TC, ticlopidine.

### 3.1 Introduction

The metabolism of methapyrilene (MP) to a glutathione adduct, via formation of an S-oxide intermediate, was described in chapter two. Much evidence exists to back up the proposition that bioactivation of molecules in this way can lead to adverse drug reactions, in particular drug-induced liver injury (DILI) (Park *et al.*, 2005; Srivastava *et al.*, 2010). A major physiological purpose of the liver is to transform low molecular weight lipophilic compounds into hydrophilic derivatives to facilitate excretion. The liver has a high capacity for phase I and phase II biotransformations. Although generally these pathways produce non-toxic, inert, readily excretable compounds, reactions catalysed by P450 isozymes can sometimes produce a metabolite that is chemically much more reactive than the parent drug, usually short lived and electrophilic in nature. These electrophilic or free radical species (if not deactivated) can cause chemical modification of cellular proteins, potentially eliciting hepatotoxicity, e.g. acetaminophen quinone imine (Park *et al.*, 2005); hypersensitivity, e.g. sulphamethoxazole nitrosoarene (Callan *et al.*, 2009); or carcinogenicity, e.g. aflatoxin oxidation products (Guengerich *et al.*, 2002).

Formation of these potentially damaging species leads to an adaptive defence response in the cell, involving nuclear translocation of transcription factors which orchestrate transcription of cellular defence genes (Kensler *et al.*, 2007). The upregulated hepatic enzymes include phase II enzymes, heme oxygenase (Takakusa *et al.*, 2008), and those involved in the formation and conjugation of glutathione (GSH), such as glutamate cysteine ligase, the rate limiting enzyme in GSH synthesis (Randle *et al.*, 2008). Synthesis and depletion of GSH is a critical mechanism in the balance of deactivation of reactive metabolites and onset of toxicity (Lu, 2009; Mercer *et al.*, 2009). Electrophilic metabolites can be quenched by reaction with the

thiol group of the cysteine residue of GSH, either by spontaneous Michael addition or by an enzymatic reaction catalysed by glutathione-S-transferases. If the thioether adduct is chemically stable – which is not invariably the case (Boots *et al.*, 2005) – this prevents the electrophilic metabolites from covalently binding to cellular proteins, and hence prevents toxicity from proceeding (Anderson *et al.*, 1998). The resulting glutathione adduct can be excreted in bile, and exceptionally in urine (Johnson *et al.*, 2005), or it can be cleaved by transpeptidase to a cysteinylglycine adduct (Josch *et al.*, 1998) which yields variously cysteine and mercapturate adducts that are excreted in a similar way (Hinchman *et al.*, 1994; Hinchman *et al.*, 1991). It is the linkage and balance of bioactivation and bioinactivation which ultimately determines whether or not a reactive metabolite produces a cytotoxic effect.

The propensity of a molecule to undergo bioactivation is a function of its chemistry. Certain moieties are considered to be more susceptible than others and are labelled as structural alerts for bioactivation (Kalgutkar *et al.*, 2009). The thiophene ring has been designated as a structural alert as it can form unstable, reactive epoxides and S-oxides (Kalgutkar *et al.*, 2005). As mentioned in the previous chapter, several thiophene-containing drugs have been associated with DILI. The types of reactions vary from autoimmune hepatitis, caused by the now withdrawn diuretic tienilic acid (Eugene *et al.*, 1980), to raised serum transaminase levels seen with zileuton treatment during asthma (Watkins *et al.*, 2007). In several cases, an S-oxide or epoxide metabolite has been identified or inferred through in vitro or in vivo laboratory studies, and are thought to be responsible for the liver injury associated with the investigated drug (Graham *et al.*, 2008; Nishiya *et al.*, 2008a; O'Donnell *et al.*, 2003; Shimizu *et al.*, 2009). The in vitro systems investigated have included human (Dansette *et al.*, 1991) and rat (Dansette *et al.*, 1990) microsomes,

Supersomes (O'Donnell *et al.*, 2003), S9 fractions (Shimizu *et al.*, 2009) and cultured primary hepatocytes (Lopez-Garcia *et al.*, 2005). In vivo investigations comprise administration of test compounds to live animals, and collection of bile and urine (Dansette *et al.*, 1992; Nishiya *et al.*, 2008a; Shimizu *et al.*, 2009).

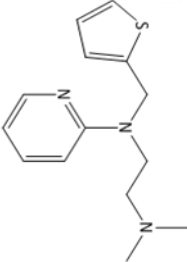
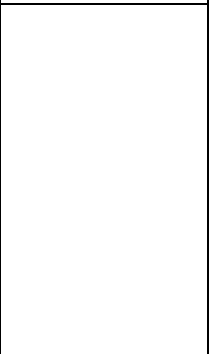
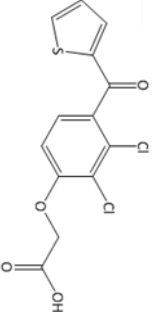

The strength of the associative link between formation of reactive drug metabolites and onset of an adverse reaction in patients is difficult to predict preclinically. Screens for irreversible protein binding and trapping of reactive intermediates can be performed, but positive results do not necessarily translate into an accurate prediction of hepatotoxicity (Kalgutkar *et al.*, 2009; Obach *et al.*, 2008). The balance between bioactivation and bioinactivation can depend on many factors, such as patient biology and genetics, dosage and co-administered drugs.

This chapter aims to elicit a link between thiophene bioactivation and an endpoint of liver cell toxicity, using suspensions of freshly isolated rat hepatocytes. These hepatocytes retain good metabolic capacity and their susceptibilities to toxins have been shown to correlate well with in vivo models in predicting hepatotoxicity (O'Brien *et al.*, 2004). The formation of GSH adducts was taken as a biomarker for thiophene-compound bioactivation (Gan *et al.*, 2009), and loss of cell membrane integrity as a measured hepatotoxicity endpoint.

The following questions were addressed:

- Are thiophene molecules bioactivated by isolated rat hepatocytes?
- Do thiophene molecules elicit cytotoxicity in isolated rat hepatocytes?
- Does modulation of bioactivation or bioinactivation attenuate or enhance toxicity?

Investigated compounds are detailed in table 3.1.

Compound	Structure	Reason to Study	Previously reported reactive metabolites	References
Methapyriline		<p>An H<sub>1</sub> receptor antagonist antihistamine drug. Withdrawn from the market following reports of rat hepatic carcinomas in rats. Also a periportal hepatotoxin in rats.</p>	<p>Forms an S-oxide intermediate trapped as a GSH adduct in rat liver microsomes</p>	<p>(Graham <i>et al.</i>, 2008; Lijinsky <i>et al.</i>, 1980)</p>
2-Phenylthiophene		<p>A model thiophene compound. Metabolism has been investigated by Dansette <i>et al.</i>, 2005. No cytotoxicity studies have been reported.</p>	<p>Forms both S-oxide and epoxide intermediates in rat liver microsomes.</p>	<p>(Dansette <i>et al.</i>, 2005)</p>
Tienilic Acid		<p>A diuretic hypotensive drug. Withdrawn in 1980 due to cases of autoimmune hepatitis. Upregulation of defence genes has been reported upon tienilic acid exposure in the rat, and centrilobular necrosis has been observed in rats pre-treated with the glutathione synthesis inhibitor buthionine sulphoximine. Tienilic acid is toxic to isolated rat hepatocytes at high concentrations</p>	<p>Thought to form an S-oxide intermediate both in vivo and in vitro.</p>	<p>(Zimmernan <i>et al.</i>, 1984); (Nishiya <i>et al.</i>, 2008a); (Higaki <i>et al.</i>, 1989; Takagi <i>et al.</i>, 1991)</p>
Ticlopidine		<p>A P2Y<sub>12</sub> receptor antagonist and platelet aggregation inhibitor. Carries a black-box warning due to cases of agranulocytosis, but has also been implicated in adverse hepatic reactions. No cytotoxicity studies have been reported.</p>	<p>Previously shown to form both S-oxide and epoxide intermediates.</p>	<p>(Farid <i>et al.</i>, 2010; Grieco <i>et al.</i>, 1998; Lim <i>et al.</i>, 2008; Liu <i>et al.</i>, 2001; Shimizu <i>et al.</i>, 2009)</p>

**Table 3.1.** Details of compounds investigated.

## **3.2 Materials and Methods.**

### **3.2.1 Materials**

Methapyrilene (MP), ticlopidine (TC) and 2-phenylthiophene (2-PT) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Tienilic acid (TA) was a gift from Molecular Toxicology, AstraZeneca (Alderley Park, Cheshire, UK). Collegenase A was purchased from Roche (Mannheim, Germany). Bradford reagent was purchased from Bio-Rad (Germany). Solvents were purchased from either VWR (Lutterworth, Leicestershire, UK) or Fischer Scientific (Loughborough, Leicestershire, UK). Unless otherwise stated, all the other reagents were purchased from Sigma-Aldrich.

### **3.2.2 Animals**

Adult male Wistar rats were obtained from Charles River Laboratories (Margate, Kent, UK). All experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act of 1986 and approved by the Animal Ethics Committee of the University of Liverpool.

### **3.2.3 Hepatocyte isolation procedure**

Rat hepatocytes were isolated using a modified two-step collagenase procedure based on Seglen et al. (1976). The rats (150 g – 300 g) were anaesthetized with sodium pentobarbital (Euthanal, 200 mg/ml, 1 µl/g, i.p.). The liver was first perfused via the hepatic portal vein for 9 min with wash buffer consisting of Hank's buffered salt solution containing 5.8 mM HEPES and 4.5 mM NaHCO<sub>3</sub> but containing no calcium chloride, magnesium chloride, magnesium sulphate, sodium hydrogencarbonate, or phenol red (Invitrogen, Paisley, UK). This was followed by a perfusion with 200 ml of wash buffer supplemented with 5 mM

CaCl<sub>2</sub> and 100 mg of collagenase until the liver interstitium had been digested. The flow rate was maintained at 30 ml/min and all solutions were kept at 37°C. After *in situ* digestion the liver was excised and the liver capsule was broken. The cells were combed and stirred out of the capsule. The cells were suspended in 100 ml wash buffer supplemented with DNase I (0.1 mg/ml) to prevent cell clusters and filtered through a 125-µm mesh (Lockertex, Warrington, Cheshire, UK). They were allowed to settle for 10 min at 4°C. Cells were centrifuged at 50g for 2 min, supernatant removed, and the cells resuspended in wash buffer containing DNase I. This was repeated twice with wash buffer, with final resuspension in wash buffer supplemented with 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O (incubation buffer). Viability was assessed by trypan blue exclusion with 20 µl of 0.4% trypan blue solution (Sigma-Aldrich) combined with 100 µl of cell suspension. The proportion of viable, i.e. dye-excluding, cells was determined by microscopic observation of stained cell suspension in a haemocytometer. At least 500 cells were counted for each determination. Hepatocytes were only used when viability was greater than 75%. Typically, initial viability was 85-90%. Cells were incubated with thiophene compound approximately 1h after the start of the liver perfusion.

#### **3.2.4 Hepatocyte incubations for assessment of cytotoxicity and metabolism of thiophene compounds**

Rat hepatocytes from individual isolations were incubated in suspensions ( $2 \times 10^6$  cells/ml, final volume 6 ml) with between 10-1000 µM thiophene, dissolved in either incubation buffer (MP; for buffer composition, see 3.2.3) or MeOH (TC, 2-PT, TA). The thiophene was added in a volume of 60 µl. Control incubations contained either 60 µl incubation buffer or 60 µl MeOH. Incubations were performed in 20 ml glass vials, with tops loosely screwed. Vials were placed in an orbital shaker at 37°C for 6 h, gently stirring to ensure the cells did not settle throughout the incubation. Hepatocytes were also pre-incubated with either 1-aminobenzotriazole (ABT) to inhibit P450 non-specifically or diethyl maleate (DEM) to deplete GSH.



### **3.2.5 Determination of thiophene cytotoxicity**

Following the incubation, an aliquot of the cell suspension (100  $\mu$ l) was used to assess trypan blue exclusion as detailed in 3.2.2. Thiophene incubations were assessed only in the case that control viability was at least 65%. The IC<sub>50</sub> value given in the results was the concentration of test compound that resulted in a proportion of viable cells half the level found in the vehicle-control incubations.

### **3.2.6 Preparation of samples for assessment of metabolism by LC-MS**

The incubation was terminated with an equal volume of ice-cold acetonitrile (ACN) and transferred to a 12 ml glass tube (LSL, Rochdale, UK). The mixture was stored at -20°C overnight in order to fully precipitate the proteins. It was then centrifuged (2200rpm, 10min) and the supernatant was evaporated to dryness under a vacuum. The resulting residue was reconstituted in 500 $\mu$ l H<sub>2</sub>O. The reconstituted residue was clarified by centrifugation (14000rpm, 5min), and was stored at -20°C until analysis.

### **3.2.7 Assessment of metabolism of thiophene compounds by LC-MS/MS analysis**

Aliquots of the solutions derived from hepatocyte incubations (30  $\mu$ l or 50  $\mu$ l) were chromatographed at room temperature on a Prodigy 5- $\mu$ m ODS-2 (C-18) column (150  $\times$  4.6 mm i.d.; Phenomenex, Macclesfield, Cheshire, UK) or a Gemini 5- $\mu$ m C-18 column (250  $\times$  4.6mm i.d.; Phenomenex, Macclesfield, Cheshire, UK), using the chromatographic conditions outlined in Table 3.2. In the case of 2-PT, the LC system consisted of two Jasco PU980 pumps (Jasco UK, Great Dunmow, Essex, U.K.) and a Jasco HG-980-30 mixing module. The eluent flow rate was 0.9 ml/min. Eluted compounds were monitored at 254 nm

(max. range, 0.0005 AUFS; adjusted as required) with a Jasco UV-975 spectrophotometer. Eluate split-flow to the LC-MS interface was *ca.* 40  $\mu$ l/min. A Quattro II mass spectrometer (Waters Corp, Manchester, UK) fitted with the standard co-axial electrospray source was operated using nitrogen as the nebulizing and drying gas. The interface temperature was 80 °C; electrospray capillary voltage, 3.8 kV; standard cone voltage, 30 V; and fragmenting cone voltage, 60 V. The instrument was set up for full-scanning acquisitions in the positive-ion mode as follows: scan range,  $m/z$  50-1050, with a scan time of 5s and an inter-scan delay of 100 ms. Instrument management and data processing were accomplished through MassLynx 3.5 software. For MP, TC and TA the LC system consisted of a PerkinElmer Series 200 pump and autosampler (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

**Table 3.2. Chromatographic conditions used for LC-MS/MS analysis of 2-PT, MP, TA and TC.**

Drug	Column	Eluent A	Eluent B	Flow rate	Gradient
MP	Gemini	10mM ammonium acetate	100% ACN	1ml/min	0min 100% A, 5min 100% A, 25min 30% A 30min 30% A 33min 100% A 34min 100% A
2PT	Prodigy	0.1% formic acid	100% MeOH	1ml/min	0min 100% A 5min 100% A 25min 20% A 30min 20% A 31min 100% A 32min 100% A
TA	Prodigy	0.1% formic acid	100% MeOH	1ml/min	0min 95% A 5min 95% A 20min 5% A 25min 5% A 26min 95% A 27min 95% A
TC	Prodigy	0.1% formic acid	100% MeOH	1ml/min	0min 90% A 5min 90% A 23min 25% A 29min 25% A 30min 90% A 35min 90% A

Eluate split-flow to the LC-MS interface was *ca.* 160  $\mu\text{l}/\text{min}$ . Analyses were carried out on an API 2000 mass spectrometer (Applied Biosystems, Warrington, Cheshire, UK) fitted with a TurboIonSpray source and operated in the positive mode. Q1 scans were acquired using the following conditions; curtain gas (CUR) setting, 20; ionspray voltage (IS), 5.5 kV; source temperature (TEM), 400°C; ion source gas 1 (GS1) setting, 20; ion source gas 2 (GS2) setting, 75; declustering potential (DP), 20 V; focusing potential (FP), 200 V; and entrance potential (EP), 10 V. Product ion scans were carried out to assess the fragmentation of detected glutathione adducts using parent ions  $m/z$  585 for MP,  $m/z$  636 for TA and  $m/z$  587 for TC. Conditions for these scans were as above except for the following changes and additions; DP, 71 V; FP, 370 V; EP, 11 V; collision energy, 31 eV and collision cell exit potential, 14 V. Instrument management and data processing were accomplished through Analyst 1.4 software.

### **3.2.8. Statistics**

All results are expressed as mean  $\pm$  standard error of the mean (SEM). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. A Students unpaired t-test was used for comparing two groups when normality was indicated and a Mann-Whitney U test was used for non-parametric data. All calculations were performed using StatsDirect statistical software (Research Solutions, Cambridge, U.K.), results were considered to be significant when  $P$  values were less than 0.05.  $\text{IC}_{50}$  calculations were carried out using GraFit 3.01 software (Erithacus Software Ltd., Surrey, UK.).

### 3.3. Results

#### 3.3.1 Cytotoxicity of thiophene-containing compounds in rat hepatocytes suspensions

##### Methapyrilene

Methapyrilene (MP) exhibited dose-dependant toxicity as assessed by trypan blue exclusion, with a significant decrease in cell viability observed at 50 $\mu$ M (Fig. 3.2A). Cell viability was decreased to 21% by a concentration of 500 $\mu$ M MP, with an IC<sub>50</sub> value of 152 $\mu$ M (Table 3.3.). Preincubation with 1mM ABT for 60min attenuated the toxicity, with a significant increase in IC<sub>50</sub> to 646 $\mu$ M. A significant attenuation was observed at 500 $\mu$ M MP, when addition of ABT produced an increase in cell viability from 21% to 75% (Fig. 3.3A). Depletion of GSH by a 30-min preincubation with 1mM DEM reduced the IC<sub>50</sub> to 88 $\mu$ M, but no significant decrease in cell viability was observed (Fig. 3.3A).

##### 2-Phenylthiophene

Significant toxicity was only observed at higher concentrations (500 $\mu$ M and 1mM) of 2-PT, which exhibited an IC<sub>50</sub> value of 553 $\mu$ M (Fig 3.2B). Preincubation with 1mM ABT produced a significant rise in cell viability at these two concentrations but overall, preincubation with ABT had little effect, producing a non significant fall in the IC<sub>50</sub> to 478 $\mu$ M (Table 3.2). However, pre-incubation with DEM produced a significant decrease in IC<sub>50</sub> compared to drug alone, from 553 $\mu$ M to 153 $\mu$ M (Table 3.3).

### **Tienilic Acid**

TA was the least toxic compound in this system, with the highest  $IC_{50}$  value of  $563\mu\text{M}$ . Significant toxicity was observed at  $500\mu\text{M}$  and  $1\text{mM}$  (Fig. 3.2D). Neither preincubation with ABT or DEM had any significant effect on toxicity (Fig. 3.3D). However, whilst ABT had little effect on the  $IC_{50}$  of TA ( $598\mu\text{M}$ ), preincubation with DEM produced a reduction to  $316\mu\text{M}$  (Table 3.3)

### **Ticlopidine**

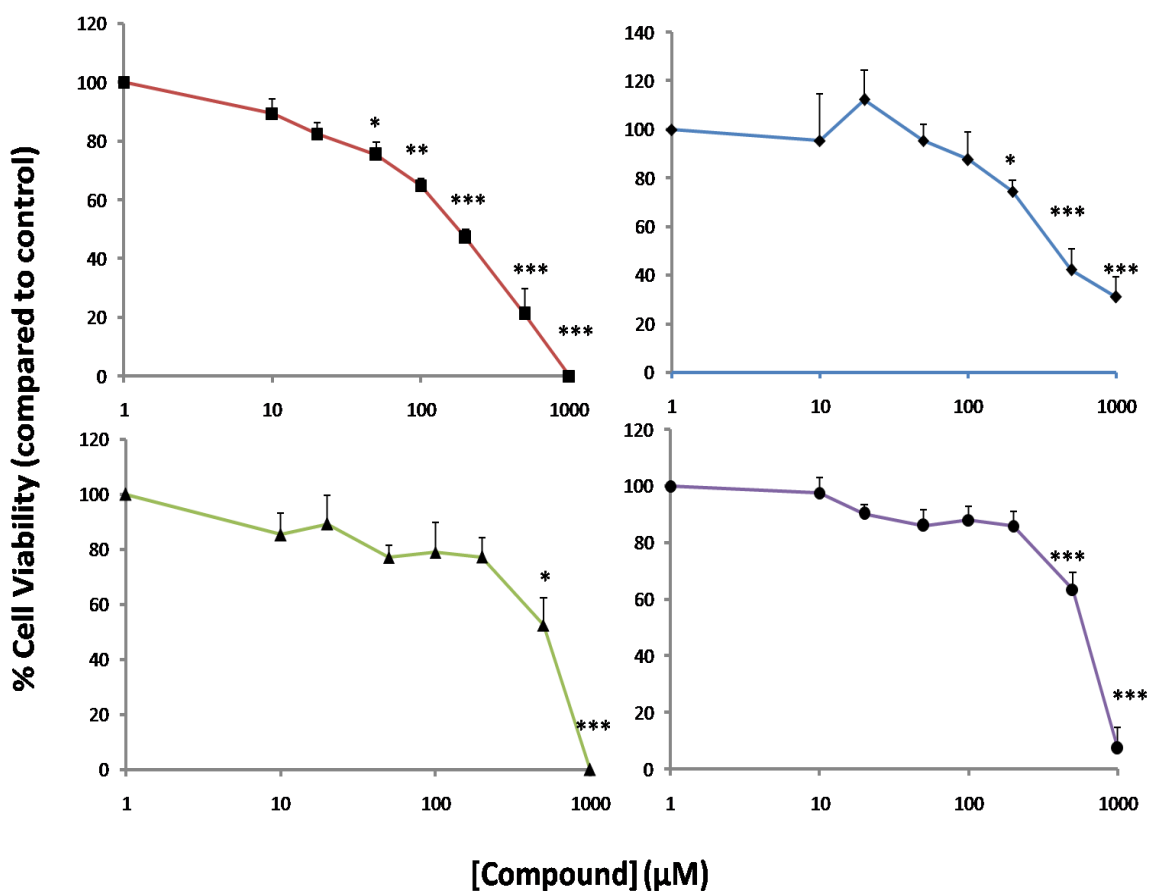
Like 2-PT, TC was only significantly toxic to hepatocytes at  $500\mu\text{M}$  and  $1\text{mM}$  (Fig 3.2C). Preincubation with ABT seemed to attenuate toxicity at lower drug concentrations, but at higher concentrations actually produced a significant decrease in toxicity (Fig. 3.3C), resulting in a reduction in  $IC_{50}$  values from  $352\mu\text{M}$  for TC alone to  $278\mu\text{M}$  for TC plus ABT (Table 3.2). DEM produced no significant fall in cell viability, but did reduce the  $IC_{50}$  value for TC to  $226\mu\text{M}$ .

**Table 3.3.** IC<sub>50</sub> values for MP, 2-PT, TC and TA from incubation with male rat hepatocytes: thiophene alone and in the presence of 1mM ABT or 1mM DEM.

Compound (95% Confidence Interval)	IC <sub>50</sub> Value (μM) †		
	Alone	+ABT	+DEM
MP	152	646*	88
	(111 to 206)	(552 to 755)	(55 to 138)
2-PT	553	478	153*
	(392 to 777)	(347 to 657)	(91 to 255)
TC	352	287*	226
	(226 to 546)	(239 to 345)	(169 to 302)
TA	563	598	316
	(485 to 651)	(458 to 779)	(167 to 594)

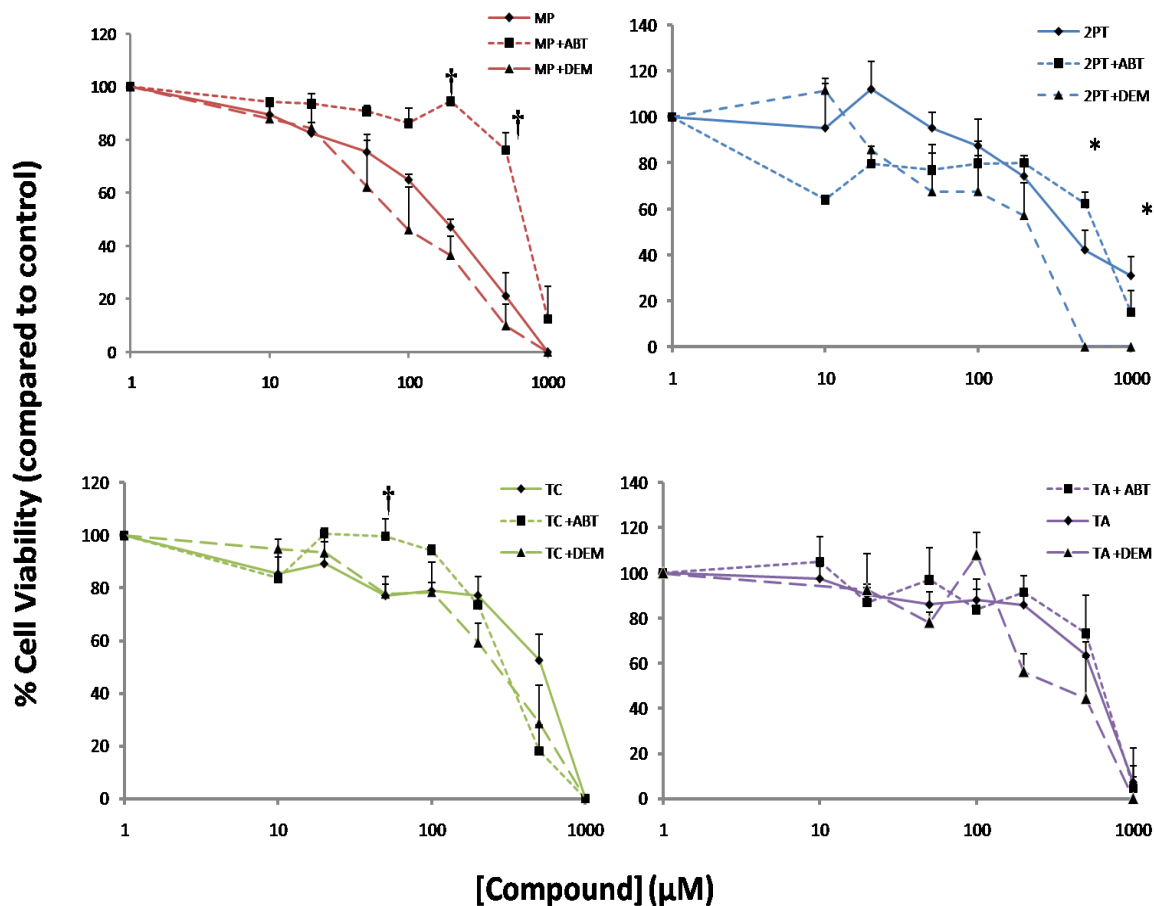
† Concentration of test compound that resulted in a proportion of viable cells half the level found in the vehicle-control incubations. The IC<sub>50</sub> was determined used Graph Pad Prism Software (GraphPad Software, La Jolla, CA, USA.).

\* 95% Confidence interval indicates significant difference in IC<sub>50</sub> value compared to drug alone.



**Figure 3.2.** Cytotoxicity of (A) MP (■); (B) 2-PT (◆); (C) TC (▲) and (D) TA (●) in isolated male rat hepatocytes following a 6h incubation at 37°C in a shaking water bath. Data points indicate mean of 3-8 separate isolations. Error bars represent standard error of the mean. Control viabilities at the end of incubation time were at least 65%.

\* P <0.05, \*\* P<0.005, \*\*\* P<0.0005.



**Figure 3.3.** Graphs showing effects of preincubation with 1mM ABT (non-specific cytochrome P450 inhibitor) and 1mM DEM (glutathione depletor) on toxicity of (A) MP; (B) 2-PT; (C) TC and (D) TA in isolated male rat hepatocytes. Data points indicate mean of 3-8 separate isolations. Error bars represent standard error of the mean. \*P < 0.05 for 1mM ABT preincubation versus drug alone. † P < 0.05 for 1mM DEM preincubation versus drug alone.



### 3.3.2 Formation of Thiophene Glutathione Adducts in Rat Hepatocyte Suspensions

#### Methapyrilene

A full Q1 scan (Fig 3.4A) did not immediately reveal any metabolites of MP following incubation with rat hepatocytes. Extracting the mass chromatogram for  $[M+1]^+$  ( $m/z$  585) of the MP glutathione adduct described in chapter two (MP[O]SG), revealed a small peak at retention time 13.89min (Fig 3.4B). A product ion scan of  $m/z$  585 did not produce any obvious peaks (Fig 3.4C), but evaluation of the spectrum between 15.071 and 17.912min, revealed an MS/MS fragmentation pattern indicative of MP[O]SG:  $m/z$  540  $[585\text{-HN}(\text{CH}_3)_2]^+$ ,  $m/z$  277.8  $[585\text{-GSH}]^+$  (retro-Michael elimination of GSH),  $m/z$  456  $[585\text{-129}]^+$  (neutral loss of pyroglutamate from the GSH residue) and  $m/z$  233  $[540\text{-GSH}]^+$  (Fig 3.4C).

#### 2-Phenylthiophene

Chromatographic analysis at 254nm revealed that 2-PT (1mM) was extensively metabolised in rat hepatocytes suspensions (Fig 3.5A). The three definitely identified metabolites were two GSH adducts of monooxygenated 2PT (2PT[O]SG) ( $[M+1]^+$  at  $m/z$  484), one major (retention time 11.76min) and one minor (retention time 10.4min), and appreciable quantities of the S-oxide dimer ( $m/z$  353, retention time 16.69min) (Fig 3.5B & C). The MS fragmentation pattern of the minor adduct (rt 10.4) suggested a GSH adduct of 2-PT thiophene S-oxide due to the presence of the following fragments:  $m/z$  436  $[484\text{-S=O}]^+$ ,  $m/z$  366  $[484\text{-129}]^+$  (neutral loss of pyroglutamate from the GSH residue),  $m/z$  307  $[436\text{-129}]^+$ ,  $m/z$  177  $[484\text{-GSH}]^+$  (Fig 3.5E). The major adduct was therefore assigned to a GSH adduct of 2-PT

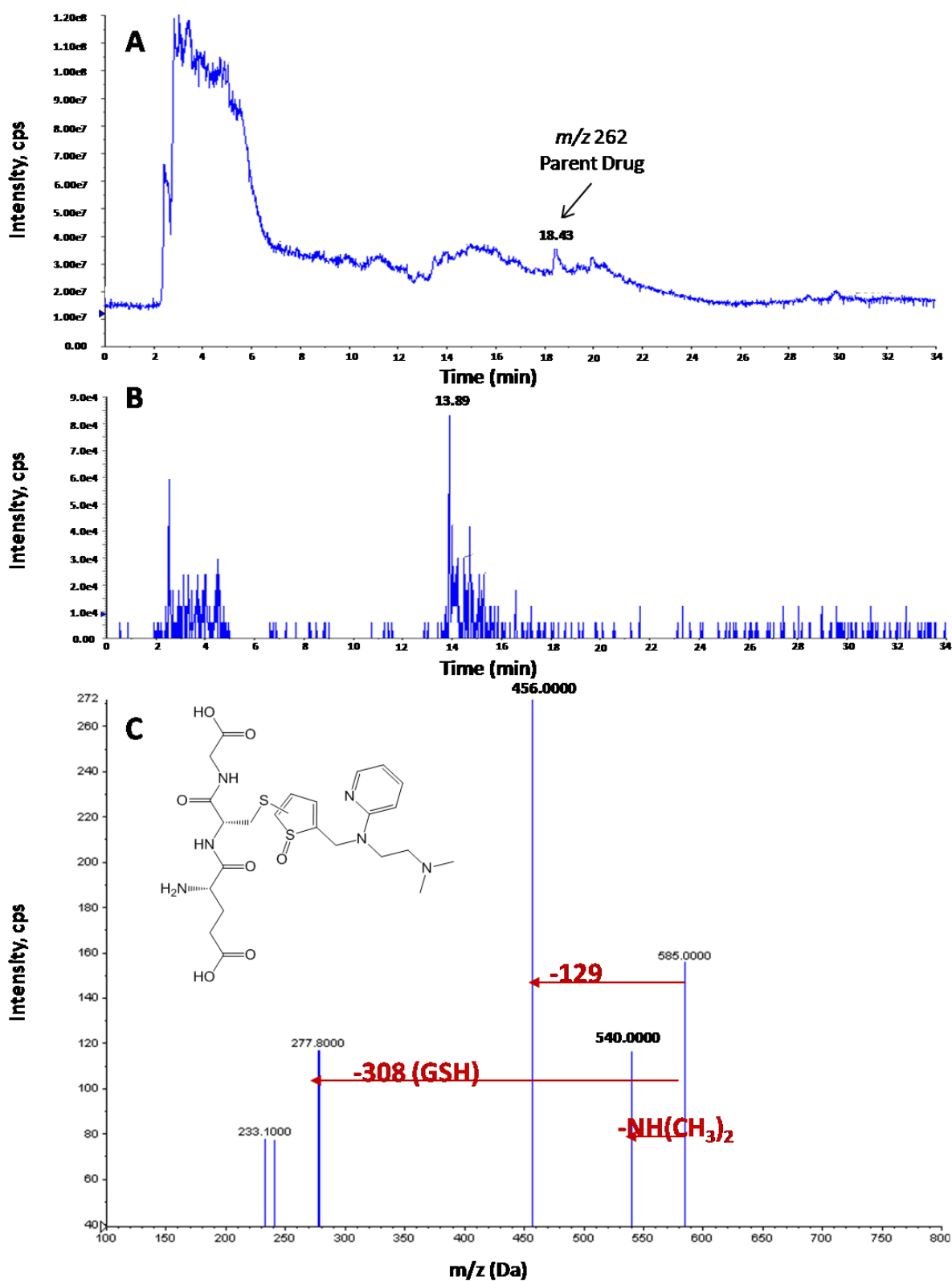
thiophene epoxide, as evidenced by the following fragments:  $m/z$  466  $[484-H_2O]^+$ ,  $m/z$  391  $[466-75]^+$  (loss of glycine from the GSH residue),  $m/z$  391  $[466-129]^+$  (neutral loss of pyroglutamate from the GSH residue),  $m/z$  308  $[GSH]^+$ ,  $m/z$  177  $[484-GSH]^+$  (Fig 3.5D). These assignments were based on those made by (Dansette *et al.*, 2005), which were founded on a combination of data from mass spectrometry and NMR spectroscopy.

### Tienilic Acid

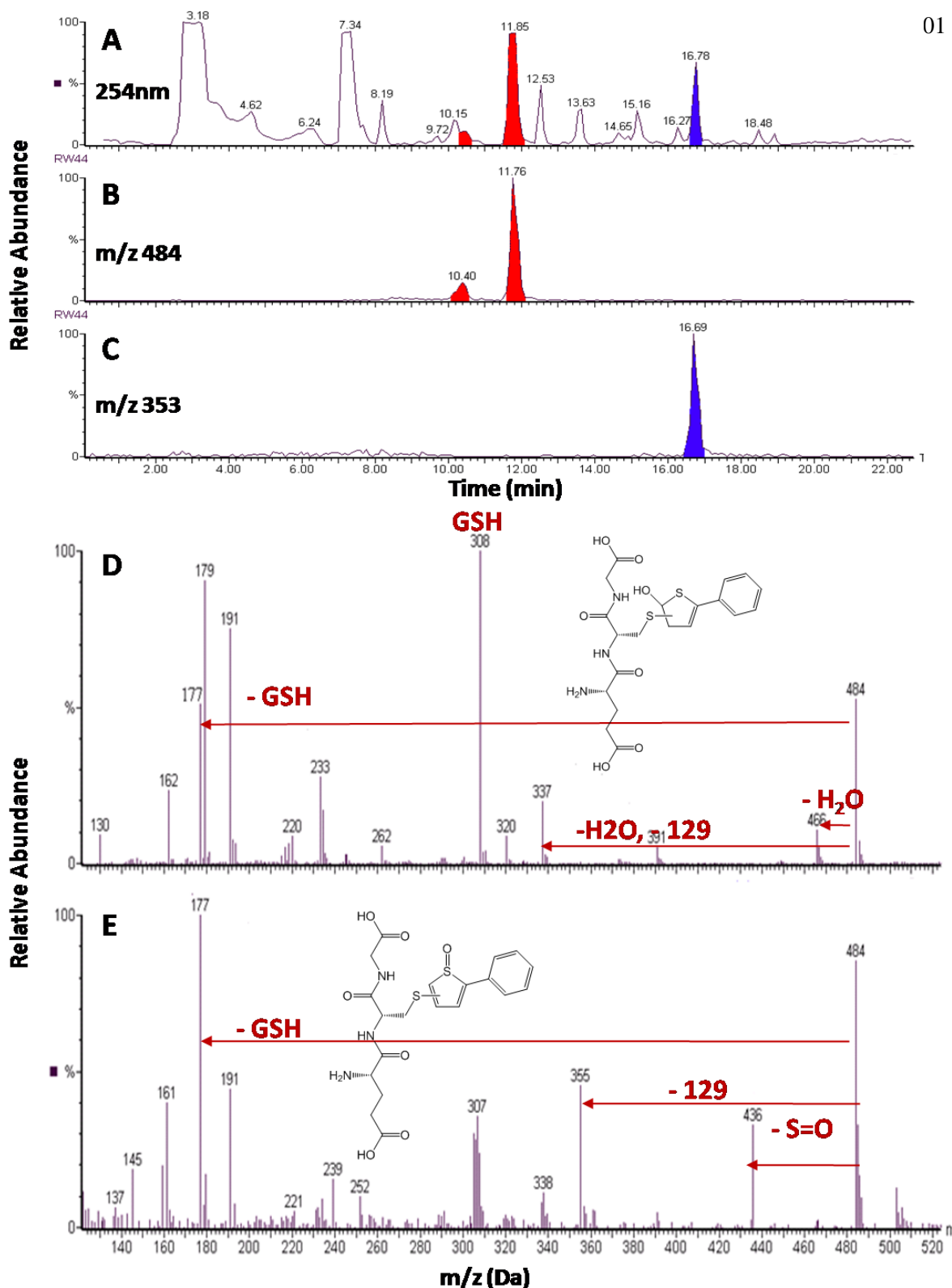
Several peaks were visible in the Q1 scan of an incubation of tienilic acid and rat hepatocytes (Fig 3.6A). The most prominent peak, at retention time 20.41min, was identified as the parent drug ( $[M+1]^+$  at  $m/z$  331). A monooxygenated metabolite ( $m/z$  347) was also found at retention time 21.5min. The MS spectrum of the peak with retention time 18.69min demonstrated an abundant ion at  $m/z$  636. This corresponded to a non-oxygenated glutathione adduct of tienilic acid in rat bile (Nishiya *et al.*, 2008a). A product ion scan of  $m/z$  636 gave a single peak with retention time 19.57min (Fig 3.6B). The MS/MS spectrum of this peak had the following fragments, suggestive of a glutathione adduct (Fig 3.6C);  $m/z$  618.8  $[636-H_2O]^+$ ,  $m/z$  561.0  $[636-72]^+$  (loss of glycine from the GSH residue),  $m/z$  506.9  $[636-129]^+$  (neutral loss of pyroglutamate from the GSH residue),  $m/z$  489.9  $[636-COOH(CH_2)_2CH_2NH_2CONH]^+$ ,  $m/z$  360.9  $[636-COOH(CH_2)_2CH_2NH_2CONHCH_2CH_3CONHCH_2COOH]^+$  and  $m/z$  246.6  $[636-COOHCH_2OC_6H_2(Cl)_2CO]^+$ .

## Ticlopidine

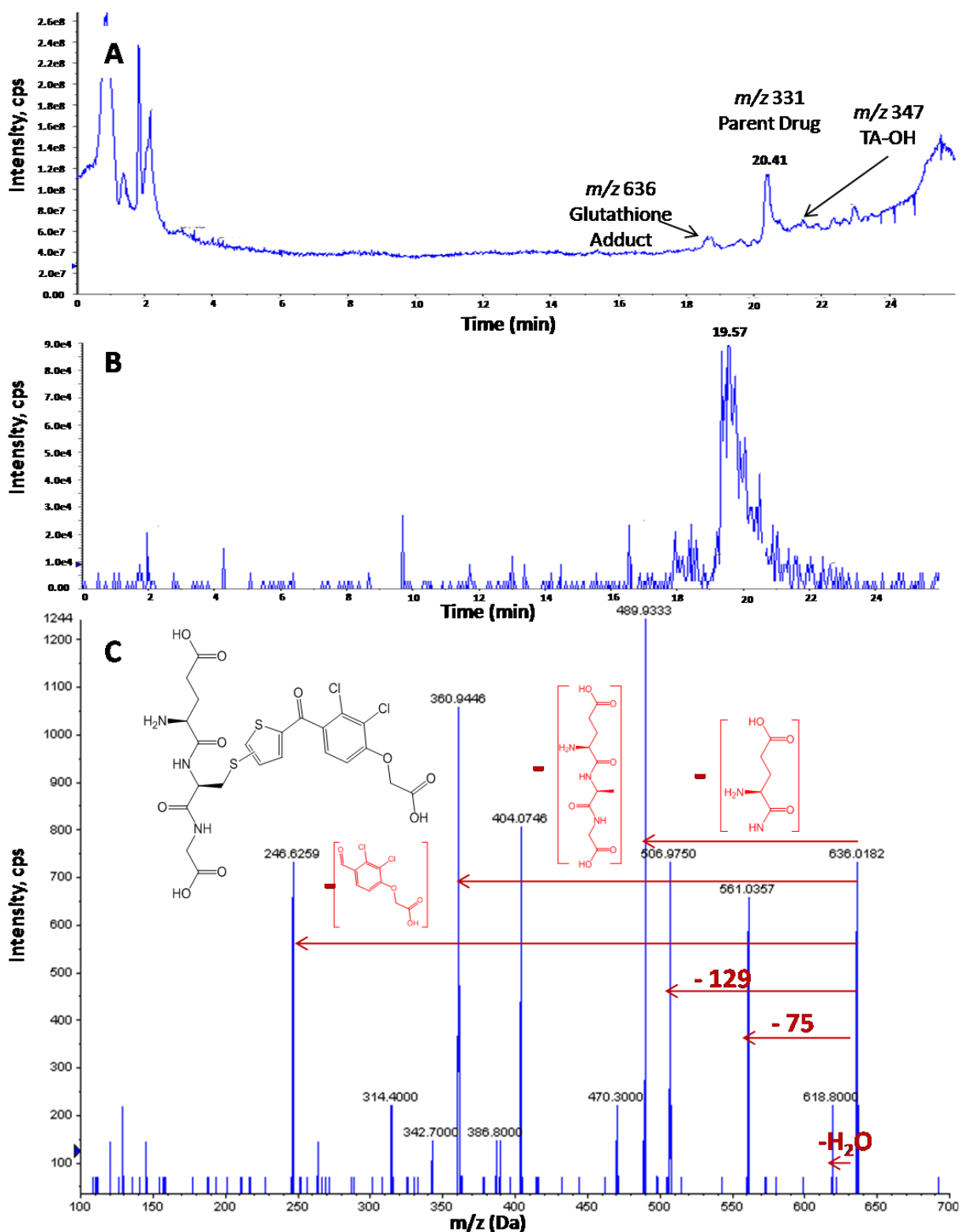
TC was extensively metabolised in the rat hepatocytes suspension (Fig 3.7A). The largest peak in the total ion chromatogram of the Q1 scan, with retention time 30.66min, was assigned to parent drug ( $[M+1]^+$  at  $m/z$  264). Peaks attributable to TC metabolites were found. The peak with retention time 23.19min was assigned to an oxygenated metabolite ( $m/z$  278), but whether this was the lactam reported by Shimizu *et al.* (2009) was not investigated. A peak of  $m/z$  296 was also seen, with a retention time of 21.90min. A thiophene ring-opened thioketone carboxylate metabolite yielding an ion at  $m/z$  296 has been described previously (Shimizu *et al.*, 2009). The two adjacent peaks with retention times 12.8min and 13.77min both displayed  $m/z$  587 in their MS spectra. This  $m/z$  value is equal to that of a glutathione adduct of monooxygenated TC (TC[O]SG). A product ion scan for  $m/z$  587 produced two distinct adjacent peaks with retention times 14.26min and 14.85min (Fig 3.7B). The MS/MS spectrum for the first peak (rt 14.26min) displayed a fragmentation pattern corresponding to a glutathione adduct formed via an S-oxide of TC. This was surmised from the presence of a fragment with  $m/z$  539.2,  $[587-S=O]^+$  (Dansette *et al.*, 2005). Other fragments in the spectrum were indicative of a glutathione adduct:  $m/z$  569.3  $[587-H_2O]^+$ ,  $m/z$  458  $[587-129]^+$  (neutral loss of pyroglutamate from the GSH residue),  $m/z$  409  $[539-S=O]^+$  and  $m/z$  280  $[587-GSH]^+$  (Fig 3.7C). The MS/MS spectrum of the second peak (rt 14.85min) did not display a fragment concordant with loss of sulphoxide, instead only the fragments at  $m/z$  457.8  $[587-129]^+$  and  $m/z$  308  $[GSH]^+$ , were indicative of a glutathione adduct (Fig 3.7D). It is possible that this adduct was formed via an epoxide of the thiophene ring.



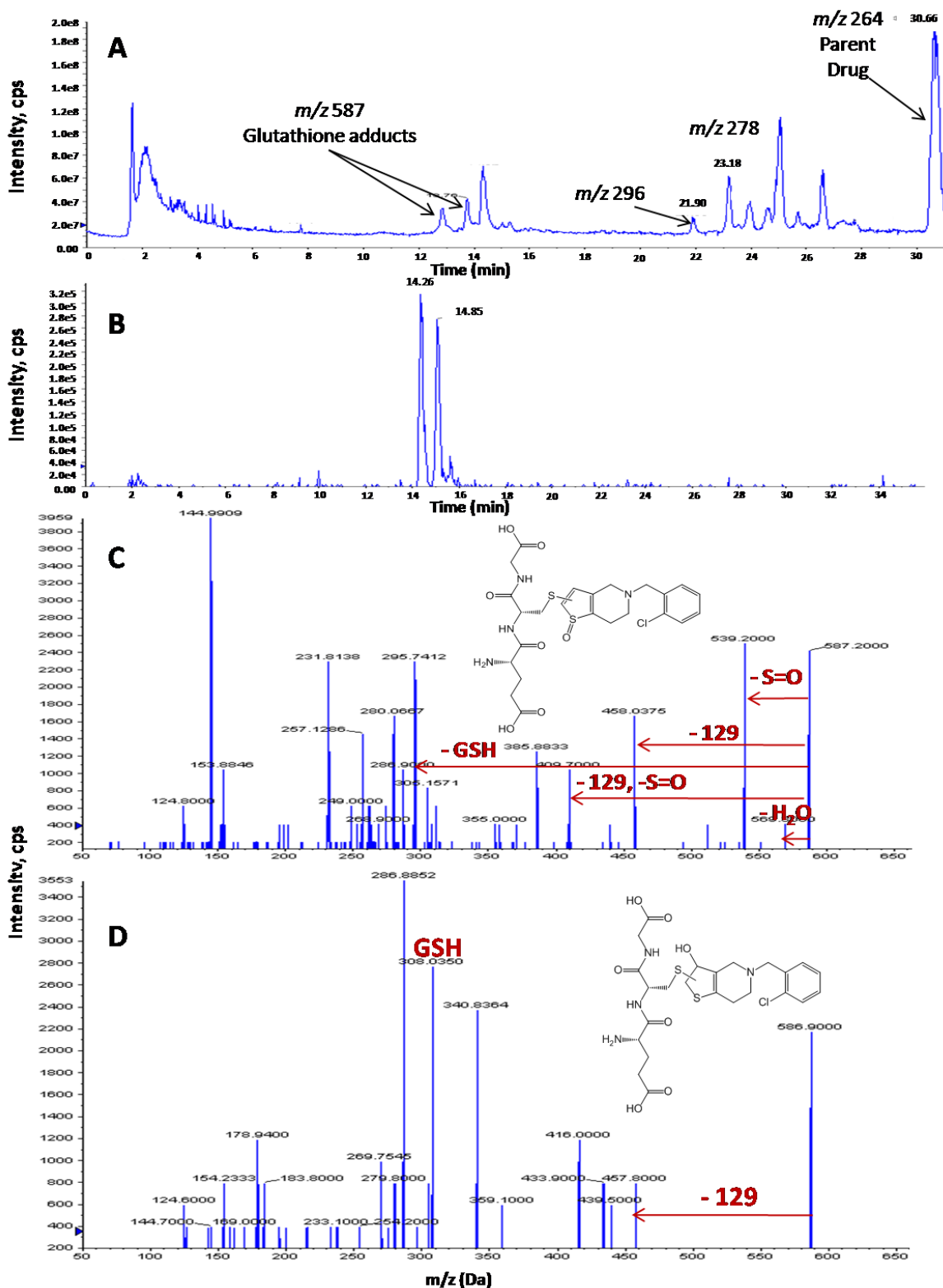
**Figure 3.4.** Mass chromatograms and spectrum from incubations of 100 $\mu$ M MP with freshly isolated rat hepatocytes (6h, 37 $^{\circ}$ C). Figure shows (A) Total ion current chromatogram with parent drug peak at retention time 18.43 min; (B) Extracted ion chromatogram of  $m/z$  585. The peak at 13.89min represents an oxygenated glutathione adduct of MP; (C) Product ion spectrum of  $m/z$  585 showing fragmentation pattern concordant with previously reported S-oxide GSH adduct of MP (Chapter 2).



**Figure 3.5** UV chromatogram and mass chromatograms and spectra from incubations of 1mM 2-PT with freshly isolated rat hepatocytes (6h, 37°C). (A) UV chromatogram showing two GSH adducts highlighted in red and S-oxide dimer highlighted in blue; (B) Extracted ion chromatogram of m/z 484. Peaks at 10.4min and 11.76min represent oxygenated glutathione adducts of 2-PT. (C) Extracted ion chromatogram of m/z 353. Single peak represents 2-PT S-oxide dimer. (D) MS spectrum of peak rt 11.79 exhibiting fragmentation consistent with an hydroxyl GSH adduct of 2-PT formed via an epoxide of the thiophene ring. (E) MS spectrum of peak rt 10.40min exhibiting fragmentation consistent with an S-oxide GSH adduct of 2-PT. Assignments were based on those made by Dansette *et al.* (2005).



**Figure 3.6** Mass chromatograms and spectrum from incubations of 100 $\mu$ M TA with freshly isolated rat hepatocytes (6h, 37°C). Figure shows (A) Total ion current chromatogram annotated to show parent drug, a GSH adduct and a hydroxy TA metabolite (B) Product ion chromatogram of  $m/z$  636. The peak at 19.57min represents a glutathione adduct of TA; (C) Product ion of spectrum of  $m/z$  636. Fragmentation pattern is in agreement with a non-oxygenated GSH adduct of TA.



**Figure 3.7** Mass chromatograms and spectra from incubations of 100µM TC with freshly isolated rat hepatocytes (6h, 37°C). Figure shows (A) Total ion current chromatogram annotated to show parent drug and two oxygenated GSH adducts. (B) Product ion chromatogram of  $m/z$  587. The two major peaks represent glutathione adducts of TC; (C) Product ion spectrum of peak with rt 14.26. Fragmentation pattern is in agreement with a GSH adduct of TC *S*-oxide. (D) Product ion spectrum of peak with rt 14.85. Fragmentation pattern consistent with a GSH adduct of hydroxy TA.

### 3.4 Discussion

The work described in this chapter aimed to address several questions. The first of these concerned the bioactivation of the thiophene moieties in isolated rat hepatocytes. This model has been used successfully in many investigations of the relationships between drug metabolism/bioactivation and hepatotoxicity (Castell *et al.*, 1997; Graham *et al.*, 2008; Lauer *et al.*, 2009; Williams *et al.*, 2007). GSH adducts of all four compounds were detected, suggesting that reactive intermediates were formed from each. Although glutathione conjugates can be converted to mercapturates via an entirely intrahepatic pathway (Hinchman *et al.*, 1994; Srivastava *et al.*, 2010), the relatively low hepatic  $\gamma$ -glutamyltranspeptidase activity in rats will tend to limit degradation of the conjugates by these isolated hepatocytes (Josch *et al.*, 1998), and particularly perhaps at high drug concentrations (Hinchman *et al.*, 1991). In addition, further metabolism by dipeptidase of any cysteinylglycine intermediate produced by  $\gamma$ -glutamyltranspeptidase, a cell surface enzyme, would require uptake into the hepatocytes (Josch *et al.*, 1998); a process that might be compromised in suspensions of isolated cells. Therefore it is probable that GSH adduct recovered from a rat hepatocyte incubation is representative of the total amount formed. Chen *et al.* (2002) noted that raloxifene, a fully substituted benzothiophene, was eliminated in rat bile and urine as a GSH adduct and mercapturate, respectively, but they found only the GSH adduct in rat hepatocytes.

A thiophene *S*-oxide glutathione adduct of MP with a similar fragmentation pattern to the one portrayed in this chapter was produced in rat liver microsomes (Chapter two). The same metabolite has also been found in rat bile (Graham, 2007). Two



oxygenated glutathione adducts of 2-PT are described in this chapter. The more abundant adduct appears to have been formed via an epoxide of the thiophene ring, and the minor adduct via an *S*-oxide. Work by Dansette *et al.* (2005) using rat liver microsomes from  $\beta$ -naphthoflavone induced rats and human CYP1A1 Supersomes has previously detected similar metabolites. This was the first evidence that P450 could catalyse both *S*-oxidation and epoxidation of a single thiophene. In the isolated rat hepatocyte model used here, TC was also metabolised to two glutathione adducts via an epoxide and *S*-oxide. Both adducts have been reported previously in experiments with human liver microsomes and GSH supplementation (Lim *et al.*, 2008). The thiophene *S*-oxide GSH adduct of TC has also been found in rat hepatic S9 fraction and was identified as the main biliary adduct in rats following TC administration (Shimizu *et al.*, 2009). TA produced a non-oxygenated GSH adduct as detected previously in the bile and urine of TA-treated rats (Nishiya *et al.*, 2008a). TA has previously been reported as being oxidised by P450. Investigations using deuterated TA in CYP2C9 Supersomes and rat liver microsomes from clofibrate-treated rats detected a mono-oxygenated GSH TA adduct with  $[M+1]^+$  at  $m/z$  654 (Belghazi *et al.*, 2001). Both formation of an *S*-oxide and epoxide intermediate were postulated as possible routes of GSH adduct formation. The authors also reported finding a GSH adduct similar to that reported in this chapter ( $m/z$  636) and surmised that it had been formed via dehydration and rearomatisation of the mono-oxygenated GSH adduct. This deduction may provide an explanation for the non-oxygenated GSH adduct of TA described here.

The metabolism of all the compounds described in this chapter have been reported previously either *in vivo* and *ex vivo* (MP, TA and MP) or *in vitro* alone (2-PT). The

findings reported here represent the first comprehensive *ex vivo* study of how bioactivation and bioinactivation of thiophene compounds is linked to cytotoxicity. When incubated in the absence of ABT or DEM, MP exhibited the highest cytotoxicity. Both TC and TA displayed  $IC_{50}$  values of over  $500\mu\text{M}$ . Addition of ABT (1 mM) and DEM (1 mM) to incubations produced varied responses. ABT is a mechanism-based inactivator of P450 (Ortiz de Montellano *et al.*, 1981) that is generally regarded as a non-specific inhibitor of P450 isoforms but it nevertheless demonstrates considerable selectivity for inhibition of the major human isoforms (Emoto *et al.*, 2005; Linder *et al.*, 2009). Thus 1 mM ABT effectively destroys CYP2A6 and CYP3A4 in human liver microsomes but eliminates only approximately 40% of CYP2C9 activity (Emoto *et al.*, 2005; Linder *et al.*, 2009). Selective inhibition by ABT of the isoforms in rat liver microsomes has not been reported – CYP4F1 and CYP4F4 are equally susceptible (Xu *et al.*, 2004)– but is a reasonable expectation. There is some evidence of selective inhibition in the livers of rats dosed chronically with ABT (Isayama *et al.*, 2003) but the relevance of these findings to isolated hepatocytes is uncertain. While ABT is invaluable for identifying a P450-mediated contribution to metabolism and hepatocytotoxicity *in vitro* (Graham *et al.*, 2008; Martin *et al.*, 2009; Williams *et al.*, 2007), it cannot be assumed that any remaining enzymatic activity or biological effect after ABT pretreatment is non-P450 mediated. DEM is an avid substrate for glutathione S-transferase in rat liver (Boyland *et al.*, 1967), and 1 mM DEM depletes the GSH in isolated male rat hepatocytes by approximately 75-90% after 30-60 min (Dicker *et al.*, 1986; Larrauri *et al.*, 1987). DEM enhances the cytotoxicity of diverse chemicals that are bioactivated in rat hepatocytes (Jurima-Romet *et al.*, 1991; Nakagawa *et al.*, 2009; Nakagawa *et al.*, 1992). Higher concentrations (2-5 mM) are hepatocytotoxic

(Miccadei *et al.*, 1988; Tirmenstein *et al.*, 2000) but not even 90% depletion of GSH by 1 mM DEM is sufficient in itself to cause ATP depletion (Nakagawa *et al.*, 2009), lipid peroxidation (Tirmenstein *et al.*, 2000) or cell injury as measured either by release of lactate dehydrogenase (Tirmenstein *et al.*, 2000) or trypan blue exclusion (Nakagawa *et al.*, 1992). Therefore it is unlikely the toxicity of MP (Graham *et al.*, 2008) and TA (Takagi *et al.*, 1991) in rat hepatocytes is caused directly by the GSH depletion produced by these drugs.

The most striking difference in toxicity was exhibited in MP incubations, where a significant attenuation of toxicity was observed at 200 $\mu$ M and 500 $\mu$ M following general inhibition of P450 by ABT, suggesting that bioactivation is a vital step in the hepatotoxicity of MP. This was also evident in incubations of MP and DEM. Pre-depletion of GSH significantly reduced the IC<sub>50</sub> of MP from 152 $\mu$ M to 88 $\mu$ M. This implies that the balance between bioactivation and bioinactivation is vital to protection from MP-induced hepatotoxicity. The P450 isoform responsible for bioactivation of MP is not yet known. Enzyme inhibition and induction work proposed a role for CYP2C11 (Ratra *et al.*, 1998), but this has not been corroborated. The hepatotoxic potential of MP in the rat is well established, and the toxicological consequences of metabolic activation of MP have been described previously (Mercer *et al.*, 2009). Chronic (150m/kg/day) dosing of MP leads to zonal GSH depletion in the periportal region, and elevated levels of serum ALT, GLDH, AST and ALP. Downstream functional effects included the induction of heme-oxygenase and glutamate cysteine ligase, both proteins associated with cell defence. The findings reported in this chapter conform with the published work on the metabolism and toxicity of MP in rat models (Graham *et al.*, 2008; Mercer *et al.*, 2009).

Inhibition of P450 by ABT had little effect on the  $IC_{50}$  of 2-PT, but did produce a significant increase in viability at higher concentrations. Pre-depletion of GSH with DEM significantly decreased the  $IC_{50}$  of 2-PT to  $153\mu\text{M}$ . The toxicity of 2-PT has not previously been described, either in animal or human models. If we use 2-PT as a paradigm compound for thiophene drugs, these results imply that oxidation of the thiophene ring to an *S*-oxide or epoxide have toxicological consequences. Whilst both metabolites are described in rat hepatocytes in this chapter and previously in rat liver microsomes (Dansette *et al.*, 2005), metabolism by human CYP1A1 Supersomes altered the ratio of adduct formation in favour of the *S*-oxide GSH adduct (Dansette *et al.*, 2005), suggesting a role for CYP1A1 in thiophene *S*-oxide formation. The results from incubations with DEM highlight the crucial role of GSH in detoxification and cell protection.

The hepatotoxicity of TC has not been investigated extensively; the focus tends to lie on the potential for TC to cause agranulocytosis and thrombocytopenia (Liu *et al.*, 2000; Nishiya *et al.*, 2009), or the ability to inhibit CYP2C19 by mechanism-based inhibition (Nishiya *et al.*, 2009). The findings in this chapter therefore present the first investigation into TC hepatotoxicity in a rat model. Concentration-dependant toxicity was observed with a  $IC_{50}$  of  $352\mu\text{M}$ . The presence of ABT actually aggravated the toxicity, suggesting that either parent drug or a metabolite not produced by an inhibited enzyme may be responsible for toxicity. For example, if metabolism to lactam or hydroxyl metabolites is favoured under normal conditions, and addition of 1mM ABT selectively inhibits this route of metabolism, then oxidation to *S*-oxide or epoxide may instead become favoured, producing more reactive metabolite. Depletion of GSH with DEM also caused a greater decrease in

cell viability compared to TC alone. These results suggest that in these conditions, TC is successfully detoxified by GSH conjugation, therefore inhibition of P450 does not attenuate toxicity, whereas depletion of GSH enhances toxicity by removing a route of detoxification. This proposition is supported by the presence of two GSH adducts observed in incubations of rat hepatocytes with TC alone as described in this chapter.

The results of incubations of TA presented here suggest it was the least hepatocytotoxic compound tested, with the highest  $IC_{50}$ . Inhibition of P450 by preincubation with ABT had little effect on the toxicity, perhaps because the particular isoenzyme responsible for bioactivation was not inhibited, or because all reactive intermediate formed in incubations of TA alone is successfully detoxified under incubation conditions. This explanation would satisfy the resultant enhancement of TA toxicity observed in incubations containing DEM. Notably, Acosta et al. (1982) reported that TA at concentrations between 1  $\mu$ M and 1 mM was “minimally toxic” in hepatocytes isolated from neonatal rats; in which basal hepatic P450 activities are lower than those in adult rats (Acosta *et al.*, 1982; Lupp *et al.*, 2008). Pre-depletion of GSH caused an increase in toxicity compared to TA alone, as exhibited by the reduction in  $IC_{50}$  from 563 $\mu$ M to 316 $\mu$ M. The data suggests a similar picture to that observed in TC incubations in that inhibition of P450 does not attenuate the toxicity, but depletion of GSH causes a greater decrease in cell viability than drug alone. This indicates a functioning route of detoxification via GSH conjugation, which is disrupted by predepletion of GSH, leaving the cell vulnerable to damage by reactive intermediates. The toxicity of TA has been investigated previously in isolated rat hepatocyte suspensions using LDH leakage as an endpoint (Higaki *et al.*, 1989). TA (3mM) was incubated with hepatocytes and found to

reduce cell viability to 0% after 60min incubation time. When compared to three other diuretics not containing the thiophene moiety, it was found to be the most toxic in the model. TA hepatotoxicity has also been assessed in vivo (Nishiya *et al.*, 2008b). TA was administered to Sprague-Dawley rats both alone and in combination with the glutamate cysteine ligase inhibitor, buthionine sulphoximine (BSO). When administered alone, the effects of TA were limited to gene expression changes and variations in bilirubin and bile acid concentrations observed at the highest dose of TA (1g/kg) from 3h to 24h following dose. In combination with BSO, striking elevations in serum alanine aminotransferase and alkaline phosphatase were observed, as well as centrilobular necrosis. This in vivo data correlates well with the ex vivo data presented here: high concentrations of TA are required to produce evident hepatotoxicity, however depletion of GSH markedly increases the toxicity by removing the ability for deactivation/detoxification of the reactive intermediate.

The findings described in this chapter suggest that the combination of isolated rat hepatocytes and LC-MS is an excellent methodology for assessing the formation of GSH adducts from thiophene-containing compounds. Gan *et al.* (2009) have shown using human liver microsomes that there appears to be a general trend between the extent of thiol adduct formation from drugs and the potential for drug-induced toxicity when dose and total daily reactive metabolite burdens are considered. The rat hepatocyte model could be improved in terms of elucidating hepatotoxic potential by assessing earlier end points of cytotoxicity other than loss of membrane integrity as is indicated by the trypan blue exclusion assay. However, the cell membrane of a rat hepatocyte can be rendered 93-100% permeable to trypan blue by toxin treatment

which does not produce gross damage to the cell surface and causes only low ( $\leq 22\%$ ) leakage of lactate dehydrogenase and minimal disruption of intracellular morphology (McEwen *et al.*, 1985). Whilst this model proves useful as an animal *ex vivo* model for assessing bioactivation and hepatotoxic potential, prediction of drug-induced liver injury in the patient population is still incredibly difficult (Obach *et al.*, 2008; Kalgutkar *et al.*, 2009). Parallel investigations in different animal and human systems should be used to complement each other and produce a broader picture of hepatotoxic potential. The following chapter looks at the consequences of thiophene bioactivation by P450 isoenzymes for toxicity in a human liver endothelial cell line.

## References

- Acosta, D, Mitchell, DB, Santone, KS, Bock, A, Lewis, W (1982) Lack of cytotoxicity of ticrynafen in primary cultures of rat liver cells. *Toxicol Lett* **10**(4): 385-388.
- Anderson, K, Andrews, R, Yin, L, McLeod, R, MacDonald, C, Hayes, JD, Grant, MH (1998) Cytotoxicity of xenobiotics and expression of glutathione-S-transferases in immortalised rat hepatocyte cell lines. *Hum Exp Toxicol* **17**(3): 131-137.
- Belghazi, M, Jean, P, Poli, S, Schmitter, JM, Mansuy, D, Dansette, PM (2001) Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv Exp Med Biol* **500**: 139-144.
- Boots, AW, Balk, JM, Bast, A, Haenen, GR (2005) The reversibility of the glutathionyl-queracetin adduct spreads oxidized queracetin-induced toxicity. *Biochem Biophys Res Commun* **338**(2): 923-929.
- Boyland, E, Chasseaud, LF (1967) Enzyme-catalysed conjugations of glutathione with unsaturated compounds. *Biochem J* **104**(1): 95-102.
- Callan, HE, Jenkins, RE, Maggs, JL, Lavergne, SN, Clarke, SE, Naisbitt, DJ, Park, BK (2009) Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. *Chem Res Toxicol* **22**(5): 937-948.
- Castell, JV, Gomez-Lechon, MJ, Ponsoda, X, Bort, R (1997) The use of cultured hepatocytes to investigate the mechanisms of drug hepatotoxicity. *Cell Biol Toxicol* **13**(4-5): 331-338.
- Dansette, PM, Amar, C, Smith, C, Pons, C, Mansuy, D (1990) Oxidative activation of the thiophene ring by hepatic enzymes. Hydroxylation and formation of electrophilic metabolites during metabolism of tienilic acid and its isomer by rat liver microsomes. *Biochem Pharmacol* **39**(5): 911-918.
- Dansette, PM, Amar, C, Valadon, P, Pons, C, Beaune, PH, Mansuy, D (1991) Hydroxylation and formation of electrophilic metabolites of tienilic acid and its isomer by human liver microsomes. Catalysis by a cytochrome P450 IIC different from that responsible for mephenytoin hydroxylation. *Biochem Pharmacol* **41**(4): 553-560.
- Dansette, PM, Bertho, G, Mansuy, D (2005) First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives. *Biochem Biophys Res Commun* **338**(1): 450-455.
- Dansette, PM, Thang, DC, el Amri, H, Mansuy, D (1992) Evidence for thiophene-S-oxide as a primary reactive metabolite of thiophene in vivo: formation of a dihydrothiophene sulfoxide mercapturic acid. *Biochem Biophys Res Commun* **186**(3): 1624-1630.
- Dicker, E, Cederbaum, AI (1986) Inhibition of the low-Km mitochondrial aldehyde dehydrogenase by diethyl maleate and phorone in vivo and in vitro. Implications for formaldehyde metabolism. *Biochem J* **240**(3): 821-827.
- Emoto, C, Murase, S, Sawada, Y, Iwasaki, K (2005) In vitro inhibitory effect of 1-aminobenzotriazole on drug oxidations in human liver microsomes: a comparison with SKF-525A. *Drug Metab Pharmacokinet* **20**(5): 351-357.



Eugene, C, Patriarche, C, Bergue, A, Quevauvilliers, J (1980) [Tienilic acid as a likely cause of "auto immune" active hepatitis (author's transl)]. *Nouv Presse Med* **9**(27): 1885-1887.

Farid, NA, Kurihara, A, Wrighton, SA (2010) Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. *J Clin Pharmacol* **50**(2): 126-142.

Gan, J, Ruan, Q, He, B, Zhu, M, Shyu, WC, Humphreys, WG (2009) In vitro screening of 50 highly prescribed drugs for thiol adduct formation--comparison of potential for drug-induced toxicity and extent of adduct formation. *Chem Res Toxicol* **22**(4): 690-698.

Graham, E PhD Thesis, University of Liverpool, Liverpool, 2007.

Graham, EE, Walsh, RJ, Hirst, CM, Maggs, JL, Martin, S, Wild, MJ, Wilson, ID, Harding, JR, Kenna, JG, Peter, RM, Williams, DP, Park, BK (2008) Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* **326**(2): 657-671.

Grieco, A, Vecchio, FM, Greco, AV, Gasbarrini, G (1998) Cholestatic hepatitis due to ticlopidine: clinical and histological recovery after drug withdrawal. Case report and review of the literature. *Eur J Gastroenterol Hepatol* **10**(8): 713-715.

Guengerich, FP, Arneson, KO, Williams, KM, Deng, Z, Harris, TM (2002) Reaction of aflatoxin B(1) oxidation products with lysine. *Chem Res Toxicol* **15**(6): 780-792.

Higaki, J, Harada, H, Tonda, K, Hirata, M (1989) Chemical structure and toxicity of diuretics in isolated hepatocytes. *Pharmacol Toxicol* **65**(1): 21-24.

Hinchman, CA, Ballatori, N (1994) Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J Toxicol Environ Health* **41**(4): 387-409.

Hinchman, CA, Matsumoto, H, Simmons, TW, Ballatori, N (1991) Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem* **266**(33): 22179-22185.

Isayama, F, Froh, M, Bradford, BU, McKim, SE, Kadiiska, MB, Connor, HD, Mason, RP, Koop, DR, Wheeler, MD, Arteel, GE (2003) The CYP inhibitor 1-aminobenzotriazole does not prevent oxidative stress associated with alcohol-induced liver injury in rats and mice. *Free Radic Biol Med* **35**(12): 1568-1581.

Johnson, KA, Plumb, R (2005) Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *J Pharm Biomed Anal* **39**(3-4): 805-810.

Josch, C, Sies, H, Akerboom, TP (1998) Hepatic mercapturic acid formation: involvement of cytosolic cysteinylglycine S-conjugate dipeptidase activity. *Biochem Pharmacol* **56**(6): 763-771.

Jurima-Romet, M, Huang, HS, Paul, CJ, Thomas, BH (1991) Enalapril cytotoxicity in primary cultures of rat hepatocytes. II. Role of glutathione. *Toxicol Lett* **58**(3): 269-277.

Kalgutkar, AS, Didiuk, MT (2009) Structural alerts, reactive metabolites, and protein covalent binding: how reliable are these attributes as predictors of drug toxicity? *Chem Biodivers* **6**(11): 2115-2137.

Kalgutkar, AS, Gardner, I, Obach, RS, Shaffer, CL, Callegari, E, Henne, KR, Mutlib, AE, Dalvie, DK, Lee, JS, Nakai, Y, O'Donnell, JP, Boer, J, Harriman, SP (2005) A comprehensive listing of bioactivation pathways of organic functional groups. *Curr Drug Metab* **6**(3): 161-225.

Kensler, TW, Wakabayashi, N, Biswal, S (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* **47**: 89-116.

Larrauri, A, Lopez, P, Gomez-Lechon, MJ, Castell, JV (1987) A cytochemical stain for glutathione in rat hepatocytes cultured on plastic. *J Histochem Cytochem* **35**(2): 271-274.

Lauer, B, Tuschl, G, Kling, M, Mueller, SO (2009) Species-specific toxicity of diclofenac and troglitazone in primary human and rat hepatocytes. *Chem Biol Interact* **179**(1): 17-24.

Lijinsky, W, Reuber, MD, Blackwell, BN (1980) Liver tumors induced in rats by oral administration of the antihistaminic methapyrilene hydrochloride. *Science* **209**(4458): 817-819.

Lim, HK, Chen, J, Cook, K, Sensenhauser, C, Silva, J, Evans, DC (2008) A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom* **22**(8): 1295-1311.

Linder, CD, Renaud, NA, Hutzler, JM (2009) Is 1-aminobenzotriazole an appropriate in vitro tool as a nonspecific cytochrome P450 inactivator? *Drug Metab Dispos* **37**(1): 10-13.

Liu, DQ, Hop, CE, Beconi, MG, Mao, A, Chiu, SH (2001) Use of on-line hydrogen/deuterium exchange to facilitate metabolite identification. *Rapid Commun Mass Spectrom* **15**(19): 1832-1839.

Liu, ZC, Uetrecht, JP (2000) Metabolism of ticlopidine by activated neutrophils: implications for ticlopidine-induced agranulocytosis. *Drug Metab Dispos* **28**(7): 726-730.

Lopez-Garcia, MP, Dansette, PM, Coloma, J (2005) Kinetics of tienilic acid bioactivation and functional generation of drug-protein adducts in intact rat hepatocytes. *Biochem Pharmacol* **70**(12): 1870-1882.

Lu, SC (2009) Regulation of glutathione synthesis. *Mol Aspects Med* **30**(1-2): 42-59.

Lupp, A, Glockner, R, Etzrodt, J, Muller, D (2008) Precision-cut liver slices from rats of different ages: basal cytochrome P450-dependent monooxygenase activities and inducibility. *Anal Bioanal Chem* **392**(6): 1173-1184.

Martin, JW, Chan, K, Mabury, SA, O'Brien, PJ (2009) Bioactivation of fluorotelomer alcohols in isolated rat hepatocytes. *Chem Biol Interact* **177**(3): 196-203.

McEwen, BF, Arion, WJ (1985) Permeabilization of rat hepatocytes with *Staphylococcus aureus* alpha-toxin. *J Cell Biol* **100**(6): 1922-1929.

Mercer, AE, Regan, SL, Hirst, CM, Graham, EE, Antoine, DJ, Benson, CA, Williams, DP, Foster, J, Kenna, JG, Park, BK (2009) Functional and toxicological consequences of metabolic bioactivation of methapyrilene via thiophene S-oxidation: Induction of cell defence, apoptosis and hepatic necrosis. *Toxicol Appl Pharmacol* **239**(3): 297-305.

Miccadei, S, Kyle, ME, Gilfor, D, Farber, JL (1988) Toxic consequence of the abrupt depletion of glutathione in cultured rat hepatocytes. *Arch Biochem Biophys* **265**(2): 311-320.

Nakagawa, Y, Suzuki, T, Nakajima, K, Ishii, H, Ogata, A (2009) Biotransformation and cytotoxic effects of hydroxychavicol, an intermediate of safrole metabolism, in isolated rat hepatocytes. *Chem Biol Interact* **180**(1): 89-97.

Nakagawa, Y, Tayama, S, Moore, GA, Moldeus, P (1992) Relationship between metabolism and cytotoxicity of ortho-phenylphenol in isolated rat hepatocytes. *Biochem Pharmacol* **43**(7): 1431-1437.

Nishiya, T, Kato, M, Suzuki, T, Maru, C, Kataoka, H, Hattori, C, Mori, K, Jindo, T, Tanaka, Y, Manabe, S (2008a) Involvement of cytochrome P450-mediated metabolism in tienilic acid hepatotoxicity in rats. *Toxicol Lett* **183**(1-3): 81-89.

Nishiya, T, Mori, K, Hattori, C, Kai, K, Kataoka, H, Masubuchi, N, Jindo, T, Manabe, S (2008b) The crucial protective role of glutathione against tienilic acid hepatotoxicity in rats. *Toxicol Appl Pharmacol* **232**(2): 280-291.

Nishiya, Y, Hagihara, K, Kurihara, A, Okudaira, N, Farid, NA, Okazaki, O, Ikeda, T (2009) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**(11): 836-843.

O'Brien, PJ, Siraki, AG, Tafazoli, S, K.Chan (2004) *Liver Diseases*.

O'Donnell, JP, Dalvie, DK, Kalgutkar, AS, Obach, RS (2003) Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31**(11): 1369-1377.

Obach, RS, Kalgutkar, AS, Soglia, JR, Zhao, SX (2008) Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem Res Toxicol* **21**(9): 1814-1822.

Ortiz de Montellano, PR, Mathews, JM (1981) Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. Isolation of an NN-bridged benzyne-protoporphyrin IX adduct. *Biochem J* **195**(3): 761-764.

Park, BK, Kitteringham, NR, Maggs, JL, Pirmohamed, M, Williams, DP (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* **45**: 177-202.

Randle, LE, Goldring, CE, Benson, CA, Metcalfe, PN, Kitteringham, NR, Park, BK, Williams, DP (2008) Investigation of the effect of a panel of model hepatotoxins on the Nrf2-Keap1 defence response pathway in CD-1 mice. *Toxicology* **243**(3): 249-260.

Ratra, GS, Cottrell, S, Powell, CJ (1998) Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* **46**(1): 185-196.

Shimizu, S, Atsumi, R, Nakazawa, T, Fujimaki, Y, Sudo, K, Okazaki, O (2009) Metabolism of ticlopidine in rats: identification of the main biliary metabolite as a glutathione conjugate of ticlopidine S-oxide. *Drug Metab Dispos* **37**(9): 1904-1915.

Srivastava, A, Maggs, JL, Antoine, DJ, Williams, DP, Smith, DA, Park, BK (2010) Role of reactive metabolites in drug-induced hepatotoxicity. *Handb Exp Pharmacol*(196): 165-194.

Takagi, S, Takayama, S, Onodera, T (1991) Hepatotoxicity of DR-3438, tienilic acid, indacrinone and furosemide studied in vitro. *Toxicol Lett* **55**(3): 287-293.

Takakusa, H, Masumoto, H, Mitsuru, A, Okazaki, O, Sudo, K (2008) Markers of electrophilic stress caused by chemically reactive metabolites in human hepatocytes. *Drug Metab Dispos* **36**(5): 816-823.

Tirmenstein, MA, Nicholls-Grzemeski, FA, Zhang, JG, Fariss, MW (2000) Glutathione depletion and the production of reactive oxygen species in isolated hepatocyte suspensions. *Chem Biol Interact* **127**(3): 201-217.

Watkins, PB, Dube, LM, Walton-Bowen, K, Cameron, CM, Kasten, LE (2007) Clinical pattern of zileuton-associated liver injury: results of a 12-month study in patients with chronic asthma. *Drug Saf* **30**(9): 805-815.

Williams, DP, Antoine, DJ, Butler, PJ, Jones, R, Randle, L, Payne, A, Howard, M, Gardner, I, Blagg, J, Park, BK (2007) The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: a chemical and biochemical definition of the toxicophore. *J Pharmacol Exp Ther* **322**(3): 1208-1220.

Xu, F, Falck, JR, Ortiz de Montellano, PR, Kroetz, DL (2004) Catalytic activity and isoform-specific inhibition of rat cytochrome p450 4F enzymes. *J Pharmacol Exp Ther* **308**(3): 887-895.

Zimmerman, HJ, Lewis, JH, Ishak, KG, Maddrey, WC (1984) Ticrynafen-associated hepatic injury: analysis of 340 cases. *Hepatology* **4**(2): 315-323.



## CHAPTER FOUR

---

AN INVESTIGATION OF  
BIOACTIVATION AND TOXICITY OF  
THIOPHENES IN THLE-CYP CELL LINES

The work described in this chapter was carried out in collaboration with Molecular Toxicology, Alderley Park, Astra Zeneca, UK.

## Contents

4.1 Introduction.....	120
4.2 Materials and Methods.....	123
4.2.1 Materials.....	123
4.2.2 THLE-CYP Cell Culture.....	123
4.2.3 THLE-CYP Cell Incubation Regime.....	124
4.2.4 Assessment of Toxicity of Thiophenes in THLE-CYP Cell Lines .....	124
4.2.5 Preparation of Samples for LC-MS nalysis.....	125
4.2.6 Analysis of Thiophene Metabolism in THLE-CYP Cell Lines.....	125
4.3 Results.....	127
4.3.1 Toxicity of Thiophene Compounds in THLE-CYP Cell Lines.....	127
4.3.2 Bioactivation of Thiophene Compounds in THLE-CYP Cell Lines.....	127
4.4 Discussion.....	135

## Abbreviations

2-PT, 2-phenylthiophene; ACN, acetonitrile; P450, cytochrome P450 mixed function oxidase; DILI, drug-induced liver injury; GSH, glutathione; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; MeOH, methanol; MP, methapyrilene; MS, mass spectrometry; rt, retention time; TA, tienilic acid; THLE-CYP, SV40 large tumour antigen-immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes.

#### 4.1 Introduction

The previous chapter described a rat hepatocyte model for assessment of thiophene bioactivation and toxicity which could potentially be used in preclinical investigations of drug-induced liver injury (DILI). However, the complexity of DILI calls for a battery of complimentary assays and models in order to assess the many different aspects involved, such as mitochondrial dysfunction (Labbe *et al.*, 2008), drug metabolism (Park *et al.*, 2005), P450 inhibition (Fowler *et al.*, 2008), cell defence systems (Coppie *et al.*, 2010) and involvement of the immune system (Lavergne *et al.*, 2008). The model described in the previous chapter and many other cell-based models use short-term cytotoxicity as a predictor for hepatotoxic potential (Dambach *et al.*, 2005). An approach that exploits this indicator uses cultured cells, whether immortalised cell lines or primary cultures from isolated hepatocytes. The use of human cells has obvious benefits over animal cell models as they are potentially more reflective of patients (Gomez-Lechon *et al.*, 2007). Whereas immortalised cell lines lose their metabolic capacity to a greater or lesser extent, primary cultured hepatocytes maintain many physiological functions when cultured under appropriate conditions, including expression of drug metabolising enzymes (Hewitt *et al.*, 2007).

In order to overcome the loss of metabolic enzyme expression in immortalised cell lines and in order to develop a viable alternative to isolated hepatocytes several cell lines have been transfected with cDNA encoding drug metabolising enzymes. Cell lines used have included human HepG2 cells (Goldring *et al.*, 2006), murine hepatoma cells (Puga *et al.*, 1990) and Chinese hamster V97 lung cells (Doehmer, 1993). However, the P450 expression in these cells is often transient and of low level when compared to hepatocytes. Recently, a human liver epithelial cell line (THLE-5b), has been stably transfected with cDNA of P450s using simian virus 40 and a cytomegalovirus promoter. Whilst these cells have a hepatocyte-like phenotype, expressing high levels of Phase 2 metabolising enzymes they do not express high levels of P450. When transfected as described above, the THLE-CYP cell lines express



comparable levels of P450 to human hepatocytes (Mace *et al.*, 1997). By engineering several cell lines expressing single human P450s, there is scope for investigating the role of discrete P450 enzymes in the metabolism and cytotoxicity of drugs.

Investigations have found that 90% of all drugs are metabolised by human P450 enzymes (Watkins, 1990). Six isoforms are responsible for 95% of this metabolism (CYP3A4, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP1A1/2) with CYP3A4 being responsible for 65% of this total (Zimmerman, 1999). In terms of their involvement in DILI, several P450's have been linked to formation of reactive metabolites. For example, CYP2E1 has been implicated in the formation of N-acetyl benzoquinone imine from paracetamol (Lee *et al.*, 1996), along with CYP3A4 and CYP1A2 (Laine *et al.*, 2009).

Investigations into the role of specific P450 enzymes in the bioactivation of thiophene drugs and non-drug xenobiotics have shown the involvement of several isoforms. Monooxygenated GSH adducts of 2-phenylthiophene (2-PT; Table 3.1) formed via both a thiophene *S*-oxide and thiophene epoxide have been observed in incubations of 2-PT with CYP1A1 Supersomes (Dansette *et al.*, 2005). Significant toxicity was only observed in freshly isolated rat hepatocytes at higher concentrations (500 $\mu$ M and 1mM) of 2-PT (chapter 3).

Tienilic acid (TA) has been shown to be metabolized by recombinant CYP2C9 to a thiophene *S*-oxide (Belghazi *et al.*, 2001), and CYP2C9 has been shown to be inactivated by reactive metabolites of TA (Lopez-Garcia *et al.*, 2005; Lopez-Garcia *et al.*, 1994). In many patients suffering from TA-induced hepatitis, anti-LKM<sub>2</sub> antibodies directed against CYP2C9 were observed (Beaune *et al.*, 1987; Homberg *et al.*, 1984); which suggested a role for CYP2C9 in the hepatotoxicity associated with TA. Suprofen, a thiophene-containing anti-inflammatory drug associated with nephrotoxicity, has also been shown to be a suicide inhibitor of CYP2C9, due to the formation of a thiophene epoxide intermediate (O'Donnell

*et al.*, 2003). TA was the least toxic thiophene examined in isolated rat hepatocytes. Significant toxicity was observed at 500 $\mu$ M and 1mM (chapter 3).

Suicide inhibition of P450 enzymes has also been observed with ticlopidine, clopidogrel and prasugrel, thiophene-containing P<sub>Y212</sub> antagonists. Ticlopidine and clopidogrel are mechanism-based inhibitors of CYP2C19, whereas their thiolactone metabolites inhibit CYP2C19 only in a concentration-dependent manner (Nishiya *et al.*, 2009b). CYP2B6 was inactivated by ticlopidine and clopidogrel and the thiolactone metabolites of ticlopidine, clopidogrel and prasugrel (Nishiya *et al.*, 2009a). A thiophene *S*-oxide dimer metabolite of ticlopidine has also been observed in incubations with CYP2C19 (Ha-Duong *et al.*, 2001), indicating that this enzyme is responsible for bioactivation of ticlopidine.

The P450 enzyme responsible for bioactivation of methapyrilene remains elusive. Previous investigations in the rat (Ratra *et al.*, 1998a; Wrighton *et al.*, 1991) have suggested a role for CYP2C11, the male rat ortholog of human CYP2C9, but this has not been confirmed. Methapyrilene is toxic to freshly isolated rat hepatocytes (chapter 3) and cultured primary rat hepatocytes (Beekman *et al.*, 2006) but not to mouse hepatocytes (Graham *et al.*, 2008).

The use of a panel of THLE cell lines expressing single P450 representing some of the most prevalent isoforms involved in drug metabolism presents an opportunity to address the role of human P450 isoforms in the metabolism and cytotoxicity of thiophene compounds, and to evaluate the place of such an assay in preclinical DILI investigations.

## 4.2 Materials and Methods

### 4.2.1 Materials

PMFR-4 medium was purchased from Millipore (Watford, UK). GlutaMAX (dipeptide substitute for L-glutamine), G418 (geneticin), foetal bovine serum (FBS) and trypsin-EDTA were purchased from Invitrogen (Paisley, UK). Methapyrilene (MP), and 2-phenylthiophene (2-PT) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Tienilic acid (TA) was a gift from Molecular Toxicology, AstraZeneca, (Alderley Park, Cheshire, UK). Solvents were purchased from either VWR (Lutterworth, Leicestershire, UK) or Fischer Scientific (Loughborough, Leicestershire, UK). Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. THLE-CYP cell lines were obtained by AstraZeneca under license from Nestec Ltd., Switzerland.

### 4.2.2 THLE-CYP Cell Culture

PMFR-4 medium was supplemented with the following reagents, values in parentheses refer to final concentration: GlutaMAX (2mM), ITS liquid media supplement (1.75 $\mu$ M), hydrocortisone (0.2 $\mu$ M), epidermal growth factor (5ng/ml), bovine pituitary extract (15 $\mu$ g/ml), retinoic acid (0.33nM), FBS (3%) and G418 (150 $\mu$ g/ml).

Cells were thawed from a vial contained  $1 \times 10^6$  cells, and seeded in a BioCoat T75 flask (BD Biosciences, San Jose, CA, USA) in freezing medium (10% DMSO in 1:1 PMFR-4:FBS). Cells were left to attach for 4 hours at 37°C in 5% CO<sub>2</sub>, and medium was removed and replaced with fresh FBS-containing medium. Cells were then incubated at 37°C in 5% CO<sub>2</sub> and medium was replaced every 2-3 days until a confluent monolayer was formed.

#### 4.2.3 THLE-CYP Cell Incubation Regime

To perform the toxicity assay, cells ( $15 \times 10^3$  cells per 200  $\mu$ l well), were firstly plated in 96-well plates using the following procedure. Medium was removed and cells were washed thoroughly with Hank's balanced salt solution (HBSS) (without magnesium, calcium or Phenol Red; Invitrogen, UK). HBSS was then removed by aspiration and 1ml 0.025% trypsin-EDTA was added. Cell detachment usually took place after 2 min and was confirmed under a microscope. Medium (8ml) was added to harvest the cells. An aliquot (50  $\mu$ l) of cells was combined with 50  $\mu$ l 0.4% trypan blue solution and the cell number per millilitre was determined. Cells were diluted to a concentration of  $75 \times 10^3$  cells/ml and 200  $\mu$ l was added to each well (15000 cells per well). Cells were left to adhere for 24h (37°C, 5% CO<sub>2</sub>).

Stock solutions of compounds (MP, TA, 2-PT, chlorpromazine) were made up in 0.4% DMSO at a concentration of 250mM. Serial dilutions were performed using PMFR-4 medium (without FBS supplement) to achieve required concentrations. DMSO concentration was maintained throughout at 0.4%. Following cell adherence, the medium was removed and replaced with drug-containing medium. Incubations were performed in triplicate. Assessment of toxicity was performed at either 24h or 72h. In the case of 72-h exposure, medium was replaced with fresh drug-containing medium at 48h. Chlorpromazine was used as a positive control for toxicity measurements (Greer et al., 2010).

#### 4.2.4 Assessment of Toxicity of Thiophenes in THLE-CYP Cell Lines

Toxicity was determined using the CellTiter 96<sup>®</sup> Aqueous One Cell Proliferation Assay Kit (Promega, Southampton, UK). Medium was combined with 3-(4,5-dimethylthiazol-2-yl)-5-

(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution at a ratio of 5:1 (v/v). Following the drug exposure period, drug-containing medium was removed (retained for LC-MS analysis), and replaced with 120µl of medium:MTS mixture. MTS was added to 3 three empty wells to act as blanks. Following 1-h incubation, 100µl of medium was removed and pipetted into a non-collagen coated 96-well plate, ensuring no bubbles were formed. Absorbance was read at 490nm on a Wallac EnVision Spectrophotometer (PerkinElmer).

#### **4.2.5 Preparation of Samples for LC-MS Analysis**

An equal volume of ice-cold ACN was added to the cell supernatant from 500-µM incubations of each thiophene compound removed prior to MTS assay. Samples were kept at -80°C until needed. Prior to LC-MS analysis, samples were centrifuged at 14000rpm for 5min and the supernatant was retained for analysis.

#### **4.2.6 Analysis of Thiophene Metabolism in THLE-CYP Cell Lines**

Aliquots of the solution (20-100µl) were chromatographed at room temperature on either a Prodigy 5-µm ODS-2 (C-18) column (150 × 4.6 mm i.d.; Phenomenex, Macclesfield, Cheshire, UK) or a Gemini 5-µm C-18 column (250 x 4.6mm i.d.; Phenomenex, Macclesfield, Cheshire, UK), using the chromatographic conditions outlined in table 4.1. Analyses were carried out on an API 2000 mass spectrometer (Applied Biosystems, Warrington, Cheshire, UK) operated in the positive mode. Q1 scans were acquired using the following conditions: curtain gas (CUR) setting, 20; ionspray voltage (IS), 5.5 kV; temperature (TEM), 400°C; ion source gas 1 (GS1) setting, 20; ion source gas 2 (GS2) setting, 75; declustering potential (DP), 20 V; focusing potential (FP), 200 V and entrance potential (EP), 10 V.

**Table 4.1. Chromatographic conditions used for LC-MS analysis of MP, 2-PT and TA.**

<b>Thiophene</b>	<b>Column</b>	<b>Eluent A</b>	<b>Eluent B</b>	<b>Flow rate</b>	<b>Gradient</b>
MP	Gemini	10mM ammonium acetate	100% ACN	1ml/min	0min 100% A, 5min 100% A, 25min 30% A 30min 30% A 33min 100% A 34min 100% A
2-PT	Prodigy	0.1% formic acid	100% MeOH	1ml/min	0min 100% A 5min 100% A 25min 20% A 30min 20% A 31min 100% A
TA	Prodigy	0.1% formic acid	100% MeOH	1ml/min	0min 90% A 5min 90% A 23min 25% A 29min 25% A 30min 90% A 35min 90% A

## 4.3 Results

### 4.3.1 Toxicity of Thiophene Compounds in THLE-CYP Cell Lines

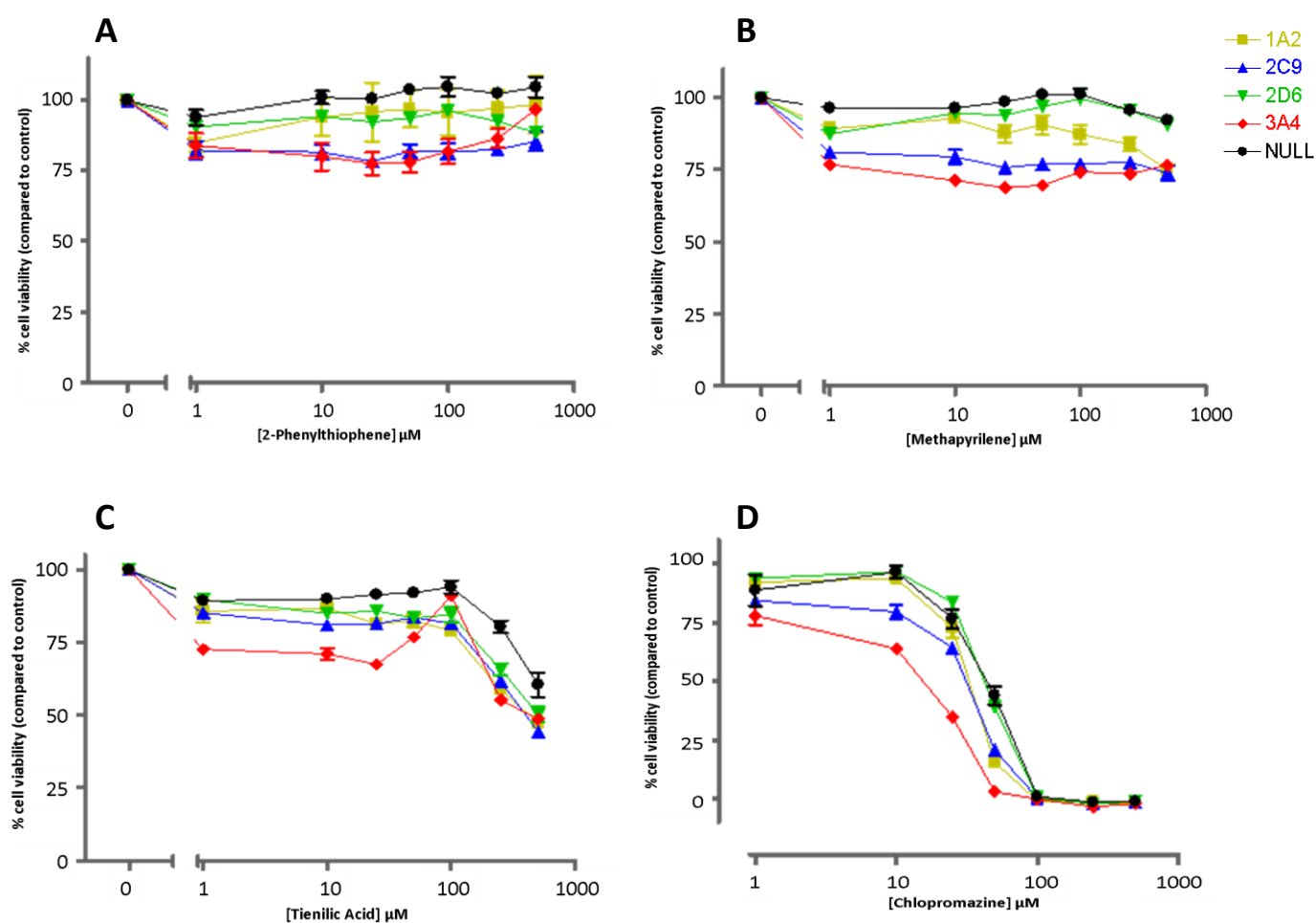
Following a 24-hr exposure period, only TA produced any decrease in viability in any cell line: approximately a 50% reduction in viability was observed in all cell lines, including the NULL cell line (no P450 expressed), at 500 $\mu$ M. No P450-dependent toxicity was observed for any of the thiophene compounds, although the CYP3A4 cell line appears to show consistently lower viability for all four compounds (including the positive control, chlorpromazine) (Fig 4.1). Elongating the exposure period to 72hr did not affect the toxicity of 2-PT in any cell lines (Fig 4.2A). Cell viability of CYP3A4, CYP2D6 and CYP1A2 cell lines was reduced by 500 $\mu$ M MP following 72-hr exposure, hinting at possible slow developing, P450-dependant, toxicity at high doses of MP (Fig 4.2B). The toxicity of TA was exacerbated in all cell lines by prolonged exposure (72hr) (Fig 4.2C). As reported by Greer et al. (2010), chlorpromazine was toxic to NULL cells and all of the THLE-CYP cell lines.

### 4.3.2 Bioactivation of Thiophene Compounds in THLE-CYP Cell Lines

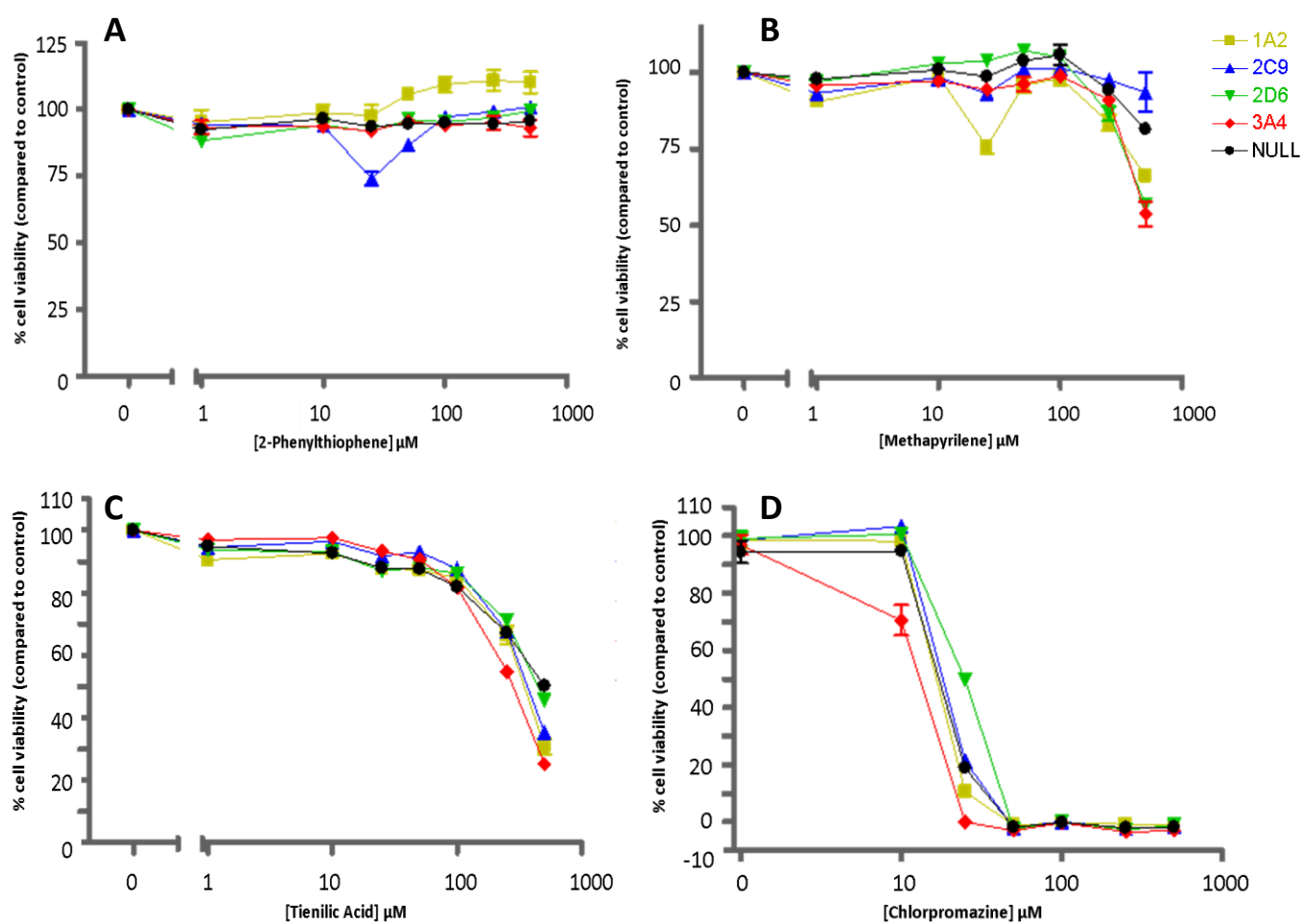
Details of metabolites observed can be seen in table 4.2. Incubations of 500 $\mu$ M 2-PT in the CYP1A2 cell line produced the only detected GSH adduct after both 24-h and 72-h incubations (Fig 4.3A & B). This adduct (m/z 484) was previously described in this work (chapter 3) and in incubations of human CYP1A1 Supersomes and rat liver microsomes (Dansette *et al.*, 2005), where an *S*-oxide dimer was also reported. The same *S*-oxide dimer (m/z 353) was only observed following a 24-hr incubation of 500 $\mu$ M 2-PT with THLE cells expressing CYP2C9 (Fig 4.3C). Conversely, the *S*-oxide dimer was not observed in 72-h incubations with the CYP2C9 cell line (Fig 4.3D). Incubations of all cell lines with TA (both 24h and 72h) failed to produce a GSH adduct, neither an oxygenated (m/z 652) nor a non-

oxygenated adduct (m/z 636) as reported in chapter 3. Hydroxyl TA (OH-TA, m/z 347) was not observed in any incubation. A previously reported oxygenated GSH adduct of methapyrilene (chapter 2 and 3) (Graham *et al.*, 2008) was not identified in 24-h or 72-h incubations of MP in any THLE-CYP cell line. However, both hydroxyl MP (m/z 278) (Fig 4.4) and desmethyl MP (m/z 248) (Fig 4.5) were found in all cell lines, after either 24h, 72h or both (Table 4.2).



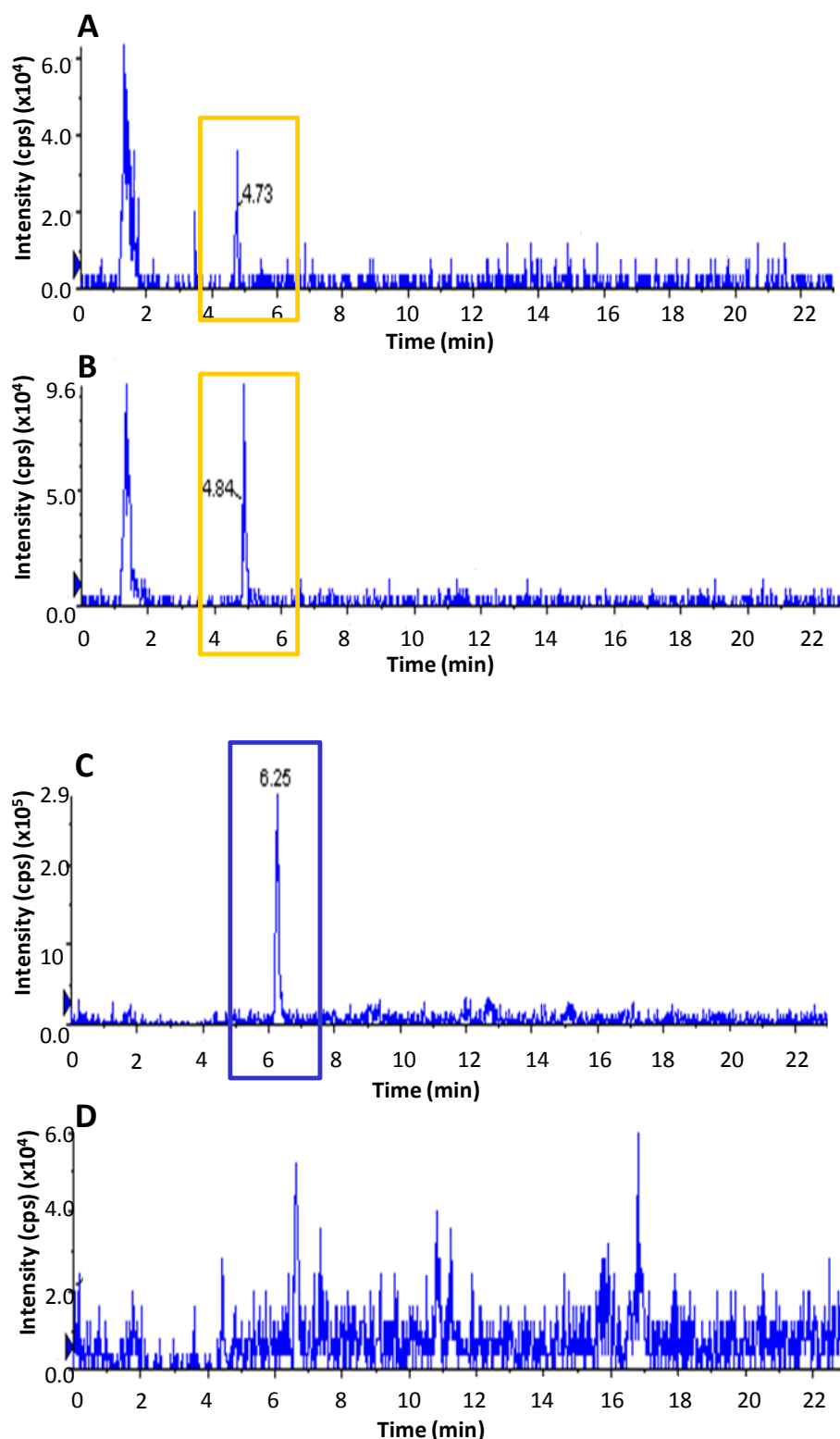


**Figure 4.1** Dose response curves showing % cell viability compared to control in five THLE-CYP cell lines (NULL, CYP1A2, CYP2C9, CYP2D6 and CYP3A4) following 24-hr exposure to (A) 2-PT, (B) MP, (C) TA and (D) chlorpromazine. Cell viability was assessed using the CellTiter 96® Aqueous One Cell Proliferation Assay Kit (Promega, Southampton, UK). Data points show mean of at least three triplicate assays and error bars indicate standard error of the mean.

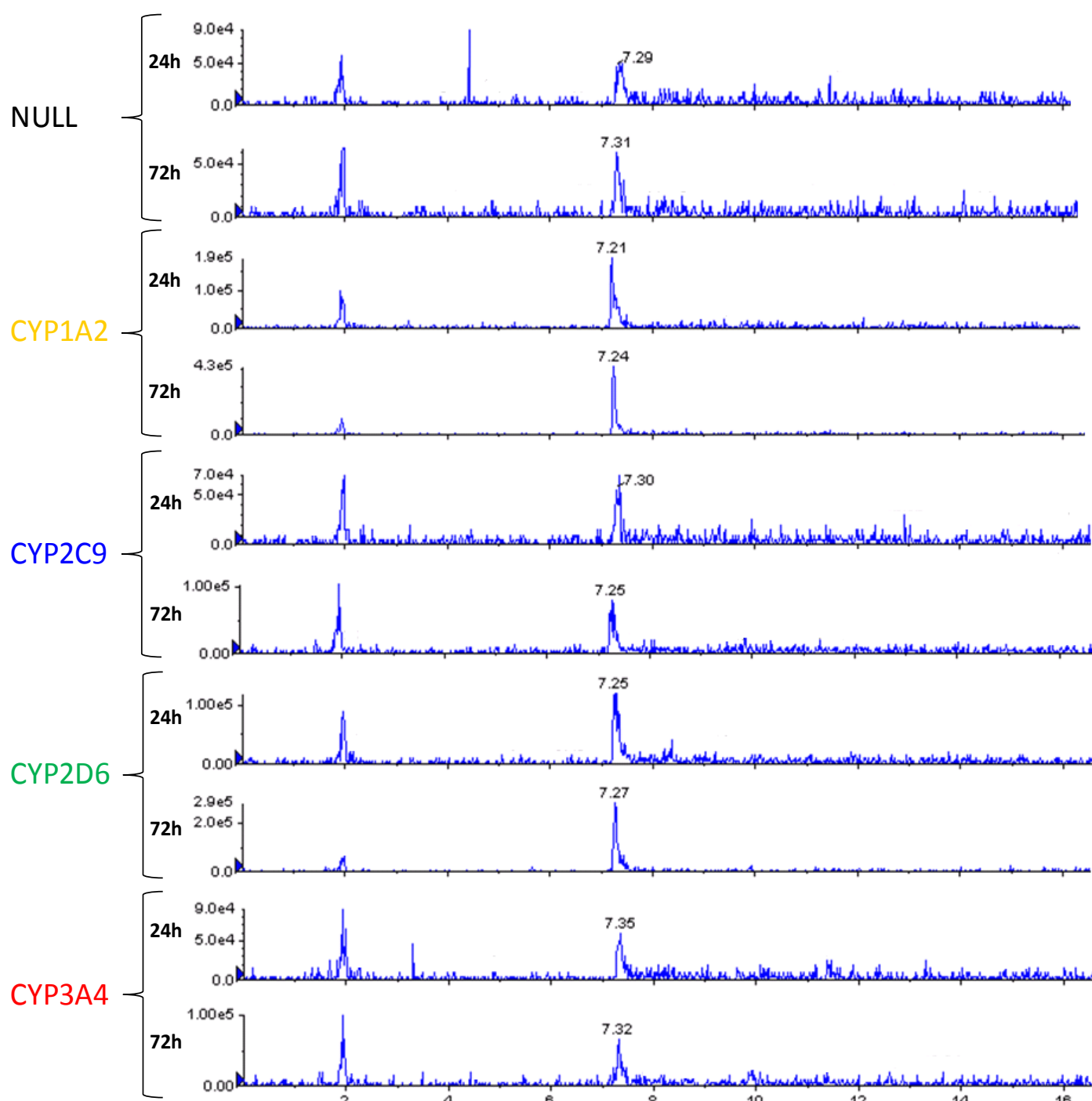


**Figure 4.2** Dose response curves showing % cell viability compared to control in five THLE-CYP cell lines (NULL, CYP1A2, CYP2C9, CYP2D6 and CYP3A4) following 72-hr exposure to (A) 2-PT, (B) MP, (C) TA and (D) chlorpromazine. Cell viability was assessed using the CellTiter 96® Aqueous One Cell Proliferation Assay Kit (Promega, Southampton, UK). Data points show mean of at least three triplicate assays and error bars indicate standard error of the mean.

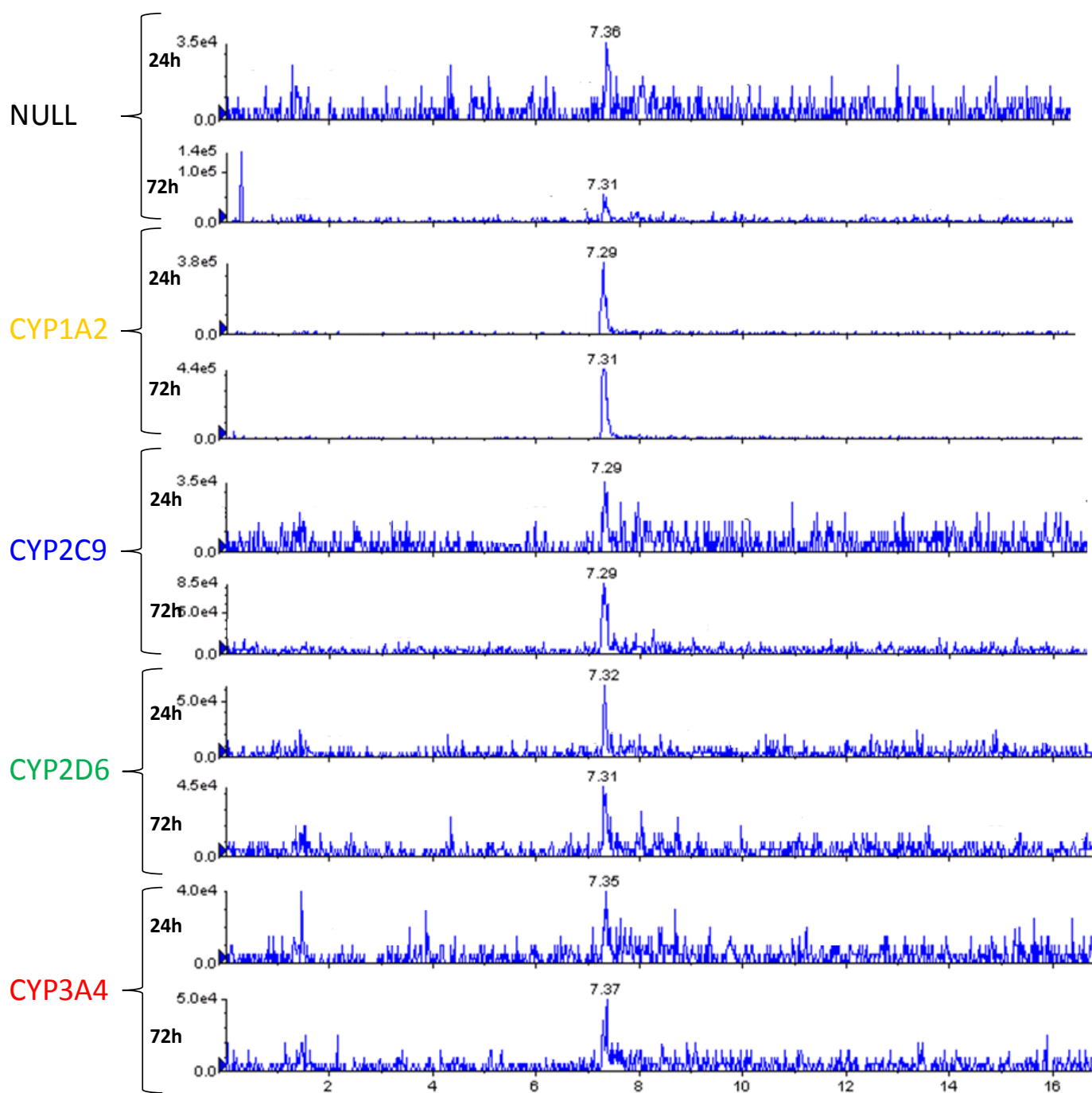




**Figure 4.3** Extracted mass chromatograms from incubations of 500 $\mu$ M 2-PT with THLE-CYP cell lines. (A) Extracted mass chromatogram of m/z 484 from 24-h incubation of 500 $\mu$ M 2-PT with THLE-CYP1A2 cell line showing presence of GSH adduct with rt 4.73min; (B) Extracted mass chromatogram of m/z 484 from 72-h incubation of 500 $\mu$ M 2-PT with THLE-CYP1A2 cell line showing presence of GSH adduct with rt 4.84min; (C) Extracted mass chromatogram of m/z 353 from 24-h incubation of 500 $\mu$ M 2-PT with THLE-CYP2C9 cell line showing presence of *S*-oxide dimer with rt 6.25min; (D) Extracted mass chromatogram of m/z 353 from 72-h incubation of 500 $\mu$ M 2-PT with THLE-CYP2C9 cell line showing lack of *S*-oxide dimer.



**Figure 4.4** Extracted mass chromatograms of  $m/z$  278 (hydroxyl MP) from incubations (24h and 72h) of 500 $\mu$ M MP with five THLE-CYP cell lines: NULL (no P450 expressed), CYP1A2, CYP2C9, CYP2D6 and CYP3A4. Hydroxyl MP was observed in all cell lines with an  $rt$  of between 7.25min – 7.35min.



**Figure 4.5** Extracted mass chromatograms of m/z 248 (desmethyl MP) from incubations (24h and 72h) of 500µM MP with five THLE-CYP cell lines: **NULL** (no P450 expressed), **CYP1A2**, **CYP2C9**, **CYP2D6** and **CYP3A4**. Desmethyl MP was observed in all cell lines with an rt of between 7.29min – 7.37min.

#### 4.4 Discussion

The work described in this chapter aimed to evaluate the role of several P450 enzymes in the metabolism and toxicity of thiophene-containing drugs and non-drug xenobiotics. A panel of THLE cell lines expressing single human P450s or none (NULL, CYP1A2, CYP2C9, CYP2D6 and CYP3A4) were exposed to either MP, 2PT or TA for a period of 24h or 72h and the cytotoxicity and metabolism of those compounds was assessed.

Following 24-h exposure, only TA showed any cytotoxicity. In all cell lines, 250 $\mu$ M TA reduced cell viability by approximately 25% compared to control. However, the observed toxicity was not accompanied by the formation of any of the drug's known metabolites. Although a 72-h exposure to TA increased the susceptibility of the cells to cytotoxicity, again, no metabolites were observed in any cell line. The sensitivity of the NULL cell line to TA suggests that the toxicity observed was not due to formation of metabolites. The lack of TA metabolism in the CYP2C9 cell line is puzzling. As mentioned previously, CYP2C9 has been implicated in the bioactivation of TA (Belghazi *et al.*, 2001), so the presence of GSH adducts would be expected in the THLE-CYP2C9 cell line. It may be the case that the MS methods were not sensitive enough to detect GSH adducts. It could also be possible that the majority of reactive metabolites formed were bound to cellular proteins and therefore not detected as GSH adducts, reactive metabolites of TA have previously been shown to bind to and inactivate human CYP2C9 (Lopez-Garcia *et al.*, 2005), however, as this did not appear to be the case in the rat hepatocyte incubations described in chapter 3. GSH adducts of TA have successfully been identified previously (Lim *et al.*, 2008), so such a phenomenon is only likely to occur due to lack of GSH or GST enzymes in these cell lines, which has not been explored. However, an investigation into the role of Phase 2 metabolising enzymes on MDMA detoxification successfully defined a role for COMT and GST enzymes using THLE cells (Antolino Lobo *et al.*, 2010).

No toxicity was observed following 24-h exposure to MP. Longer exposure (72h) led to a decrease in cell viability in CYP1A2, CYP2D6 and CYP3A4 cell lines at the highest concentration of MP (500 $\mu$ M). As with TA, this toxicity was not accompanied by the formation of any reactive intermediates stabilized as GSH adducts. However, unlike TA, two metabolites of methapyrilene, desmethyl MP and hydroxyl MP, were observed in all cell lines at both 24h and 72h. These metabolites are formed by rat hepatocytes (Graham *et al.*, 2008). The lack of toxicity of MP in the NULL cell line suggests a role for certain P450 enzymes in the reduction of cell viability that was observed. It may be the case that human P450 enzymes do not bioactivate MP to a reactive metabolite. Through its years in clinical use, no human adverse events were reported, and investigations into MP's hepatotoxicity and carcinogenicity have mainly been performed in the rat (Craig *et al.*, 2006; Graham *et al.*, 2008; Kammerer *et al.*, 1986; Lijinsky *et al.*, 1980; Ratra *et al.*, 1998b; Ratra *et al.*, 2000). The toxicity seen in these THLE-CYP cell lines could be due to parent drug effects. As with TA, it may be the case that any reactive metabolite formed favoured protein covalent binding as opposed to GSH adduct formation. MP has previously been shown to form covalent adducts with cellular proteins (Graham, 2007).

The least cytotoxic compound tested was 2-PT. No decrease in cell viability was observed in any cell line, following either 24-h or 72-h exposure. Despite this, 2-PT was the only thiophene to produce a detectable GSH adduct in this system, in the CYP1A2 cell line. Following both 24- and 72-h exposure, an oxygenated GSH adduct of 2-PT (m/z 484) was observed. An S-oxide dimer was also visible in the mass chromatographic analysis of a 24-h incubation of CYP1A2 cell line with 2-PT. Previous work with human Supersomes has shown a role for human CYP1A1 in formation of GSH adducts of 2-PT via both thiophene S-oxide and thiophene epoxide intermediates (Dansette *et al.*, 2005). Human CYP1A1 and CYP1A2 share 72% amino acid sequence identity (Goldstone *et al.*, 2006), and have previously been shown to metabolise similar substrates (Arlt *et al.*, 2004; Stiborova *et al.*,



2005). The lack of toxicity in the THLE-CYP1A2 cell line associated with formation of thiophene *S*-oxide and epoxide intermediates could be due to a number of factors. Although, as described in the previous chapter, 2-PT cytotoxicity was detected in incubations with freshly isolated rat hepatocytes, there were many differences between the conditions and methods used in that work and the procedures described in this chapter. The cytotoxicity 'end point' used for the work reported in chapter three was a loss of membrane integrity as assessed by the trypan blue assay, whereas in this investigation, mitochondrial viability was assessed using a tetrazolium dye assay (Promega, Southampton, UK). A comparison of cell viability assays showed that use of tetrazolium dye assay can overestimate the number of viable cells compared with trypan blue determination (Wang *et al.*, 2010). It could also be that formation of GSH adducts conferred protection from reactive metabolites, and that in the case of 2-PT, any thiophene *S*-oxide or epoxide intermediate formed was successfully detoxified and that the GSH adducts detected represent a portion of reactive intermediate, large enough that damage from any residual intermediate does not result in observable cytotoxicity.

Comparisons between metabolic and toxicological results obtained in this chapter and the previous chapter is difficult for several reasons. Method, length of exposure and the end point of the experiment were different in both cases. It is however interesting to note the homology of the human enzymes investigated in this chapter and their rat counterparts. Rat CYP1A2 is 88% homologous to the human form. The rat form of human CYP2C9 is known as CYP2C11 and is 87% homologous. Rat CYP2D4 is 75% homologous with human CYP2D6 and the rat counterpart of human CYP3A4 is called CYP3A9 and is 88% homologous.

The results gained with these cell lines do not enable extrapolation to link a single P450 enzyme with the bioactivation and toxicity of any of the compounds investigated. Single-P450 cells have an obvious potential limitation in respect of investigating the metabolic origins of toxicity: cells expressing single P450 will not reveal a cytotoxicity that depends on bioactivation by two or more P450 acting sequentially. The extent to which this is a

significant limitation on use of THLE-CYP cells for metabolism/toxicity screening is unknown, however carbamazepine has been shown to undergo sequential P450-catalyzed bioactivation reactions in HLM incubations (Bu et al., 2007), but the number and operational sequence of the P450s involved are unknown. The lack of detection of GSH adducts might have been a result of intracellular covalent binding as mentioned above, or because the amount of adduct formed was too small to be detected by the MS methods described in this chapter. It could also have been due to low metabolic capacity of the cells, either in phase 1 or phase 2 metabolism. However, the phase 1 metabolic capacity of the THLE-CYP cells was assessed by Molecular Toxicology (AstraZeneca, UK) using probe substrates and the P450-Glo™ luminescence assay (Promega, Southampton, UK). The probe substrates used were phenacetin (CYP1A2), diclofenac (CYP2C9), bufuranol (CYP2D6) and verapamil (CYP3A4), and confirmed P450 activity in all cell lines. Phase 2 enzyme activity has also been described in THLE cell lines (Antolino Lobo *et al.*, 2010).

Despite the low cytotoxicity of thiophene compounds observed here, THLE-CYP cells have previously been shown to display cytotoxicity with chlorpromazine (Greer *et al.*, 2010). Chlorpromazine was found to be toxic to THLE-1A2, -2C9, -2D6, -3A4 and -null cells at following 24h exposure, but it was much more toxic to THLE-3A4 cells than the other cell types over 72h.

The findings reported in this chapter have indicated that a detailed assessment of the toxicological usefulness of THLE-CYP cells will require, firstly, an understanding of the role of their GSH and GST in detoxification of reactive metabolites. GSH depletion is an important factor in hepatotoxicity (Comporti *et al.*, 1991) and has been observed in investigations into mechanisms of several hepatotoxins (Kitteringham *et al.*, 2000), and detection of GSH depletion may be key in to the diagnosis of an altered redox state or of formation of reactive metabolites. Serum biomarkers to assess tissue GSH levels and help to reveal the effects of drug administration on GSH biochemistry would be an excellent tool in preclinical evaluation, and could potentially be extrapolated to the clinic.

## References

- Antolino Lobo, I, Meulenbelt, J, Nijmeijer, SM, Scherpenisse, P, van den Berg, M, van Duursen, MB (2010) Differential roles of phase I and phase II enzymes in 3,4-methylenedioxyamphetamine (MDMA)-induced cytotoxicity. *Drug Metab Dispos.*
- Arlt, VM, Hewer, A, Sorg, BL, Schmeiser, HH, Phillips, DH, Stiborova, M (2004) 3-aminobenzanthrone, a human metabolite of the environmental pollutant 3-nitrobenzanthrone, forms DNA adducts after metabolic activation by human and rat liver microsomes: evidence for activation by cytochrome P450 1A1 and P450 1A2. *Chem Res Toxicol* **17**(8): 1092-1101.
- Beaune, P, Dansette, PM, Mansuy, D, Kiffel, L, Finck, M, Amar, C, Leroux, JP, Homberg, JC (1987) Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P-450 that hydroxylates the drug. *Proc Natl Acad Sci U S A* **84**(2): 551-555.
- Beekman, JM, Boess, F, Hildebrand, H, Kalkuhl, A, Suter, L (2006) Gene expression analysis of the hepatotoxicant methapyrilene in primary rat hepatocytes: an interlaboratory study. *Environ Health Perspect* **114**(1): 92-99.
- Belghazi, M, Jean, P, Poli, S, Schmitter, JM, Mansuy, D, Dansette, PM (2001) Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv Exp Med Biol* **500**: 139-144.
- Bu, HZ, Zhao, P, Dalvie, DK, Pool, WF (2007) Identification of primary and sequential bioactivation pathways of carbamazepine in human liver microsomes using liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **21**(20): 3317-3322.
- Comporti, M, Maellaro, E, Del Bello, B, Casini, AF (1991) Glutathione depletion: its effects on other antioxidant systems and hepatocellular damage. *Xenobiotica* **21**(8): 1067-1076.
- Copple, IM, Goldring, CE, Kitteringham, NR, Park, BK (2010) The keap1-nrf2 cellular defense pathway: mechanisms of regulation and role in protection against drug-induced toxicity. *Handb Exp Pharmacol*(196): 233-266.
- Craig, A, Sidaway, J, Holmes, E, Orton, T, Jackson, D, Rowlinson, R, Nickson, J, Tonge, R, Wilson, I, Nicholson, J (2006) Systems toxicology: integrated genomic, proteomic and metabolomic analysis of methapyrilene induced hepatotoxicity in the rat. *J Proteome Res* **5**(7): 1586-1601.
- Dambach, DM, Andrews, BA, Moulin, F (2005) New technologies and screening strategies for hepatotoxicity: use of in vitro models. *Toxicol Pathol* **33**(1): 17-26.
- Dansette, PM, Bertho, G, Mansuy, D (2005) First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives. *Biochem Biophys Res Commun* **338**(1): 450-455.
- Doehmer, J (1993) V79 Chinese hamster cells genetically engineered for cytochrome P450 and their use in mutagenicity and metabolism studies. *Toxicology* **82**(1-3): 105-118.
- Fowler, S, Zhang, H (2008) In vitro evaluation of reversible and irreversible cytochrome P450 inhibition: current status on methodologies and their utility for predicting drug-drug interactions. *AAPS J* **10**(2): 410-424.

Goldring, CE, Kitteringham, NR, Jenkins, R, Lovatt, CA, Randle, LE, Abdullah, A, Owen, A, Liu, X, Butler, PJ, Williams, DP, Metcalfe, P, Berens, C, Hillen, W, Foster, B, Simpson, A, McLellan, L, Park, BK (2006) Development of a transactivator in hepatoma cells that allows expression of phase I, phase II, and chemical defense genes. *Am J Physiol Cell Physiol* **290**(1): C104-115.

Goldstone, HM, Stegeman, JJ (2006) A revised evolutionary history of the CYP1A subfamily: gene duplication, gene conversion, and positive selection. *J Mol Evol* **62**(6): 708-717.

Gomez-Lechon, MJ, Castell, JV, Donato, MT (2007) Hepatocytes--the choice to investigate drug metabolism and toxicity in man: in vitro variability as a reflection of in vivo. *Chem Biol Interact* **168**(1): 30-50.

Graham, E PhD Thesis, University of Liverpool, Liverpool, 2007.

Graham, EE, Walsh, RJ, Hirst, CM, Maggs, JL, Martin, S, Wild, MJ, Wilson, ID, Harding, JR, Kenna, JG, Peter, RM, Williams, DP, Park, BK (2008) Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* **326**(2): 657-671.

Greer, ML, Barber, J, Eakins, J, Kenna, JG (2010) Cell based approaches for evaluation of drug-induced liver injury. *Toxicology* **268**(3): 125-131.

Ha-Duong, NT, Dijols, S, Macherey, AC, Goldstein, JA, Dansette, PM, Mansuy, D (2001) Ticlopidine as a selective mechanism-based inhibitor of human cytochrome P450 2C19. *Biochemistry* **40**(40): 12112-12122.

Hewitt, NJ, Lechon, MJ, Houston, JB, Hallifax, D, Brown, HS, Maurel, P, Kenna, JG, Gustavsson, L, Lohmann, C, Skonberg, C, Guillouzo, A, Tuschl, G, Li, AP, LeCluyse, E, Groothuis, GM, Hengstler, JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39**(1): 159-234.

Homberg, JC, Andre, C, Abuaf, N (1984) A new anti-liver-kidney microsome antibody (anti-LKM2) in tienilic acid-induced hepatitis. *Clin Exp Immunol* **55**(3): 561-570.

Kammerer, RC, Schmitz, DA (1986) Metabolism of methapyrilene by rat-liver homogenate. *Xenobiotica* **16**(7): 671-680.

Kitteringham, NR, Powell, H, Clement, YN, Dodd, CC, Tetley, JN, Pirmohamed, M, Smith, DA, McLellan, LI, Kevin Park, B (2000) Hepatocellular response to chemical stress in CD-1 mice: induction of early genes and gamma-glutamylcysteine synthetase. *Hepatology* **32**(2): 321-333.

Labbe, G, Pessayre, D, Fromenty, B (2008) Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. *Fundam Clin Pharmacol* **22**(4): 335-353.

Laine, JE, Auriola, S, Pasanen, M, Juvonen, RO (2009) Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. *Xenobiotica* **39**(1): 11-21.

Lavergne, SN, Park, BK, Naisbitt, DJ (2008) The roles of drug metabolism in the pathogenesis of T-cell-mediated drug hypersensitivity. *Curr Opin Allergy Clin Immunol* **8**(4): 299-307.

Lee, SS, Buters, JT, Pineau, T, Fernandez-Salguero, P, Gonzalez, FJ (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* **271**(20): 12063-12067.

Lijinsky, W, Reuber, MD, Blackwell, BN (1980) Liver tumors induced in rats by oral administration of the antihistaminic methapyrilene hydrochloride. *Science* **209**(4458): 817-819.

Lim, HK, Chen, J, Cook, K, Sensenhauser, C, Silva, J, Evans, DC (2008) A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom* **22**(8): 1295-1311.

Lopez-Garcia, MP, Dansette, PM, Coloma, J (2005) Kinetics of tienilic acid bioactivation and functional generation of drug-protein adducts in intact rat hepatocytes. *Biochem Pharmacol* **70**(12): 1870-1882.

Lopez-Garcia, MP, Dansette, PM, Mansuy, D (1994) Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry* **33**(1): 166-175.

Mace, K, Aguilar, F, Wang, JS, Vautravers, P, Gomez-Lechon, M, Gonzalez, FJ, Groopman, J, Harris, CC, Pfeifer, AM (1997) Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* **18**(7): 1291-1297.

Nishiya, Y, Hagihara, K, Ito, T, Tajima, M, Miura, S, Kurihara, A, Farid, NA, Ikeda, T (2009a) Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab Dispos* **37**(3): 589-593.

Nishiya, Y, Hagihara, K, Kurihara, A, Okudaira, N, Farid, NA, Okazaki, O, Ikeda, T (2009b) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**(11): 836-843.

O'Donnell, JP, Dalvie, DK, Kalgutkar, AS, Obach, RS (2003) Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31**(11): 1369-1377.

Park, BK, Kitteringham, NR, Maggs, JL, Pirmohamed, M, Williams, DP (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* **45**: 177-202.

Puga, A, Raychaudhuri, B, Salata, K, Zhang, YH, Nebert, DW (1990) Stable expression of mouse Cyp1a1 and human CYP1A2 cDNAs transfected into mouse hepatoma cells lacking detectable P450 enzyme activity. *DNA Cell Biol* **9**(6): 425-436.

Ratra, GS, Cottrell, S, Powell, CJ (1998a) Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* **46**(1): 185-196.

Ratra, GS, Morgan, WA, Mullervy, J, Powell, CJ, Wright, MC (1998b) Methapyrilene hepatotoxicity is associated with oxidative stress, mitochondrial dysfunction and is prevented by the Ca<sup>2+</sup> channel blocker verapamil. *Toxicology* **130**(2-3): 79-93.

Ratra, GS, Powell, CJ, Park, BK, Maggs, JL, Cottrell, S (2000) Methapyrilene hepatotoxicity is associated with increased hepatic glutathione, the formation of glucuronide conjugates, and enterohepatic recirculation. *Chem Biol Interact* **129**(3): 279-295.

Stiborova, M, Sopko, B, Hodek, P, Frei, E, Schmeiser, HH, Hudecek, J (2005) The binding of aristolochic acid I to the active site of human cytochromes P450 1A1 and 1A2 explains their potential to reductively activate this human carcinogen. *Cancer Lett* **229**(2): 193-204.

Wang, P, Henning, SM, Heber, D (2010) Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS One* **5**(4): e10202.

Watkins, PB (1990) Role of cytochromes P450 in drug metabolism and hepatotoxicity. *Semin Liver Dis* **10**(4): 235-250.

Wrighton, SA, VandenBranden, M, Brown, TJ, Van Pelt, CS, Thomas, PE, Shipley, LA (1991) Modulation of rat hepatic cytochromes P450 by chronic methapyrilene treatment. *Biochem Pharmacol* **42**(5): 1093-1097.

Zimmerman, HJ (1999) *Hepatotoxicity*. 2nd edn. Lippincott Williams & Wilkins.



## CHAPTER FIVE

---

OPHTHALMIC ACID AS A BIOMARKER  
OF GLUTATHIONE DEPLETION



## Contents

5.1 Introduction.....	146
5.2 Materials and Methods.....	151
5.2.1 Materials .....	151
5.2.2 Animals .....	151
5.2.3 Investigation into the effects of acute APAP dosing on serum OA levels in male C57BL6 mice .....	151
5.2.4. Investigation into the effects of chronic APAP dosing on serum OA levels in male Wistar rats using chronic infusion. ....	152
5.2.5. Investigation into the effects of acute DEM dosing on serum OA levels and hepatic GSH levels in male Wistar rats using plasma sampling and liver biopsy.....	153
5.2.6. Investigation into the serum kinetics of OA in vivo in the rat .....	154
5.2.7 Determination of stability of OA in rat serum and tissue homogenates .....	154
5.2.8 Determination of OA and 2-AB concentrations via LC-MS/MRM.....	155
5.3.9 Determination of hepatic GSH concentrations .....	156
5.3.10 Determination of serum alanine transferase (ALT) activity .....	157
5.3.11. Statistical Analysis.....	157
5.3. Results.....	158
5.3.1. Investigation into the effects of acute APAP dosing on serum OA levels in male C57BL6 mice .....	158
5.3.2. Investigation into the effects of chronic APAP dosing on serum OA levels in male Wistar rats using chronic infusion .....	158
5.3.3 Investigation into the effects of acute DEM dosing on serum OA levels and hepatic GSH levels in male Wistar rats using plasma sampling and liver biopsy.....	159
5.3.4. Investigation into the serum kinetics of OA in vivo in the rat .....	159
5.3.5 Determination of stability of OA in rat serum and tissue homogenates .....	160
5.4. Discussion .....	167

## Abbreviations

2-AB, 2-aminobutyrate; ALT, serum alanine transferase; APAP, acetaminophen; DEM, diethylmaleate; DILI, drug induced liver injury; GCL, glutamate cysteine ligase; GSH, glutathione; LC-MS, liquid chromatography mass spectrometry; MRM, multiple reaction monitoring; OA, ophthalmic acid; S-hex, S-hexylglutathione.

## 5.1 Introduction

The previous two chapters have focused on formation of glutathione (GSH) adducts as a marker of bioactivation of thiophene molecules. The potential of GSH adducts and their metabolites to serve as biomarkers of exogenously and endogenously derived electrophilic intermediates, and thereby as disease and toxin-exposure biomarkers, has been reviewed (Blair, 2010). Bioactivation to reactive metabolites is often, though not invariably, associated with drug-induced liver injury (DILI) in patients (Srivastava *et al.*, 2010), usually, it is thought, by initiating primary changes within the cell from which damage and cell death can lead. These primary changes may be oxidative stress – characterised typically by lipid peroxidation and changes in thiol status – and covalent binding to macromolecules, with immediate consequences that include enzyme inhibition and mitochondrial damage (Timbrell, 2000). Although some reactive oxygen species play a physiological role in intracellular signalling (Forman *et al.*, 2010), the appearance of excessive reactive oxygen species and of organic electrophiles such as reactive drug metabolites provokes a defensive response from the cell (Nguyen *et al.*, 2009; Copple *et al.*, 2010). This response is centred on the upregulation of phase-II metabolism enzymes and those responsible for production and maintenance of GSH, enabling the cell to restore its redox and electrophile status. GSH is critical to maintenance of a cell's redox and electrophile status (Forman *et al.*, 2009), playing a protective role via three mechanisms: conjugation to reactive species by glutathione-S-transferases, non-enzymic reaction to form GSH adducts and donation of protons to reactive metabolites or free radicals. GSH is found at high concentrations (5mM) in the liver and in addition to performing a variety of other functions (Cho *et al.*, 1984; Pallardo *et al.*, 2009) must be available within a cell to quench excessive electrophilic species and prevent oxidative stress; thereby averting covalent modifications – adduction and oxidation – of critical cellular proteins (Park *et al.*, 2005).

GSH arises within the cell via synthesis from its precursor L-amino acids (Bloch *et al.*, 1947): glutamate, cysteine and glycine. It is formed in a two-step process catalysed by two discrete enzymes. The first and rate-limiting step involves the conjugation of glutamate and cysteine to form  $\gamma$ -glutamylcysteine by glutamate cysteine ligase (GCL) (Meister *et al.*, 1983). This enzyme is regulated by feedback inhibition from GSH. The second step, catalysed by glutathione synthetase, is the addition of glycine to the cysteine residue forming GSH (See fig 5.6 for an overview of GSH metabolism). The free sulphhydryl group on the cysteine residue is the crucial moiety in GSH biochemistry. It can act as an electron donor, a reductant and as a scavenger of free radicals. Nucleophilic addition or substitution reactions of the GS<sup>-</sup> thiolate ion (Graminski *et al.*, 1989), catalysed by glutathione transferases, lead to formation of glutathione S-conjugates, excreted principally in bile but also in urine (Johnson *et al.*, 2005), whereas reactions involving reduction catalysed by enzymes such as GSH peroxidase give rise to glutathione disulphide. The use of *N*-acetylcystein-S-yl (mercapturate) metabolites of GSH adducts as non-invasive biomarkers of electrophile exposure has been reviewed (Jian *et al.*, 2009). GSH peroxidase enzymes utilise reduced GSH to reduce hydrogen peroxide, lipid peroxides and organic hydroperoxides, protecting DNA, proteins and lipids from oxidative damage (Awasthi *et al.*, 1975; Hou *et al.*, 1996; Sies *et al.*, 1997). To maintain a reducing environment within the cell, glutathione reductase utilises NADPH to convert glutathione disulphide back to the reduced form.

For a number of drugs for which glutathione conjugate formation takes place, the reactive metabolites do not appear to exert toxicity as long as the cellular pool of glutathione is not depleted. Toxicity occurs when the cellular pool of GSH is depleted to below a critical threshold, due to overdose or saturation of other clearance pathways, as is the case with the analgesic, acetaminophen (APAP).

There is a lack of an accurate and convenient preclinical test for this toxicity threshold. A serum biomarker reflective or indeed predictive of hepatic GSH depletion would be an ideal tool both in drug safety studies and potentially in the clinic. The potential of biomarkers to improve the progress of clinical research and to reduce attrition in drug development is becoming widely acknowledged. Biomarkers, in this context, can be defined as changes in DNA, mRNA, protein or metabolite levels that correlate with particular disease states or are reflective of drug treatment. An ideal biomarker would be detected in biological material obtainable by non-invasive or minimally invasive procedures, such as saliva, blood or urine, and be amenable to rapid, specific and sensitive assay, as well as being proportionately reflective of the tissue injury sustained. Ideally, it should be predictive of toxicity, and therefore, in human subjects, allow avoidance of the onset of any irreversible injury. In order to be useful when bridging studies in basic science, drug development and in the clinic, the ideal biomarker should be transferable across *in vitro*, *in vivo* and clinical systems.

A recent metabolomic study identified a potential biomarker of hepatic GSH depletion in mouse serum by capillary electrophoresis and mass spectrometry (Soga *et al.*, 2006). Male C57BL6 mice were fasted overnight prior to anaesthetising with pentobarbital sodium and administration of 150mg/kg APAP (*i.p.*). The authors detected a significant increase in the abundance of an unknown ion at  $m/z$  290.135 in the serum and liver of the APAP-treated animals. Through tandem mass spectrometry, they defined this ion as ophthalmic acid (OA), an analogue of GSH. The mass deficit between the unknown ion and GSH was 17.958Da, concordant with replacement of the sulphhydryl group on the cysteine residue with the methyl group of 2-aminobutyrate (2-AB). The authors suggest that this increase in the serum concentration of OA is due, firstly, to depletion of the GSH pool, removing the feedback inhibition on GCL and increasing the activity of this enzyme. They then propose that due to a decreased cysteine pool, GCL instead recruits 2-AB and forms  $\gamma$ -glutamylaminobutyrate, the precursor for OA, which is then effluxed out of the hepatocyte (Fig 5.6). Through the use

of diethylmaleate (DEM), they were able to reproduce this effect, i.e. a rise in serum OA reflective of a hepatic GSH decrease. Administration of the GCL inhibitor buthionine sulphoximine depleted hepatic GSH, but was not accompanied by an increase in hepatic OA, implying that GCL is also the crucial enzyme in formation of the latter. They concluded that monitoring serum OA levels could facilitate the detection of several pathologies – not just DILI – such as Alzheimer's, Parkinson's, cardiac infarction and diabetes, in which oxidative stress is thought to play an important role.

OA was first isolated from calf lens in 1956 (Waley, 1956) and was discovered to be synthesised via a two-step process that is similar to the biosynthesis of GSH (Cliffe *et al.*, 1958; Cliffe *et al.*, 1961). Subsequent studies detected OA in the lens of several other species (Tsuboi *et al.*, 1984) and later in rat liver (Kasai *et al.*, 1989). Little research has been conducted into the physiological role of OA in the liver, with the majority of investigations focusing on GSH transport processes. OA has been shown to stimulate GSH efflux from freshly isolated rat hepatocytes (Garcia-Ruiz *et al.*, 1992) and to both stimulate and inhibit GSH transport across bile canalicular membranes (Ballatori *et al.*, 1994). As mentioned previously, GSH and OA are synthesised in a two-step process from glutamate, either cysteine or 2-AB, and glycine (Meister, 1989). Glutamate is synthesised from glutamine and 2-ketoglutarate, and is recycled via the  $\gamma$ -glutamyl cycle. Glycine is formed from serine and tetrahydrofolate. Cysteine is synthesised from the essential amino acid methionine via homocysteine and cystathionine. 2-AB is also formed from methionine, via excision of 2-ketobutyrate from cystathionine. This is then transaminated with either serine or threonine to create 2-aminobutyrate. Both OA and GSH are reliant on the activity of GCL and glutathione synthetase. The  $K_m$  values of the rate-limiting enzyme, GCL, towards 2-AB and cysteine are 1.0mM and 0.3mM, respectively (Seelig *et al.*, 1985).

The aim of the work described in this chapter was to evaluate the usefulness and limitations of serum OA concentration as a biomarker of hepatic GSH consumption

using models of hepatic GSH depletion and OA stability. Serum OA and hepatic OA were measured, and where possible, compared to hepatic GSH and a classic biomarker of liver injury, serum alanine transaminase (ALT) activity. In kinetic and stability studies, 2-AB concentrations were also monitored.

The following models were used:

- Acute APAP exposure in male C57BL6 mice
- Chronic APAP exposure in male Wistar rats using an infusion pump
- Acute exposure of anaesthetised male Wistar rats to DEM
- Definition of serum kinetics of OA in male Wistar rats following a single intravenous dose
- Stability of OA in serum and tissue homogenates from untreated male Wistar rats.

The GSH depleting agents used were APAP and DEM. APAP is often used as a model hepatotoxin. It is bioactivated by P450 enzymes to a reactive metabolite, N-acetyl p-benzoquinone imine, which is detoxified via GSH conjugation (Mitchell *et al.*, 1973a). In overdose, this route of elimination results in depletion of hepatic GSH, which may herald the onset of APAP-induced injury (Mitchell *et al.*, 1973b). Indeed, treating APAP overdose with N-acetylcysteine to replace cysteine and therefore restore GSH concentrations can prevent liver injury from proceeding (Prescott *et al.*, 1977). Acute dosing of APAP to mice has classically been used as a model to study DILI. Rats are more resistant to APAP-induced liver injury (Moldeus, 1978), and therefore require chronic or very high dosing to produce an effect useful for studying the consequences of APAP-induced GSH depletion. DEM is also a model hepatotoxin, used mainly for its ability to deplete hepatic GSH rapidly and extensively following acute dosing (Gerard-Monnier *et al.*, 1992).

## **5.2 Materials and Methods**

### **5.2.1 Materials**

Ophthalmic acid (OA) was purchased from Bachem (Rhein, Germany). 2-Aminobutyrate (2-AB), acetaminophen (APAP), urethane, [remove] and diethylmaleate (DEM) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Bradford reagent was purchased from Bio-Rad (Germany). Solvents were purchased from either VWR (Lutterworth, Leicestershire, UK) or Fischer Scientific (Loughborough, Leicestershire, UK). Unless otherwise stated, all the other reagents were purchased from Sigma-Aldrich.

### **5.2.2 Animals**

Adult male Wistar rats and adult male C57BL6 mice were obtained from Charles River Laboratories (Margate, Kent, UK). All experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act of 1986 and approved by the Animal Ethics Committee of the University of Liverpool.

### **5.2.3 Investigation into the effects of acute APAP dosing on serum OA levels in male C57BL6 mice**

At time-point zero, male C57BL6 (20-25g) mice were dosed intraperitoneally (i.p.) with either 530mg/kg APAP or with saline vehicle (control). Table 5.1 details the dosing regime. At various time points upto 6h, animals were euthanised with rising levels of CO<sub>2</sub>. Blood was sampled via cardiac puncture, and livers were removed and snap frozen in liquid

nitrogen and then stored at -80°C until analysis. Blood samples were stored overnight at 2-4°C to allow clotting to take place. Serum was then removed and stored at -80°C until analysis.

**Table 5.1. Regime for acute APAP dosing in male C57BL6 mice**

<b>Group</b>	<b>Number of Animals</b>	<b>Dose</b>	<b>Euthanised</b>
0h	4	530mg/kg APAP	0h
0h control	4	0.9% Saline	0h
1h	4	530mg/kg APAP	1h
1h control	4	0.9% Saline	1h
2h	4	530mg/kg APAP	2h
2h control	4	0.9% Saline	2h
6h	4	530mg/kg APAP	6h
6h control	4	0.9% Saline	6h

**5.2.4. Investigation into the effects of chronic APAP dosing on serum OA levels in male Han Wistar rats using chronic infusion.**

(The study described here was carried out in conjunction with AstraZeneca, Alderley Park, UK)



Adult male Han Wistar rats were anaesthetised and cannulated via the femoral vein. Following recovery (seven days was allowed for animals to recover from surgery), vehicle (saline) (n=3) or APAP (6mg/ml) (n=3) was infused into the femoral vein cannula at a constant rate (3ml/kg/hr) for 48hr. Blood samples (300µl) were taken via the tail vein at 3h, 5h, 24h, 30h and 36h. Plasma was extracted via centrifugation (1300g, 10min) and stored at -80°C until analysis. Following termination of infusion, animals were euthanized by administration of halothane, and approximately 2ml of blood removed via the vena cava. Approximately 3mm thick slices will be taken from the left, median and caudate lobes. In addition a 3mm sample will be removed from the left lobe, frozen in liquid nitrogen, then stored at approximately -80°C for analysis of GSH.

**5.2.5. Investigation into the effects of acute DEM dosing on serum OA levels and hepatic GSH levels in male Wistar rats using plasma sampling and liver biopsy.**

Adult male Wistar rats (200-300g) were anaesthetised with urethane (1.4g/ml in saline; 1.0ml/kg i.p.) and cannulated via the trachea and carotid artery. A 3-cm incision was made in the abdomen and covered with saline-soaked gauze to allow access to the liver when required. The carotid artery cannula was filled with heparin-containing saline (250U/ml). A predose blood sample (100µl, via carotid artery) and liver sample were taken 5min before administration of DEM. DEM (1ml/kg; 1.064 g/kg) (n = 4) was administered i.p. Blood samples (100µl) were taken via the carotid artery cannula simultaneously with liver biopsy at 15min, 30min 45min and then every 15min until 2h when animals were euthanized. Liver biopsy samples (20-40mg) were snipped from the left lateral lobe and were snap frozen in liquid nitrogen and then stored at -80°C until analysed for OA and GSH levels. Plasma was extracted via centrifugation (1300g, 10min) and stored at -80°C until analysed.

### **5.2.6. Investigation into the serum kinetics of OA in vivo in the rat**

Adult male Wistar rats (200-300g) were anaesthetised with urethane (1.4g/ml in saline; 1.0ml/kg i.p.) and cannulated via the trachea, common bile duct, femoral vein and carotid artery. Carotid artery cannula was filled with heparin-containing saline (250U/ml). A predose blood sample (100µl) was taken via the carotid artery cannula 10min prior to dosing. Bile was sampled for 10min prior to dose OA (100mg/kg in 0.9% saline) (n = 4) was administered i.v. via the femoral vein. Volume of administration was kept between 200-300µl and was administered over 5min. Blood samples (100µl) were taken via the carotid artery cannula at 5min, 10min, 20min, 30min and then every 30min until 3h when animals were euthanized. The penis was ligated and bladder contents removed for analysis at the termination of study. Plasma was extracted via centrifugation (1300g, 10min). Plasma, bile and urine were then stored at -80°C until analysed.

### **5.2.7 Determination of stability of OA in rat serum and tissue homogenates**

OA (10mM in dH<sub>2</sub>O) was spiked into serum, whole liver homogenate and whole kidney homogenate from untreated male Wistar rats to give a final concentration of 100µM OA and kept at room temperature. Homogenates were obtained by homogenising approximately 40mg of tissue in cold phosphate buffer (pH 7.4, 1:4 w/v). The homogenate was then briefly sonicated (6min in three 2min blasts) before being subject to centrifugation (2200 rpm, 5min). Supernatants were retained for analysis. At various time intervals up to 24h, 10µl was removed and subjected to analysis of OA and 2-AB concentration as outlined below Spiking of OA was performed in triplicate.

### 5.2.8 Determination of OA and 2-AB concentrations via LC-MS/MRM.

#### Sample preparation

Sample (10 $\mu$ l) was diluted with 30 $\mu$ l H<sub>2</sub>O and 200 $\mu$ l acetone to precipitate proteins. S-hexylglutathione (S-hex) was used as an internal standard: 10 $\mu$ l of 12.5 $\mu$ M aqueous S-hex was added, to give a final concentration of 0.5 $\mu$ M. Standards (OA or 2-AB) were prepared for analysis in a similar way. An aqueous solution of standard (10 $\mu$ l) at 25-fold the final concentration was added to 20 $\mu$ l H<sub>2</sub>O and 10 $\mu$ l 20mg/ml bovine serum albumin. Acetone (200 $\mu$ l) was added to precipitate protein. Standards and samples were kept at -20°C for 1h, before centrifugation at 14, 000rpm for 10min. Supernatant was retained and acetone was removed under a vacuum. Finally, 200 $\mu$ l 0.1% aqueous formic acid was added to all standards and samples.

#### LC-MS/MRM Conditions

Aliquots of the standards and samples (50 $\mu$ l) were chromatographed on an Uptisphere ODB 120Å 5 $\mu$ m column (100  $\times$  2mm) (Interchim, Montluçon, France), using a gradient of 0-80% MeOH in 0.1% formic acid over 30min at a flow rate of 0.2ml/min (PerkinElmer 200 series LC pump and autosampler, PerkinElmer, UK). Column temperature was maintained at 40°C. Analyses were carried out on an API 3000 mass spectrometer (Applied Biosystems, Warrington, Cheshire, UK) operated in the positive-ion mode. Multiple reaction monitoring (MRM) acquisitions were performed using the optimized conditions outlined in table 5.2 and the following unoptimized conditions: nebuliser gas setting, 20; curtain gas (CUR) setting, 10; collision gas setting, 8; ionspray voltage (IS), 5.5 kV; source temperature (TEM), 350°C.

**Table 5.2. MRM conditions for detection of OA, 2-AB and S-hex**

Analyte	Q1 m/z	Q3 m/z	Declustering potential (V)	Focusing potential (V)	Collision cell entrance potential (V)	Collision cell exit potential (V)
OA	290.440	58.00	61	280	47	16
S-hex	392.093	246.5	36	200	23	16
2-AB	104.45	58.00	76	370	15	16

Instrument operation and data processing were performed through Analyst 1.4 software (Applied Biosystems). To determine either the OA or 2-AB concentration, peak areas of samples and standards were first normalised against the S-hex peak area. A standard curve was then constructed and used to determine OA and 2-AB concentrations in the biological samples.

### 5.3.9 Determination of hepatic GSH concentrations

Hepatic GSH levels were determined using a microtitre plate assay (Vandeputte *et al.*, 1994). Briefly, approximately 50 mg of tissue was homogenised in 5-sulphosalicylic acid (200 µl; 6.5 % w/v) and GSH stock buffer (800 µl; 143 mM NaH<sub>2</sub>PO<sub>4</sub>, 6.3mM EDTA, pH 7.4) and the protein was allowed to precipitate on ice for 10 min prior to centrifugation (18400g for 5 min). The supernatants were used to

determine the total GSH and GSSG content. Total GSH (GSH + GSSG) was determined spectrophotometrically using 5,5'-dithio-bis(2-nitrobenzoic acid), NADPH and GSH reductase at 412 nm. The results were compared to GSH standards (0-40 nmol/ml). Protein pellets were solubilised in 1M NaOH for 1h at 60°C before the protein concentration was determined (Bradford, 1976).

### **5.3.10 Determination of serum alanine transferase (ALT) activity**

Plasma was prepared by centrifugation (10000rpm, 5min) after the blood was allowed to clot at room temperature for 1 hr. ALT was measured using the ThermoTrace Infinity ALT Liquid stable reagent (Alpha Labs, Eastleigh, Hampshire, UK) according to the manufacturer's instructions.

### **5.3.11. Statistical Analysis**

All statistical analyses were carried out using StatsDirect software (StatsDirect Ltd., Altrincham, UK). Data were first assessed for normality using the Shapiro-Wilk test. Normal data were subjected to analysis of variance and Bonferroni comparison. Non-normal results were compared using the Mann-Whitney test. A value of  $p \leq 0.05$  was taken as significant in all cases.

### 5.3. Results

#### 5.3.1. Investigation into the effects of acute APAP dosing on serum OA levels in male C57BL6 mice

Animals were treated in groups of four, receiving one intraperitoneal dose of either 530mg/kg APAP (in saline) or an equivalent volume of saline. Following euthanasia at either 0h, 1h, 2h or 6h, serum was analysed for ALT activity and OA concentrations. Liver was analysed for total GSH concentration. Results from the ALT analyses show a significant elevation above control only at the 6-h time point for APAP-treated animals (Fig 5.1A). However, hepatic GSH levels in APAP-treated animals were significantly depleted immediately to 41% of the mean time-matched control value (18.50nmol/mg protein vs. 7.63nmol/mg protein), dropping to only 5% of the time-matched control value (23.15nmol/mg protein vs. 1.36nmol/mg protein) at 2h following dosing (Fig 5.1B). Serum OA concentrations in APAP-treated animals were increased in a similar pattern to serum ALT levels: only at 6h following APAP dosing were OA concentrations increased significantly compared to time-matched controls (0.6034 $\mu$ M vs. 0.0834 $\mu$ M) (Fig 5.1C).

#### 5.3.2. Investigation into the effects of chronic APAP dosing on serum OA levels in male Wistar rats using chronic infusion

During the infusion period (6mg/ml APAP at 3ml/kg/hr; 18mg/kg/hr) blood samples were taken at 3h, 5h, 24h, 30h, 36h and 48h and analysed for OA concentration. Following termination at 48h, blood was removed from the vena cava and analysed for ATL activity. Hepatic GSH concentration was also determined. Serum concentrations of OA remained within a 10nM for the first 5h of infusion. From 24h to 36h, serum OA concentrations rose from 178nM to 689nM, an increase of 387%. Following 48h of infusion, serum OA had

increased sharply from predose levels (101nM) to 6.8 $\mu$ M. Terminal serum ALT activity was also determined and compared to that of control animals (Fig 5.2). ALT activity in animals receiving APAP infusion was 689.33U/L compared to 24.5U/L in control animals. Terminal hepatic GSH in APAP-treated animals was depleted by 50% compared to controls (19.46 vs. 38.87nmol/mg protein) (Fig 5.2).

### **5.3.3 Investigation into the effects of acute DEM dosing on serum OA levels and hepatic GSH levels in male Wistar rats using plasma sampling and liver biopsy.**

Male Wistar rats were anaesthetised (1ml/100g 14% urethane i.p.) followed by cannulation via the trachea and carotid artery which allowed for frequent blood sampling from the same animal. Blood samples and liver biopsies were taken simultaneously to enable time-matched comparisons of serum OA concentrations and hepatic total GSH concentrations. Predose samples and liver biopsies were taken before animals were dosed intraperitoneally with 1ml/kg DEM. Samples and biopsies were then taken every 15min until 2h. Serum samples were analysed for OA concentration. Liver biopsies were analysed for both GSH and OA. Hepatic GSH was depleted significantly to a mean of 29% of predose value at 15min post dose and continued to stay significantly depleted over the 2h study (Fig 5.3). No significant rise was observed in either serum OA or hepatic OA concentrations over the course of the 2h study. However, hepatic OA concentrations did appear to increase over the time period from a mean of 58% of predose value at 15min to a mean of 134% of predose value at 2h (Fig 5.3).

### **5.3.4. Investigation into the serum kinetics of OA in vivo in the rat**

Following induction of anaesthesia, male Wistar rats were cannulated via the trachea, carotid artery, common bile duct and femoral vein. Animals received one dose of 100mg/kg OA via

the femoral vein to force an exaggerated increase in serum OA concentrations analogous though not necessarily proportionate to one that may potentially follow extensive hepatic GSH depletion. Blood and bile samples were then taken over 3h, as well as collection of urine. Serum, bile and urine were analysed to determine OA concentrations. Concentrations of 2-AB were also determined in serum samples. The greater part of the OA dose was found in serum, with a smaller fraction detected in bile (Fig 5.4). The levels of 2-AB were elevated above the predose concentration in serum, possibly due to metabolism of OA. OA and 2-AB were not detected in the urine.

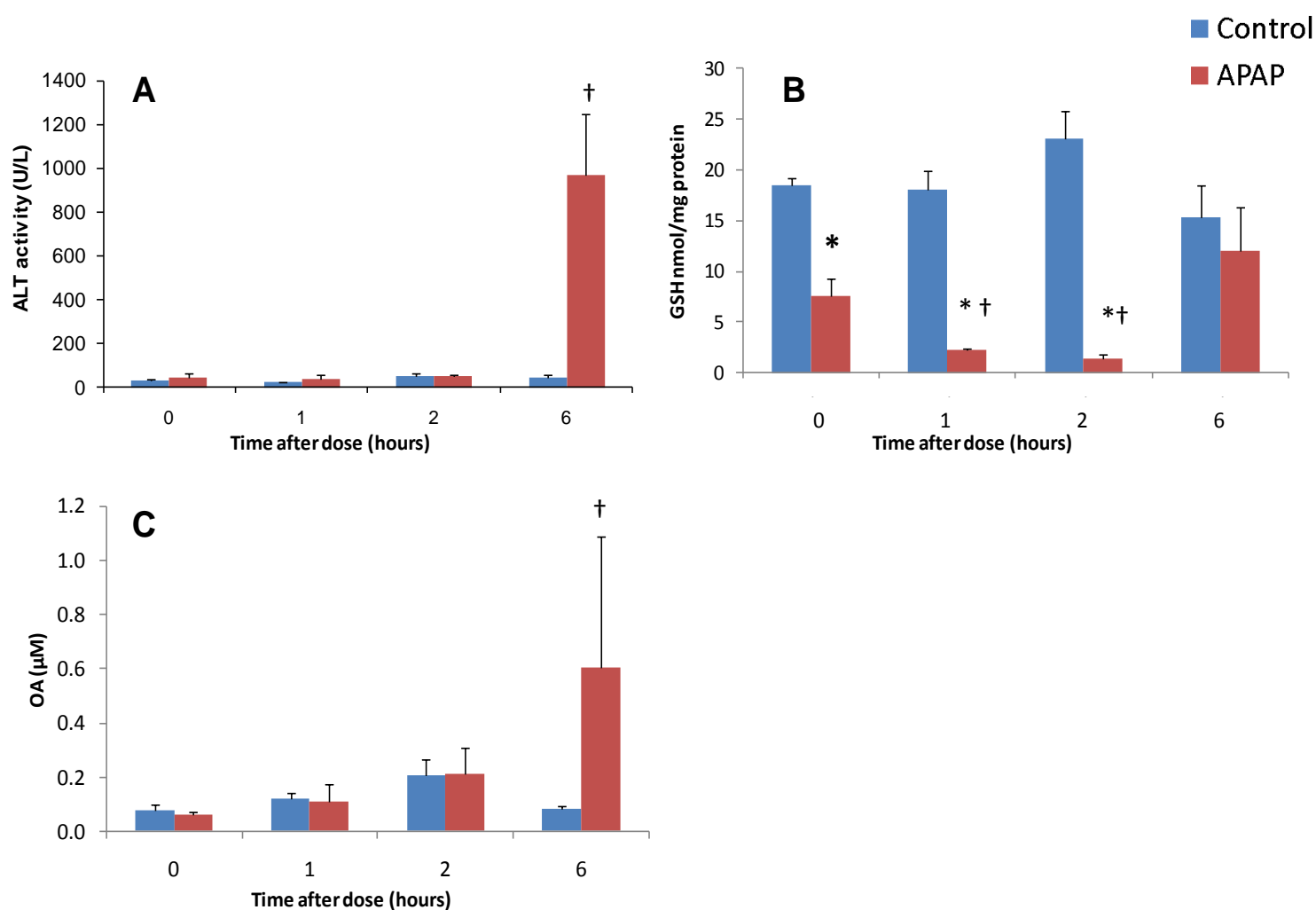
Several values for the distribution, elimination and clearance of OA can be calculated (Table 5.3). Assuming the  $C_{\max}$  to be the value obtained at 5-min sampling (238.46 $\mu$ M), the half-life of OA in rat serum is 26.18min. This allows calculation of the elimination rate constant using the formula  $K_{el} = \ln 2/t_{1/2}$ . The value for ' $K_{el}$ ' in this instance would therefore be 0.026min. Volume of distribution ( $V_d$ ) can also be estimated. Taking the average weight of the male Wistar rats in the study to be 300g, then the average dose given would be 30mg.  $C_{\max}$  (238.46 $\mu$ M) can be expressed as 0.069mg/ml, and if  $V_d = \text{total dose/plasma concentration}$ , then in this instance  $V_d = 30\text{mg} / 0.069\text{mg/ml} = 434.8\text{ml}$ , or 0.43L. These values allow calculation of initial clearance using the formula  $Cl_p = V_d \times K_{el}$ , which yields an estimate for the clearance of OA from plasma of 11.30ml/min.

### 5.3.5 Determination of stability of OA in rat serum and tissue homogenates

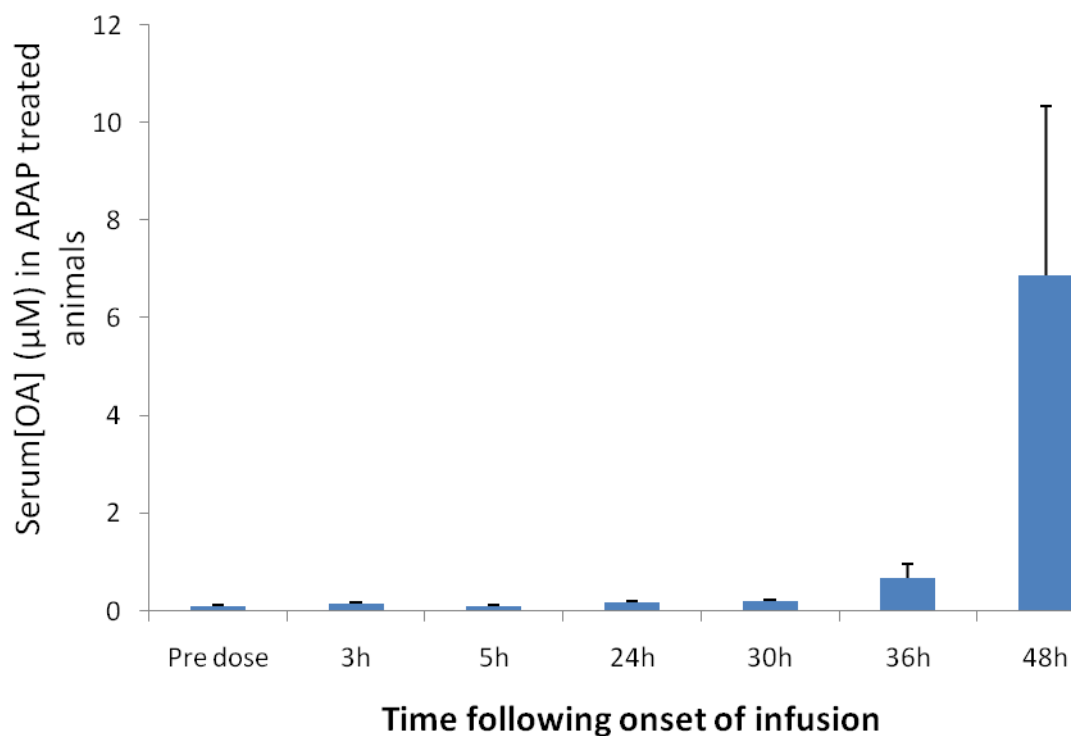
Stability of OA was assessed by 'spiking' synthetic OA into serum, liver homogenate and kidney homogenate from untreated male Wistar rats. At various time points, an aliquot was analysed for OA and a constituent amino acid, 2-AB. The levels of OA in serum remained relatively stable, within a 14.2 $\mu$ M range (19.03 – 33.23 $\mu$ M), for 24h. Concentrations in liver homogenate remained stable until 6h (range = 13.3  $\mu$ M), and then decreased at 24h to 36% of the initial value. OA was degraded very quickly in kidney homogenate even at room temperature, decreasing to 0.004% of the initial concentration within 30min of spiking (Fig accompanied by an increase in the concentration of 2-AB. Whilst the concentration of 2-AB



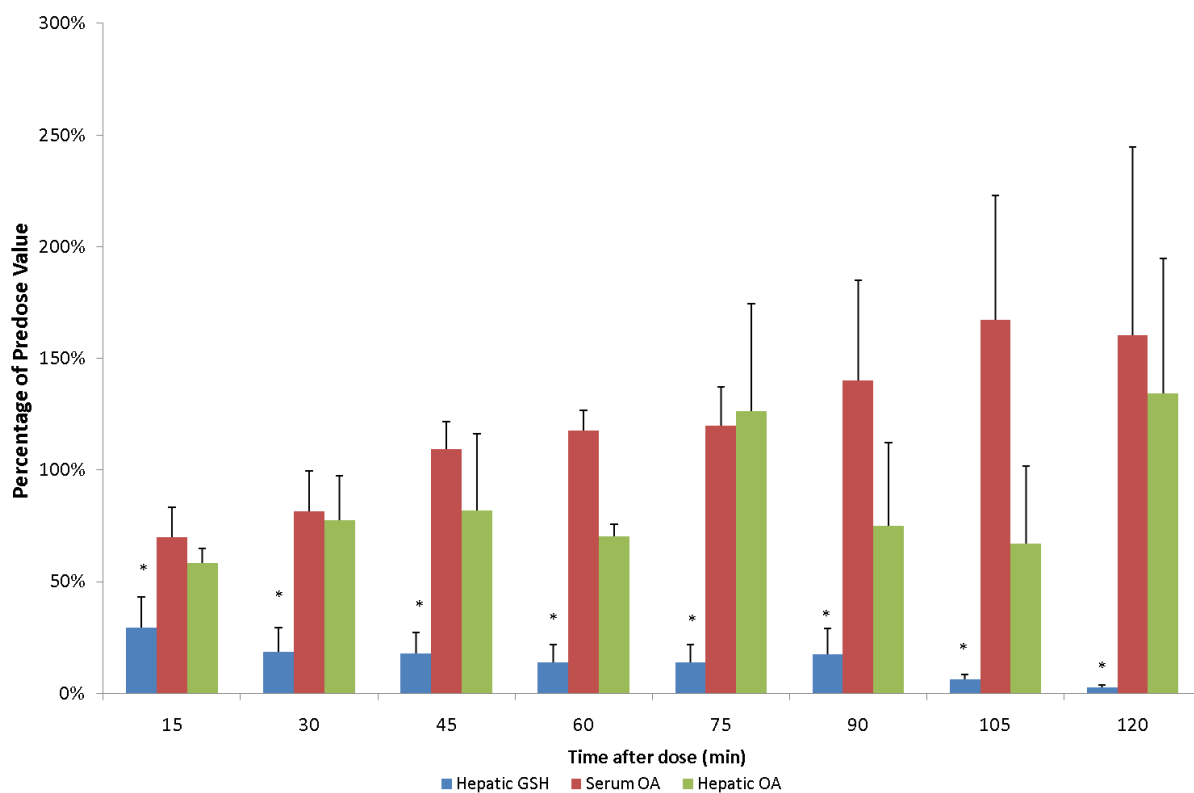
in the serum rose slightly (8.59 – 14.22 $\mu$ M over 24h), the concentration in liver homogenate rose steadily to 114% initial value (4.63 – 40.51 $\mu$ M). In kidney homogenate, the steep decline in OA concentrations was mirrored by a fast rise in 2-AB concentration, which increased from 1.13 $\mu$ M to 13.65 $\mu$ M over 30min, and then to 61.65 $\mu$ M at 24h (Fig 5.5B).



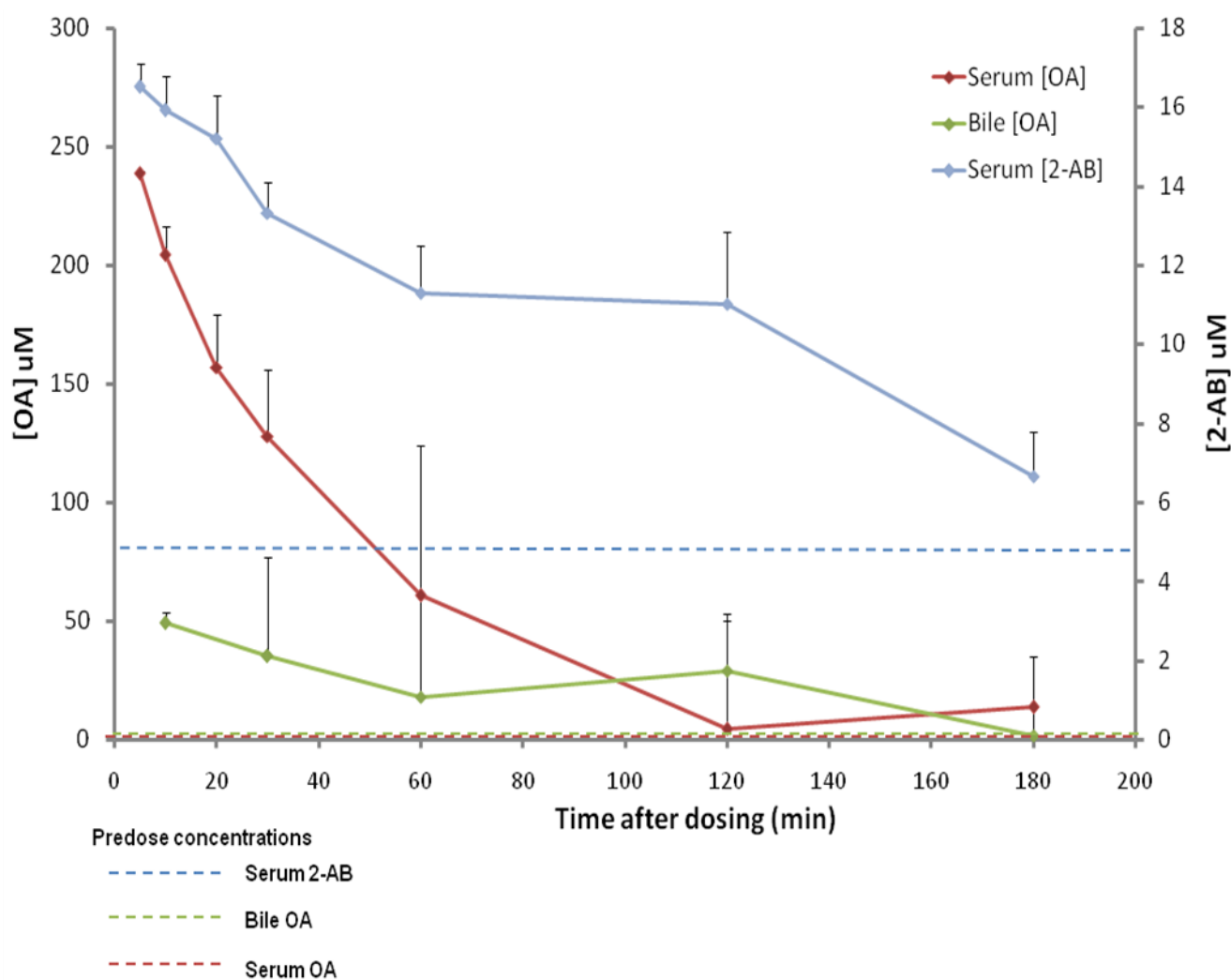
**Figure 5.1.** Effect of APAP dosing on (A) serum ALT activity; (B) total hepatic GSH concentration and (C) serum OA concentration in male C57BL6 mice. Mice were treated in groups of four, receiving either one i.p. dose of 530mg/kg APAP or equivalent volume of saline vehicle. Following euthanasia at the indicated time point, analyses were carried out on serum (ALT, OA) and liver (GSH). \*  $p \leq 0.05$  versus 0h control value, <sup>†</sup>  $p \leq 0.05$  versus time-matched control. Columns represent mean of  $n = 4$ ; error bars indicate standard error of the mean.



**Figure 5.2.** Effect of APAP infusion on serum OA concentration, serum ALT activity and hepatic GSH concentration in male Han Wistar rats. Animals were treated in groups of three, receiving either infusion at 3ml/kg/hr of 6mg/ml APAP or saline vehicle. Blood samples were taken and analysed for OA at the indicated time points. Following termination at 48h, analyses were carried out on serum (ALT, OA) and liver (GSH). Columns represent mean of  $n \leq 2$ ; error bars indicate standard error of the mean.



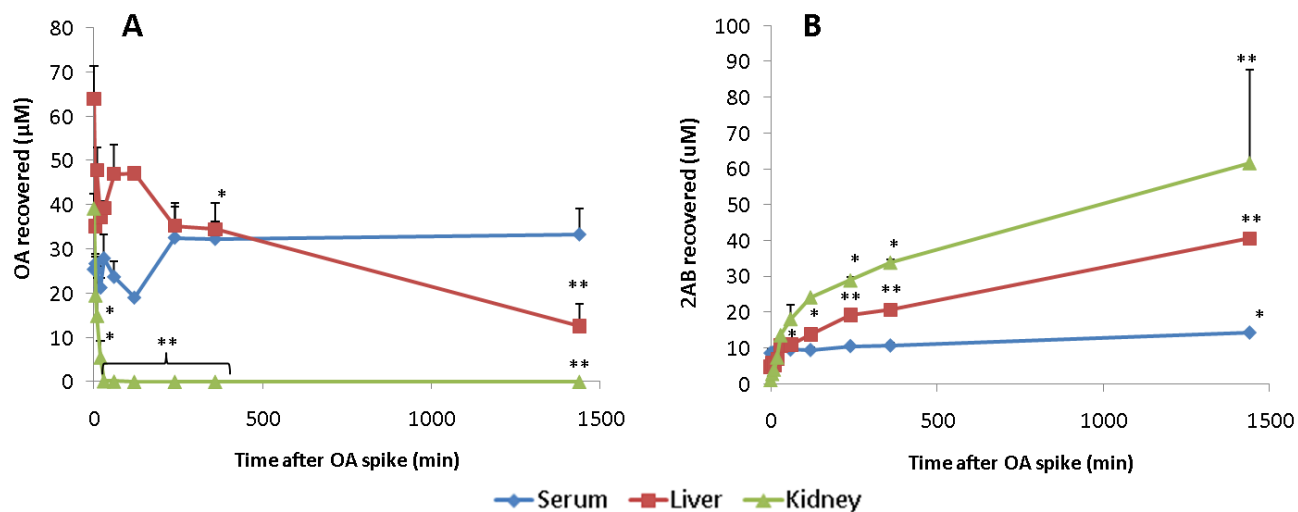
**Figure 5.3.** Effect of 1ml/kg DEM (i.p.) on serum OA, ~~and~~ hepatic OA and hepatic GSH levels in anaesthetised male Wistar rats. Animals were anaesthetised with an i.p dose of 1ml/100g 14% (w/v) urethane and cannulated via the trachea and carotid artery. Blood samples (100 $\mu$ l) were taken via the carotid artery cannula simultaneously with liver biopsy. Mean predose value (15min prior to dose) for each metabolite was taken as 100% and data are presented as percentages of this value. Columns represent mean of four separate determinations. Error bars indicate standard error of the mean. \* $p \leq 0.05$  versus predose value.



**Figure 5.4.** Concentrations of OA and 2-AB in serum and bile of male Wistar rats pre (10min prior to dose) and post administration of a 100mg/kg i.v. dose of OA. Animals were anaesthetised with an i.p dose of 1ml/100g 14% (w/v) urethane and cannulated via the trachea, carotid artery, common bile duct and femoral vein. Data points indicate mean of at least four separate determinations. Error bars indicate standard error of the mean. \*  $p \leq 0.05$  compared to 5-min value.

**Table 5.3.** Kinetics of OA in serum following 100mg/kg i.v. dose (based on Fig. 5.5)

Half-life ( $t_{1/2}$ ) (minutes)	Elimination rate constant ( $K_{el}$ ) (minutes)	Volume of distribution ( $V_d$ ) (ml)	Clearance ( $Cl_p$ ) (ml/min)
26.18	0.026	434.8	11.30



**Figure 5.5.** Concentrations of (A) OA and (B) 2-AB in serum, liver homogenate and kidney homogenate that were prepared from untreated male Wistar rats and spiked with Serum and homogenates were spiked with 10mM OA to give a final concentration of 100µM OA. OA-spiked preparations were kept in sealed containers at room temperature and aliquots (10µl) removed for analysis at various time points upto 24h. Data points represent mean of three separate determinations. Error bars indicate standard error of the mean. \*  $p \leq 0.05$ , \*\*  $p \leq 0.001$ , compared to 0-min value.

## 5.4. Discussion

The results presented in this chapter enable an evaluation of the usefulness of serum OA concentration as a biomarker of the GSH depletion associated with DILI. In vivo models were used to produce hepatic GSH depletion and serum OA concentrations were monitored for consequential perturbations.

The first of these models was acute dosing of C57BL6 male mice with 530mg/kg APAP. Hepatic GSH levels were depleted rapidly, however a significant increase in serum OA was only seen at the 6h time point, indicating poor concurrency with GSH depletion. Serum transaminase levels were also increased only at 6h following dosing. In this case, whilst serum OA levels were increased by a significant amount in animals exposed to APAP, the usefulness of this increase as a biomarker is questionable due to the delay after the immediate depletion of GSH. These results contrast with those of Soga (Soga *et al.*, 2006). Whilst an increase in serum OA levels was observed in both studies, the results presented in this chapter show a delay between initial depletion of GSH in APAP-treated mice and elevation of serum OA whereas those presented by Soga *et al.* (2006) show a maximal increase in serum OA at 1h post dosing. There are differences in study design which may go some way to providing a rationale for this difference. Whilst the same strain of mouse was studied, the dose level was markedly different: 150mg/kg (Soga *et al.*, 2006) vs. 530mg/kg. Mice in the previous study were also starved overnight prior to dosing and were anaesthetised before APAP administration with pentobarbital (Soga *et al.*, 2006) Neither of these methods was used in the work reported here. Starvation of the animals may have influenced the level of OA, as OA synthesis relies on methionine, an amino acid which must be obtained from the diet (Fig 5.6). Indeed, in rats fed with an excess of methionine (8% casein plus 3% methionine), although plasma OA was not detected, liver OA concentrations were shown to decrease compared to animals fed 8% casein alone, despite an increase in the plasma levels

of the precursor 2-AB. Liver glycine was also reduced and plasma levels of glutamate decreased (Kasai T *et al.*, 1989). Starvation has also been shown to influence  $\alpha$ -ketobutyrate dehydrogenase, leading to a loss of activity of this enzyme in the liver (Steele *et al.*, 1984). The authors suggest that inhibition of this enzyme would therefore favour the transamination of  $\alpha$ -ketobutyrate to 2-AB. The increase in 2-AB caused by fasting may go some way to explaining the immediate increase in OA observed by Soga *et al.* (2006). High levels of 2-AB caused by fasting and increased activity of GCL through depletion of GSH due to APAP administration could have led to the fast formation of OA observed in that case. Presumably, the animals in this work would have had lower levels of 2-AB due to having access to food prior to dosing. This may mean that OA would take longer to accumulate, therefore clarifying the delayed increase in serum OA levels compared to the work published previously (Soga *et al.*, 2006).

The second part of the investigations presented here was a monitoring of OA in the serum of rats receiving a chronic infusion of APAP. In this case, it was not possible to analyse the results statistically. This was because the small blood sample collected at each time point meant that quantification of OA could not be carried out on all samples at each time point, leaving insufficient data for analysis. However, it is clear that the APAP infusion process caused a huge increase in serum OA levels at the later time points, despite the relatively low dose of APAP received over the course of the study. This was reflective of the terminal hepatic GSH value, which was depleted by 50% in APAP-treated animals versus control. Nevertheless, this method did not allow an assessment of how serum OA compared to hepatic GSH over the course of the study. Therefore, although the increase in serum OA observed is interesting, conclusions cannot be drawn as to how indicative OA serum values were of hepatic GSH levels throughout the course of the study.



To address this problem, a liver biopsy was taken simultaneously with each blood sample after the rats were anaesthetised and exposed to the classic GSH depletor, DEM. However, although hepatic GSH was depleted to 25% of the predose value by 15min, neither serum nor hepatic OA levels reflected this decrease within the 2h study time, wherein hepatic GSH remained below 25% of the predose value. It may be, however, as in the C57BL6 mice administered APAP, that an elevation of serum OA levels is not seen until more than 2h following administration of DEM.

The last section of the work described in this chapter was concerned with kinetics and stability of OA. Understanding the routes of elimination and kinetics of OA in serum are essential if it is to be a viable biomarker, as this knowledge enables more intelligent sampling times as well as going some way to gaining information about the metabolism of OA. To mimic an increase in serum OA that might occur following extensive hepatic GSH consumption, a large intravenous dose of OA was administered to anaesthetised male rats. The concentration of OA was monitored in bile, serum and urine. The majority of the dose appeared in serum, with a small amount observed in bile. OA was not detected in urine. Employing the single-compartment model used in pharmacokinetic studies, values of some kinetic parameters were calculated. OA was found to have a half-life in serum of 26.18min. This gives a relatively short window of time in which serum samples would be reflective of an initial increase in OA. The absence of OA from bile and urine suggests that OA is metabolised into constituent parts during transport into these biofluids. Serum and urine were also analysed for 2-AB, a constituent amino acid of OA. Whilst small amounts of 2-AB were found in the serum, presumably due to catabolism of OA in solid tissues. No 2-AB was found in the urine. This is predictable due to the fact that the amino acids filtered by the kidney are near completely reabsorbed by the proximal tubule (Verrey *et al.*, 2009). Ex-vivo stability studies reported in this chapter show fast metabolism of OA to 2-AB in rat kidney homogenate, as well as slower breakdown in rat liver homogenate, suggesting that OA is

subject to a metabolic fate similar to that of GSH. Whereas synthesis of GSH is intracellular, its catabolism takes place in the extracellular environment as part of the  $\gamma$ -glutamyl cycle (Fig 5.6). GSH and presumably OA are transported out of the cell and the first stage of metabolism is carried out by the membrane-bound enzyme,  $\gamma$ -glutamyltranspeptidase, which recruits an amino acid and conjugates it to the glutamate residue, releasing cysteinylglycine.  $\gamma$ -Glutamyltranspeptidase is expressed on the apical surfaces of transporting epithelia, such as liver canalicular or bile ductular membranes, but activity varies from organ to organ, with kidney activity being the highest (DeLap *et al.*, 1977; Meister *et al.*, 1976). This high kidney activity would account for the absence of OA from the urine. This enzyme has very low specificity for the  $\gamma$ -glutamyl donor and it is therefore likely that it will also catalyse the release of glutamate from OA to form aminobutyrylglycine (Fig 5.6). These dipeptides are then broken down by peptidase enzymes and the amino acids are actively reabsorbed into the cell and recycled. The  $\gamma$ -glutamylaminoacid is then converted to 5-oxoproline (the cyclic form of glutamate) by  $\gamma$ -glutamylcyclotransferase. 5-oxoproline is converted into glutamate by 5-oxoprolinease, and the glutamate can then be recycled (Fig 5.6).

The observations from all the studies reported in this chapter suggest that whilst elevated serum OA levels may be an interesting by-product of hepatic GSH consumption, they are not consistently reflective of it. OA also does not conform to certain defining characteristics for a reliable biomarker of DILI: it is not predictive of liver injury and has proved not to be transferable across all animal models of hepatotoxicity. However, it could potentially be used as a reflective biomarker specific to hepatic GSH depletion. The kinetic data presented here provides information which could be exploited to calculate optimal sampling times in rats. Human parameters can be predicted with good accuracy from animal studies), suggesting that OA could be used in both preclinical and clinical safety studies to assess a compound's potential to deplete hepatic GSH levels.



**Figure 5.6.** The synthesis and metabolism of GSH and OA

**References**

Awasthi, YC, Beutler, E, Srivastava, SK (1975) Purification and properties of human erythrocyte glutathione peroxidase. *J Biol Chem* **250**(13): 5144-5149.

Ballatori, N, Dutczak, WJ (1994) Identification and characterization of high and low affinity transport systems for reduced glutathione in liver cell canalicular membranes. *J Biol Chem* **269**(31): 19731-19737.

Blair, IA (2010) Analysis of endogenous glutathione-adducts and their metabolites. *Biomed Chromatogr* **24**(1): 29-38.

Bloch, K, Anker, HS (1947) Synthesis of glutathione in isolated liver. *J Biol Chem* **169**(3): 765.

Bradford, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Cho, ES, Johnson, N, Snider, BC (1984) Tissue glutathione as a cyst(e)ine reservoir during cystine depletion in growing rats. *J Nutr* **114**(10): 1853-1862.

Cliffe, EE, Waley, SG (1958) Acidic peptides of the lens. 4. The biosynthesis of ophthalmic acid. *Biochem J* **69**(4): 649-655.

Cliffe, EE, Waley, SG (1961) Acidic peptides of the lens. 7. The preparative enzymic synthesis of ophthalmic acid. *Biochem J* **79**: 669-671.

DeLap, LW, Tate, SS, Meister, A (1977) gamma-glutamyl transpeptidase and related enzyme activities in the reproductive system of the male rat. *Life Sci* **20**(4): 673-679.

Forman, HJ, Maiorino, M, Ursini, F (2010) Signaling functions of reactive oxygen species. *Biochemistry* **49**(5): 835-842.

Forman, HJ, Zhang, H, Rinna, A (2009) Glutathione: overview of its protective roles, measurement, and biosynthesis. *Mol Aspects Med* **30**(1-2): 1-12.

Garcia-Ruiz, C, Fernandez-Checa, JC, Kaplowitz, N (1992) Bidirectional mechanism of plasma membrane transport of reduced glutathione in intact rat hepatocytes and membrane vesicles. *J Biol Chem* **267**(31): 22256-22264.

Gerard-Monnier, D, Fougeat, S, Chaudiere, J (1992) Glutathione and cysteine depletion in rats and mice following acute intoxication with diethylmaleate. *Biochem Pharmacol* **43**(3): 451-456.

Graminski, GF, Zhang, PH, Sesay, MA, Ammon, HL, Armstrong, RN (1989) Formation of the 1-(S-glutathionyl)-2,4,6-trinitrocyclohexadienate anion at the active site of glutathione S-transferase: evidence for enzymic stabilization of sigma-complex intermediates in nucleophilic aromatic substitution reactions. *Biochemistry* **28**(15): 6252-6258.

Hou, Y, Guo, Z, Li, J, Wang, PG (1996) Seleno compounds and glutathione peroxidase catalyzed decomposition of S-nitrosothiols. *Biochem Biophys Res Commun* **228**(1): 88-93.

- Jian, W, Yao, M, Zhang, D, Zhu, M (2009) Rapid detection and characterization of in vitro and urinary N-acetyl-L-cysteine conjugates using quadrupole-linear ion trap mass spectrometry and polarity switching. *Chem Res Toxicol* **22**(7): 1246-1255.
- Johnson, KA, Plumb, R (2005) Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *J Pharm Biomed Anal* **39**(3-4): 805-810.
- Kasai T, Otobe Y, Kiriyama, S (1989) Changes in Ophthalmic Acid and Free Amino Acids in Rats Fed with an Excess Methionine Diet *Agric. Biol. Chem* **53**(2).
- Kasai, T, Otobe, Y, Kiriyama, S (1989) Identification of Glutathione Analogs in Rat Liver. *Agric. Bioll. Chem* **53**(2): 549-551.
- Meister, A (1989) Metabolism and Function of Glutathione. In: *Glutathione. Chemical, Biochemical and Medical Aspects*, David Dolphin, Poulson, R, Avramovic, O (eds) Vol. Part A, pp 367-474: John Wiley & Sons, Inc.
- Meister, A, Anderson, ME (1983) Glutathione. *Annu Rev Biochem* **52**: 711-760.
- Meister, A, Tate, SS (1976) Glutathione and related gamma-glutamyl compounds: biosynthesis and utilization. *Annu Rev Biochem* **45**: 559-604.
- Mitchell, JR, Jollow, DJ, Potter, WZ, Davis, DC, Gillette, JR, Brodie, BB (1973a) Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **187**(1): 185-194.
- Mitchell, JR, Jollow, DJ, Potter, WZ, Gillette, JR, Brodie, BB (1973b) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther* **187**(1): 211-217.
- Moldeus, P (1978) Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. *Biochem Pharmacol* **27**(24): 2859-2863.
- Pallardo, FV, Markovic, J, Garcia, JL, Vina, J (2009) Role of nuclear glutathione as a key regulator of cell proliferation. *Mol Aspects Med* **30**(1-2): 77-85.
- Prescott, LF, Park, J, Ballantyne, A, Adriaenssens, P, Proudfoot, AT (1977) Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. *Lancet* **2**(8035): 432-434.
- Seelig, GF, Meister, A (1985) Glutathione biosynthesis; gamma-glutamylcysteine synthetase from rat kidney. *Methods Enzymol* **113**: 379-390.
- Sies, H, Sharov, VS, Klotz, LO, Briviba, K (1997) Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *J Biol Chem* **272**(44): 27812-27817.
- Soga, T, Baran, R, Suematsu, M, Ueno, Y, Ikeda, S, Sakurakawa, T, Kakazu, Y, Ishikawa, T, Robert, M, Nishioka, T, Tomita, M (2006) Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem* **281**(24): 16768-16776.
- Srivastava, A, Maggs, JL, Antoine, DJ, Williams, DP, Smith, DA, Park, BK (2010) Role of reactive metabolites in drug-induced hepatotoxicity. *Handb Exp Pharmacol*(196): 165-194.
- Steele, RD, Weber, H, Patterson, JI (1984) Characterization of alpha-ketobutyrate metabolism in rat tissues: effects of dietary protein and fasting. *J Nutr* **114**(4): 701-710.

Timbrell, J (2000) Principles of Biochemical Toxicology, 3rd edn. London: Taylor & Francis Ltd.

Tsuboi, S, Hirota, K, Ogata, K, Ohmori, S (1984) Ophthalmic and norophthalmic acid in lens, liver, and brain of higher animals. *Anal Biochem* **136**(2): 520-524.

Vandeputte, C, Guizon, I, Genestie-Denis, I, Vannier, B, Lorenzon, G (1994) A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol Toxicol* **10**(5-6): 415-421.

Verrey, F, Singer, D, Ramadan, T, Vuille-dit-Bille, RN, Mariotta, L, Camargo, SM (2009) Kidney amino acid transport. *Pflugers Arch* **458**(1): 53-60.

Waley, SG (1956) Acidic peptides of the lens. *Biochem J* **64**(4): 715-726.



## CHAPTER SIX

---

**GENERAL DISCUSSION**



## Contents

6.1. Discussion .....	178
6.2 Development of Methapyrilene as a Model Hepatotoxin .....	178
6.3 Thiophene Bioactivation and Toxicity in Hepatic Cell Models.....	181
6.3.1 The Use of Isolated Hepatocyte Suspensions to Study Thiophene Bioactivation and Toxicity .....	183
6.3.2 The Use of Single P450 Expressing Cell Lines to Study Thiophene Bioactivation and Toxicity .....	188
6.4 Mechanistic Biomarkers of Drug Induced Liver Injury.....	190
6.5.1 Biomarkers of Hepatic Glutathione Consumption.....	191
6.5 Conclusions.....	194
6.6.1 Concluding Remarks.....	196

## Abbreviations

2-PT, 2-phenylthiophene; ALT, serum alanine transferase; APAP, acetaminophen; DEM, diethylmaleate; DILI, drug-induced liver injury; GCL, glutamate cysteine ligase; GSH, glutathione; MP, methapyrilene; OA, ophthalmic acid; P450, cytochrome P450 mixed function oxidase; TA, tienilic acid; TC, ticlopidine; THLE-CYP, SV40 large tumour antigen-immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes.

## 6.1. Discussion

The work described in this thesis aimed to investigate if various animal and human liver models and analysis by mass spectrometry can be used to establish a link between bioactivation and toxicity of thiophene drugs, and how useful these models would be as preclinical drug safety tools. This includes the potential use of thiophene molecules with well defined chemistry as model hepatotoxins, and whether by obtaining integrated metabolic and toxicological data in these models aids in exploring structure toxicity relationships. The usefulness of ophthalmic acid (OA) as a potential biomarker of hepatic glutathione (GSH) depletion was investigated, with a view to exploring biochemical mechanism of DILI, including beginning to define the kinetics of OA in serum.

## 6.2 Development of Methapyrilene as a Model Hepatotoxin

Methapyrilene (MP) is H<sub>1</sub> receptor antagonist and was introduced in the 1950s and used commonly as a sleeping aid for 25 years until being withdrawn due to hepatocarcinogenicity in chronically dosed rats (Lijinsky *et al.*, 1980). The carcinogenic effect appears to be non-mutagenic – a negative result is obtained in the Ames test – and species specific, with no carcinomas reported in humans, hamsters, guinea pigs and mice (Brennan *et al.*, 1982; Reznik-Schuller *et al.*, 1981). As well as having carcinogenic effects, dose dependent hepatotoxicity in rats has also been reported following both acute (Cunningham *et al.*, 1995; Mercer *et al.*, 2009; Ratra *et al.*, 2000) and chronic (Cunningham *et al.*, 1995) dosing in vivo. Being rat specific, MP has been used as a model hepatotoxin in both in vivo and in vitro rat

models. Chronic in vivo dosing regimens of between 100mg/kg/day to 300mg/kg/day for three days produce mild to moderate injury, consisting of necrosis, inflammatory cell infiltration and bile duct proliferation. In vitro, MP produces concentration dependent toxicity in isolated rat hepatocytes (Graham *et al.*, 2008; Ratra *et al.*, 1998b). MP has also been used as model hepatotoxin in systems biology studies (Craig *et al.*, 2006; Man *et al.*, 2002). Despite the common use of MP in this way, any chemical basis behind the hepatotoxicity associated with MP had yet to be defined. MP has several structural alerts with the potential to act as a substrate for metabolic bioactivation (Kalgutkar *et al.*, 2005). Previous in vitro studies using rabbit liver microsomes and cyanide as a trapping agent, have identified an iminium ion as a reactive intermediate of MP (Ziegler *et al.*, 1981), but hepatotoxicity has not been noted in the rabbit, and it is unclear how this relates to the use of MP as a model hepatotoxin in the rat. Subsequent investigation showed MP toxicity to depend upon both bioactivation and presence of the thiophene ring (Graham, 2007; Graham *et al.*, 2008; Ratra *et al.*, 1998a). Whilst a GSH adduct had previously been reported in in vivo and in vitro investigations, and that the site of GSH conjugation was the thiophene ring (Graham, 2007), whether the reactive intermediate involved was an epoxide, *S*-oxide or  $\alpha,\beta$ -unsaturated aldehyde had not been determined. Previously, the  $\alpha,\beta$ -unsaturated aldehyde intermediate has only been postulated following detection of pyrazine adduct of suprofen following use of semicarbazide as a trapping agent (O'Donnell *et al.*, 2003). Epoxide and *S*-oxide intermediates are more widely reported both in vivo and in vitro, in investigations with TA (Belghazi *et al.*, 2001; Dansette *et al.*, 1990; Mansuy *et al.*, 1984; Nishiya *et al.*, 2008a) and its isomer (Dansette *et al.*, 1991), 2-PT (Dansette *et al.*, 2005) and TC (Lim *et al.*, 2008; Shimizu *et al.*, 2009; Wen *et al.*, 2008). The use of LC-MS/MS analysis following

incubation of MP with rat liver microsomes allowed us to confirm the site of oxidation as the thiophene ring (Fig 2.8). In order to elucidate if the GSH adduct detected in this system was the product of an epoxide or *S*-oxide intermediate, hydrogen/deuterium exchange was utilised. A similar method has successfully been used in other investigations to determine *S*-oxides and N-oxides from their hydroxyl counterparts (Chen *et al.*, 2009; Liu *et al.*, 2001; Medower *et al.*, 2008). Described in chapter 2, the use of hydrogen/deuterium (H/D) exchange and mass spectrometry allowed exploitation of the mass difference between the non-deuterated and deuterated GSH adduct of MP formed by rat liver microsomes to determine that the reactive intermediate was an *S*-oxide of the thiophene ring (Fig 2.9, Fig 2.10, Fig 2.11). Having defined the chemistry of MP bioactivation, this enables a fuller understanding of the mechanisms of MP induced hepatotoxicity, and the use of MP as a model hepatotoxin. The consequences of the bioactivation of MP to MP *S*-oxide have been investigated (Mercer *et al.*, 2009). Male Wistar rats receiving 150mg/kg/day for three days experienced zonal GSH depletion in the periportal region of the liver and significant increases in serum transaminase levels. Expression of proteins associated with cell defence, glutamate cysteine ligase and heme-oxygenase 1 were also increased (Mercer *et al.*, 2009). Histology also confirmed the presence of apoptotic bodies and necrotic activity as early as 3h. Apoptosis was also determined *in vitro* by assessing cytochrome *c* release and procaspase activation using isolated rat hepatocytes (Mercer *et al.*, 2009). Taken together, the chemical and biological evidence inform studies using MP as a model, and provide information about the potential of other thiophene containing molecules to illicit a similar response.

### 6.3 Thiophene Bioactivation and Toxicity in Hepatic Cell Models

Whilst microsomal incubations can provide information regarding metabolism, their use is restricted to this purpose, and indeed is limited due to the lack of co-factors present in incubations, which require addition in order to generate metabolites, e.g. NADPH and GSH. The use of whole hepatocyte incubations can overcome this problem and produce information regarding both Phase I and Phase II metabolism without the addition of co-factors. Isolated hepatocytes are recognised as a relevant model for studying metabolism, but also transporter interactions (Liu *et al.*, 1999), and toxicological potential of molecules (Tirmenstein *et al.*, 2002). Suspension or culture of isolated hepatocytes, will, under certain conditions, maintain the entire hepatic metabolising system and functional hepatocyte traits (Gomez-Lechon *et al.*, 2004; Gomez-Lechon *et al.*, 2003; O'Brien *et al.*, 2005). Correlation between human hepatocyte metabolic studies and human in vivo outcomes has been shown to be good (Ponsoda *et al.*, 2001), but this is not always the case (Fig 2.1). However, freshly isolated human hepatocytes are not readily available, and unlike isolated animal hepatocytes, are subject to marked inter-individual variability. Although cryopreservation and pooling of isolated human hepatocytes has begun to address this problem, the use of isolated animal hepatocytes offers an alternative, as fresh animal hepatocytes are more straightforward to obtain. By using suspensions, as opposed to culture, the incubation can be started soon after isolation when enzymatic capability will be highest. This competency will be maintained for up to six hours under correct suspension conditions and allows acute investigation of both chemistry of metabolism and toxicological endpoints in the same incubation (O'Brien *et al.*,

2004; O'Brien *et al.*, 2005). This would enable an integrated exploration of structure toxicity relationships.

Despite presenting simpler culture conditions, hepatoma cell lines represent a limited model for studying hepatic metabolism, due to the low expression of metabolic enzymes (Donato *et al.*, 2008). However, cell lines such as these have previously been used to investigate toxicological potential of molecules (Mersch-Sundermann *et al.*, 2004). However, the lack of metabolic competency in hepatoma cell lines means that any toxicity due to metabolism could be missed, and therefore development of transfected metabolic enzyme systems in hepatoma derived cell lines would provide a useful tool for study of the role of metabolism in hepatotoxicity. Transfected human (HepG2) and murine cell lines have been developed with both stable and inducible cDNA encoding for both Phase I and Phase II metabolising enzymes and cell defence genes (Aoyama *et al.*, 1990; Goldring *et al.*, 2006; Puga *et al.*, 1990), however, the P450 expression in these cells is often transient and of low level when compared to hepatocytes. A stable transfection with cDNA of P450s using simian virus 40 and a cytomegalovirus promoter has recently been developed using a human liver epithelial cell line (THLE-5b). These cells have a hepatocyte-like phenotype, expressing high levels of Phase 2 metabolising enzymes and when transfected, the THLE-CYP cell lines express comparable levels of P450 to human hepatocytes (Mace *et al.*, 1997). By engineering several cell lines expressing single human P450s, there is scope for investigating the role of discrete P450 enzymes in the metabolism and cytotoxicity of drugs.

Both freshly isolated rat hepatocyte suspensions and THLE-CYP cell lines were used in this work, both with the aim of evaluating them as models to investigate

metabolism dependent toxicity, but also to enhance knowledge of thiophene induced hepatotoxicity. Several thiophene containing drugs have been linked with some kind of adverse effect in the liver, including, but not limited to, those studied in this work. From the same family of platelet aggregation inhibitors as TC, clopidogrel has also been associated with hepatotoxicity in patients (Hollmuller *et al.*, 2006), as well as inhibition of certain P450 enzymes (Nishiya *et al.*, 2009a; Nishiya *et al.*, 2009b). Zileuton, used in asthma therapy, has been shown to cause an increase in serum transaminase levels in clinical practice (Watkins *et al.*, 2007). For both of these drugs, there is evidence that bioactivation may be involved. Clopidogrel has been shown to form sulfenic acid intermediates (Dansette *et al.*, 2009) and also to inhibit CYP2B6 and CYP2C19 via metabolic mechanisms (Nishiya *et al.*, 2009a; Nishiya *et al.*, 2009b). Zileuton is thought to form a 2-acetylthiophene S-oxide metabolite, capable of alkylating human serum albumin (Joshi *et al.*, 2004; Li *et al.*, 2007). The bioactivation of MP, 2-phenylthiophene (2-PT), ticlopidine (TC) and tienilic acid (TA) will be discussed in more detail below.

### **6.3.1 The Use of Isolated Hepatocyte Suspensions to Study Thiophene Bioactivation and Toxicity**

Chapter three dealt with the use of freshly isolated rat hepatocytes as a model for providing integrated information regarding bioactivation and toxicity of xenobiotics, using thiophene containing molecules as a paradigm. Freshly isolated rat hepatocytes have previously been successfully used as a model for studying bioactivation and toxicity (O'Brien *et al.*, 2005; Regan, 2009). The aim of the chapter was not only to evaluate this model, but also to provide evidence of a link between metabolism of the thiophene ring and onset of hepatocyte damage. The model was further extended

to investigate the effect of metabolism on toxicity and of GSH conjugation as a detoxification method by pre incubation with 1-aminobenzotriazole (ABT), a P450 inhibitor, and diethylmaleate, a depletor of GSH. ABT is a mechanism-based inactivator of P450 (Ortiz de Montellano *et al.*, 1981) that is regarded as a non-specific inhibitor of P450 isoforms, and while ABT is invaluable for identifying a P450-mediated contribution to metabolism and hepatocytotoxicity in vitro (Boyland *et al.*, 1967; Graham *et al.*, 2008; Martin *et al.*, 2009; Williams *et al.*, 2007), it cannot be assumed that any remaining enzymatic activity or biological effect after ABT pretreatment is non-P450 mediated. DEM (1mM) depletes the GSH in isolated male rat hepatocytes by approximately 75-90% after 30-60 min (Dicker *et al.*, 1986; Larrauri *et al.*, 1987).

The decision to choose to study 2-PT, despite it not being a therapeutic molecule was taken due to the evidence that 2-PT is bioactivated in vitro to both an epoxide and *S*-oxide intermediate. Analysis by LC-MS and NMR of incubation of 2-PT with both rat liver microsomes from  $\beta$ -naphthoflavone induced rats and human CYP1A1 supersomes confirmed presence of two oxygenated GSH adducts of 2-PT, formed via a thiophene epoxide and a thiophene *S*-oxide intermediate (Dansette *et al.*, 2005). The results obtained here from incubations of 2-PT with freshly isolated rat hepatocytes produced a similar metabolic profile (Fig 3.5). No previous studies have been carried out to investigate whether 2-PT induces hepatocytotoxicity, and whether this toxicity is metabolism dependent. Pre-depletion of GSH with DEM significantly reduced the IC<sub>50</sub> of 2-PT, bringing to light the importance of detoxification pathways in thiophene toxicity. If we use 2-PT as a paradigm compound for thiophene drugs, these results imply that oxidation of the thiophene ring to an *S*-oxide or epoxide have toxicological consequences.



As previously discussed, TA was withdrawn for the market following cases of autoimmune hepatitis, characterised by circulation of anti-LKM2 antibodies (Homberg *et al.*, 1984). Although the bioactivation of TA to a thiophene *S*-oxide intermediate has been extensively studied (Belghazi *et al.*, 2001; Dansette *et al.*, 1991; Mansuy *et al.*, 1984), as well its ability to inhibit CYP2C9 (Lopez-Garcia *et al.*, 2005; Lopez-Garcia *et al.*, 1994), linking this bioactivation to toxicity has proved more difficult. Recently, GSH was shown to be a critical component in TA hepatotoxicity in vivo (Nishiya *et al.*, 2008b). Male Sprague-Dawley rats were administered either buthionine-sulfoximine (BSO) - a glutamate cysteine ligase inhibitor, and therefore depletor of GSH - prior to TA administration, or were treated with TA alone. In groups receiving BSO, serum transaminases, bilirubin and bile acids were significantly increased over the animals receiving TA alone. A similar, although not significant, effect was also observed in ex vivo investigations in chapter three of this work. Cytotoxicity of TA to freshly isolated rat hepatocyte suspensions was increased when hepatocytes were co-incubated with the GSH depletor, diethylmaleate (DEM) (Fig 3.3 D), suggesting GSH is involved in protection from TA hepatotoxicity. This is backed up by the observation of GSH adducts in the freshly isolated rat hepatocyte model (Fig 3.6). This report is the first to describe a decrease in cell viability and to detect GSH adducts of TA in the same ex vivo incubation, therefore providing evidence of a link between TA bioactivation and hepatocyte damage, and a crucial role for GSH in protection from this injury.

MP was withdrawn from the market due to reports of rat hepatic carcinomas (Lijinsky *et al.*, 1980). The hepatotoxic effects in rats have been investigated, including its potential to cause oxidative stress and disrupt mitochondrial function (Ratra *et al.*, 1998b) and to cause zonal depletion of GSH (Mercer *et al.*, 2009). The

connection between bioactivation of MP in the rat and cytotoxicity is substantiated by several studies. Inhibition of P450 by cobalt protoporphyrin IX afforded protection against MP induced liver injury in vivo (Ratra *et al.*, 1998a). Pre-incubation with the P450 inhibitor 2-aminobenzotriazole (ABT) attenuated hepatotoxicity in freshly isolated hepatocytes incubated with MP (Graham, 2007). The results obtained in this work from suspensions of freshly isolated hepatocytes incubated with MP are in keeping with previously reported findings, both alone and following pre-incubation with both ABT and DEM (Graham, 2007; Mercer *et al.*, 2009).

The data presented on the hepatotoxicity of TC in chapter three is a novel demonstration of the cytotoxic effects of TC in a rat liver model. It is also the first report describing both hepatotoxicity of TC and formation of GSH adducts in the same model, as well as the first observation of simultaneous formation of two GSH adducts formed via both epoxidation and *S*-oxidation of the thiophene ring (Fig.3.7). The hepatotoxicity observed in freshly isolated hepatocytes was not attenuated by pre-incubation with ABT, but was increased by pre-incubation with DEM (Fig. 3.3D). This suggests that GSH is vital to protection from TC induced hepatotoxicity, presumably by conjugation to reactive metabolites. It also suggests that addition of ABT may inhibit an alternative, non-reactive route of metabolism, therefore increasing the proportion of TC being converted to a reactive metabolite to over a threshold that can be coped with by detoxification processes.

In terms of thiophene metabolism, the aim in chapter three was to determine *if* the xenobiotics were bioactivated in the rat hepatocyte model used. However, using product ion scans, it was possible to ascertain more information regarding the chemistry of bioactivation, including the identification of a GSH adduct formed from

the previously described *S*-oxide metabolite of MP (Fig 3.4), both a hydroxyl GSH adduct, formed via an epoxide intermediate and an *S*-oxide GSH adduct of 2-PT, as described by Dansette et al (2005) (Fig 3.5), and both an hydroxyl GSH adduct and *S*-oxide GSH adduct of TC (Fig 3.7), in keeping with the literature (Lim *et al.*, 2008; Shimizu *et al.*, 2009; Wen *et al.*, 2008) Previously however, these two metabolites have not been detected simultaneously. A non-oxygenated GSH adduct of TA was also detected (Fig 3.6). This GSH adduct has previously been described (Belghazi *et al.*, 2001; Nishiya *et al.*, 2008a). Here, the authors suggest that an *S*-oxide intermediate is formed, but that loss of H<sub>2</sub>O across the thiophene ring accounts for the resulting non-oxygenated GSH adduct.

The presence of previously described metabolites of all four thiophene compounds stands to provide evidence that freshly isolated hepatocyte suspensions are a useful tool in metabolic profiling and metabolism. The ability to identify metabolic profile, assess toxicological potential and understand the importance of bioactivation and detoxification by use of ABT and DEM in one incubation affords a large amount of information which when visualised as a whole, provides an integrated picture of the chemistry of reactive metabolites and the hepatotoxic potential this bioactivation may cause. However, due to the short time scale in which hepatocytes in suspension remain viable (up to 6h), they are only able to provide a picture of an acute situation and not how cells may react to molecules or their metabolites over time.

### 6.3.2 The Use of Single P450 Expressing Cell Lines to Study Thiophene Bioactivation and Toxicity

Single CYP expressing THLE cells as a method for assessing the role of bioactivation by discrete P450 enzymes in hepatocytotoxicity was evaluated in chapter four. Incubation of MP, 2-PT and TA in five cell lines (CYP1A2, CYP2C9, CYP2D6, CYP3A4 and a non CYP expressing line) and subsequent assessment of cell viability and GSH adduct formation aimed to inform about the role of the above P450 enzymes in thiophene metabolism and toxicity. Incubations (72h) with TA exhibited the most toxicity, though showed no difference in susceptibility between the cell lines (Fig 4.2C). Incubations with MP (72h) also resulted in a drop in cell viability at the highest concentrations (Fig 4.2B) in CYP3A4, CYP2D6 and CYP1A2 cell lines. The lack of toxicity of MP in this system versus the cytotoxicity observed in freshly isolated rat hepatocytes was reflected in the lack of observation of any GSH adducts. The only evidence of bioactivation for any of the drugs was the presence of a GSH adduct and *S*-oxide dimer in incubations with 2-PT and CYP1A2 and CYP2C9 cell lines, respectively. It was therefore difficult to make any meaningful connection between bioactivation and toxicity of thiophenes in these cell lines. This is particularly disappointing for the incubations of TA and MP with the CYP2C9 cell line. TA hepatotoxicity has previously been associated with CYP2C9 (Lopez-Garcia *et al.*, 1994). Metabolism of MP has also been linked to rat CYP2C11, the ortholog of human CYP2C9.

Previously, THLE-CYP1A2 cells have been used to demonstrate metabolism and protein binding of thiabendazole (Coulet *et al.*, 2000) and THLE-CYP2D6 cells have

been used to investigate metabolism of diltiazem (Molden *et al.*, 2000). P450 dependent toxicity of chlorpromazine has also been described using this model (Greer *et al.*, 2010). However, metabolite identification *and* toxicity has only been demonstrated with troglitazone in non-transfected THLE-2 cells (Saha *et al.*, 2010a). Use of other human derived cell lines for investigating P450 dependent toxicity has proved more successful. Transfection or retroviral infection of HepG2 cells with human CYP2E1, has allowed exploration of the role of CYP2E1 of ethanol induced liver disease, but metabolites were not identified (Cederbaum *et al.*, 2001). HepaRG cells, a human hepatoma derived cell line which differentiates into a hepatocyte like cell after two weeks at confluency, represent a promising model (Guillouzo *et al.*, 2007). These tumour derived cells, were found to form colonies, including formation of bile canalicular and epithelial cells following addition of dimethylsulfoxide (Aninat *et al.*, 2006). PCR analysis also revealed expression of Phase I and Phase II metabolising enzymes, and activity of enzymes was found to be inducible in a similar fashion to in primary human hepatocytes. Both metabolite identification and determination of cell viability were achieved in this cell line, and the authors suggest they are a comparable model to cultured primary human hepatocytes (Aninat *et al.*, 2006).

The data gathered on metabolism and toxicity of thiophenes in the THLE cell model, and the lack of published studies determining both metabolism and toxicity in this model suggest that predicting P450 dependant toxicity using the THLE cell model is potentially flawed method. However, useful information could be conceived about the roles of individual P450 enzymes in the metabolism or toxicity of certain drugs. Development of the HepaRG cell line could provide an alternative to the use of isolated hepatocytes in this field of study.

#### 6.4 Mechanistic Biomarkers of Drug Induced Liver Injury

As discussed previously, the mechanisms involved in DILI are varied and complex. Traditional routes of diagnosis have relied on determining certain clinical chemistry parameters and histology. Plasma levels of hepatic transaminase enzymes and other proteins, such as bilirubin, are taken to represent breakdown of cell disruption or organelle damage by either apoptotic or necrotic means. Differences in the order or level of protein or enzyme elevation are used to distinguish between cholestatic, mitochondrial or cytotoxic injury, but do not give information regarding the mechanisms behind the injury, are not predictive and are not entirely liver specific. For example, measurement of total serum ALT takes into account both ALT1 and ALT2. Whilst ALT1 is expressed highly in human liver tissue (as well as skeletal muscle and kidney), ALT2 is found in heart and skeletal muscle tissue (Lindblom *et al.*, 2007). Enzyme levels can also be elevated in other disease states, such as liver cancer, fatty liver disease and viral hepatitis (Ozer *et al.*, 2008). Ideally, a biomarker would be specific not only to hepatic injury, but reflective and preferably predictive, of the type of injury and the mechanisms behind that injury. It should also be minimally invasive, rapidly assayed, and to be useful both in preclinical safety studies and the clinic, transferable across in vitro and in vivo studies, as well as across species. Measurable changes in DNA, protein or metabolite levels could all act as biomarkers, alone or in a pattern or 'fingerprint' of DILI.

There are several novel biomarkers currently being developed as potential mechanism specific and markers reflective of DILI. High-mobility group box protein 1 (HMGB1), is a nuclear binding protein with proinflammatory activity and has been

suggested as playing a role in indicating the presence of dying cells to the immune system (Scaffidi *et al.*, 2002). Activation of the innate immune system following drug-induced hepatic necrosis is thought to play a key role in the extent of damage caused (Liu *et al.*, 2004). HMGB1 is released in two forms; a hyper-acetylated form is released from activated innate immune cells (Bonaldi *et al.*, 2003), and a hypo-acetylated form is released passively from necrotic cells (Scaffidi *et al.*, 2002). An increase in serum HMGB1 levels has been observed in endotoxin exposure and autoimmune disease. Measurable in a minimally invasive medium and reflective of mechanisms involved in injury, HMGB1 could potentially inform about the level of innate immune involvement in DILI. However, there are many other mechanisms involved in DILI for which a suitable biomarker still remains elusive.

### **6.5.1 Biomarkers of Hepatic Glutathione Consumption**

Depletion of GSH is a phenomenon observed in DILI associated with several model hepatotoxins (Kitteringham *et al.*, 2000). Conjugation of GSH to the electrophilic species following bioactivation of xenobiotics is a vital detoxification and therefore protection system within the hepatocyte. However, in certain cases where GSH is depleted below a certain threshold, this can be a factor in the onset of cell death. High concentrations of GSH (5mM) are found in the hepatocyte, and this is critical to maintaining the redox status of the cell, and when depleted, free radicals and electrophilic species cannot be effectively detoxified, and can therefore modify cellular proteins. As covered in chapter five, the synthesis of GSH is a two step process controlled by two discrete enzymes, glutamate cysteine ligase (GCL) and glutathione synthetase. This enzyme is under a negative feedback inhibition

controlled by GSH concentrations, decreases in GSH increase the activity of the enzyme. It is because of this that it is proposed that levels of the GSH analog, ophthalmic acid (OA) will rise following depletion of GSH. A metabolomic investigation (Soga *et al.*, 2006) found increased levels of a metabolite with a mass of 290amu following hepatic depletion of GSH in mice by acetaminophen (APAP) administration. This metabolite was revealed to be OA following examination by mass spectrometry. The authors suggest that depletion of GSH leads to an increase in the activity of GCL which, following a depletion in the cysteine pool, recruits 2-aminobutyrate in place of cysteine, forming the precursor to OA. Evidence for this theory is also presented in the study. Mice were administered either buthionine sulphoximine (BSO), an inhibitor of GCL, or DEM, a GSH depletor. OA serum levels increased in the mice administered DEM, but not in those administered BSO. This suggests that serum OA increases would be reflective of direct depletion of hepatic GSH via conjugation, rather than through inhibition of either GCL or glutathione synthetase. OA could therefore serve as a non-invasive biomarker of direct hepatic GSH depletion.

Chapter five aimed to explore the viability of OA as a biomarker of hepatic OA depletion. Firstly, in an attempt to replicate the results from Soga (Soga *et al.*, 2006), and therefore to validate the MS method, APAP was administered to C57BL6 in a toxic dose (530mg/kg) in order to achieve GSH depletion. Whilst an increase in serum OA was observed, it was delayed from the original GSH depletion by 5h (Fig 5.1). As discussed in chapter five, this may be due to differences in the experimental method used, specifically in that animals in the previous study were starved prior to treatment, possibly affecting enzymes related to 2-aminobutyrate metabolism (Kasai



T *et al.*, 1989; Steele *et al.*, 1984) , and potentially causing a depletion in hepatic GSH (Leeuwenburgh *et al.*, 1998).

Although chronic APAP dosing in rats by infusion did increase OA at late time points (48h, Fig. 5.2), administration of DEM to anaesthetised rats did not produce a significant increase in serum or hepatic OA within the 2h of the study time, despite a huge depletion in hepatic GSH (Fig 5.3). Kinetic studies also revealed that OA has a short half-life (35min) in rat serum, potentially impacting on sampling time (Fig. 5.4 and Table 5.3) if OA were to be developed as a biomarker. Therefore, although serum OA levels could be used alongside other investigations to provide supplementary information on the mechanisms of liver injury, alone it is unlikely to be suitable as a biomarker of GSH consumption, due to the delay between initial depletion of GSH and elevation of serum OA, and the lack of consistency in OA increase between the models presented.

As well as OA, there are several other metabolites in the glutathione metabolomic pathway which may serve as potential biomarkers of disruption of GSH status or oxidative stress. Taurine, has already been assessed as both a urine biomarker of DILI and a hepatoprotectant in rats dosed with model hepatotoxins (Waterfield *et al.*, 1993a; Waterfield *et al.*, 1993b; Waterfield *et al.*, 1993c). 5-oxoproline, has also been identified in rat biofluids following exposure to bromobenzene (Waters *et al.*, 2006).

Whilst many other biomarkers are available to analyse for a state of oxidative stress, e.g. lipid peroxides, serum GSH and oxidised GSH concentrations and hydrogen peroxide, and of liver injury in general, e.g. serum transaminase concentrations, more specific biomarkers or a 'signature' of proteins or metabolites would provide

much more information regarding the type of injury caused and, in clinic, aid in diagnosis and treatment. There is therefore currently, much focus on the use of proteomics, metabolomics and transcriptomics to provide an integrated systems biology approach for identifying possible biomarkers or to identify patterns associated with not only DILI (Amacher, 2010; Lee *et al.*, 2009), but many other types of organ or system injury and various disease states (Nam *et al.*, 2009; Rosner, 2009; Waterman *et al.*, 2010).

## 6.5 Conclusions

The thiophene ring is a potentially dangerous toxicophore, due to the ability of P450 enzymes to form thiophene epoxides and thiophene *S*-oxides. However, incorporation of a thiophene ring into a therapeutic agent does not necessarily result in a drug capable of causing DILI. Despite this, care should be taken to ensure thorough pre-clinical metabolism and safety studies on any new drugs containing a thiophene ring. The H/D exchange method utilised in chapter one to define the reactive metabolite of methapyrilene formed in rat microsomal incubations is a simple technique which when used with mass spectrometry can provide information on the chemistry of thiophene bioactivation. The well defined chemistry of reactive intermediates associated with thiophenes means that they are useful tools for studying mechanisms of DILI following bioactivation and to determine links between formation of reactive metabolites and onset of hepatocyte damage.

The freshly isolated rat hepatocyte model is an ideal tool with which to carry out investigations into structure, metabolism and toxicity as results can be evaluated in

the same incubation. This model can also be used to determine covalent binding (if radioactive substrate is available), GSH concentrations and could be expanded to examine different toxicity endpoints, such as mitochondrial function or lactose dehydrogenase leakage. There is also the potential to measure proteomic or metabolomic biomarkers, using mass spectrometry. Ideally, human hepatocytes could be used to present a more true reflection of the *in vivo* situation in the patient; however, rat hepatocytes are more readily available and subject to less variation between donors. Unfortunately, incubations with single P450 expressing THLE cells did not enable any links to be extrapolated between individual enzymes and bioactivation or toxicity of thiophene drugs. The use of chlorpromazine as a positive control for toxicity validates the data obtained in the toxicity side of the work. However, despite some toxicity observed for MP and TA, no evidence of bioactivation was detected. Whether this a false negative is not known and would require further investigation, taking into account several factors, such as a 'mis-hit' of the P450 enzymes involved in metabolism, the range of drug concentrations examined or the methods involved in incubation or in analysis by mass spectrometry. For example, other studies using THLE cells have used a larger incubation volume and number of cells in metabolism studies than was utilised in this work (Saha *et al.*, 2010b). Other human derived cell lines may be more appropriate for elucidating information on the mechanisms of DILI, such as the HepaRG cell line, shown to differentiate to a hepatocyte like phenotype, expressing metabolic enzymes at a similar level to isolated human hepatocytes (Aninat *et al.*, 2006; Guillouzo *et al.*, 2007).

Biomarkers have the potential to expand knowledge of mechanisms of DILI and to enhance preclinical safety studies, as well as translating to the clinic as diagnostic tools. In vivo investigations using depletors of hepatic GSH determined that elevation of serum OA is not a consistent product of hepatic GSH consumption and that there is a delay between decrease in GSH and increase in OA. However, despite the unsuitability of OA as a singular biomarker of GSH depletion, the kinetic and stability studies did provide evidence that OA is subject to the same routes of metabolism of GSH, via breakdown by  $\gamma$ -glutamyltranspeptidase on kidney cell membrane. Metabolic pathways could as yet be exploited to reveal a novel biomarker.

### 6.6.1 Concluding Remarks

The original aim of this thesis was to answer the following questions:

- How mass spectrometry can be used to provide information on the chemistry of thiophene bioactivation?
- Are thiophene GSH adducts formed in certain animal and human in vitro models, and if so can they be linked to any observed toxicity?
- Can serum OA levels be exploited as a biomarker of hepatic GSH depletion?

The overall scope of this work reveals the usefulness of H/D exchange in combination with mass spectrometry to define thiophene *S*-oxide and epoxide

intermediates, as well as the use of LC-MS/MS to identify these adducts in vitro studies. The freshly isolated rat hepatocyte model was a useful tool for providing integrated metabolic and toxicological data of thiophene containing molecules and has the potential to be expanded to include data on covalent binding and levels of DILI biomarkers. The single CYP expressing THLE cell model was not as useful in this case, but has been used in other studies to explore the role of discrete P450 enzymes in toxicity (Greer *et al.*, 2010) and metabolism (Coulet *et al.*, 2000; Molden *et al.*, 2000). Whilst it is unfortunate that serum OA did not reflect hepatic OA in such a way that it could be easily exploited as a biomarker, this does help us to understand that the plethora of potential biomarkers uncovered by proteomic, metabolomic and transcriptomic studies need to be investigated in depth in order to understand their applications across different species and systems.

## References

- Amacher DE (2010). The discovery and development of proteomic safety biomarkers for the detection of drug-induced liver toxicity. *Toxicol Appl Pharmacol* **245**(1): 134-142.
- Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, *et al.* (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos* **34**(1): 75-83.
- Aoyama T, Yamano S, Guzelian PS, Gelboin HV, Gonzalez FJ (1990). Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B1. *Proc Natl Acad Sci U S A* **87**(12): 4790-4793.
- Belghazi M, Jean P, Poli S, Schmitter JM, Mansuy D, Dansette PM (2001). Use of isotopes and LC-MS-ESI-TOF for mechanistic studies of tienilic acid metabolic activation. *Adv Exp Med Biol* **500**: 139-144.
- Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, *et al.* (2003). Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J* **22**(20): 5551-5560.
- Boyland E, Chasseaud LF (1967). Enzyme-catalysed conjugations of glutathione with unsaturated compounds. *Biochem J* **104**(1): 95-102.
- Brennan LM, Creasia DA (1982). The effects of methapyrilene hydrochloride on hepatocarcinogenicity and pentobarbital-induced sleeping time in rats and mice. *Toxicology and applied pharmacology* **66**(2): 252-258.
- Cederbaum AI, Wu D, Mari M, Bai J (2001). CYP2E1-dependent toxicity and oxidative stress in HepG2 cells. *Free Radic Biol Med* **31**(12): 1539-1543.
- Chen G, Daaro I, Pramanik BN, Piwinski JJ (2009). Structural characterization of in vitro rat liver microsomal metabolites of antihistamine desloratadine using LTQ-Orbitrap hybrid mass spectrometer in combination with online hydrogen/deuterium exchange HR-LC/MS. *J Mass Spectrom* **44**(2): 203-213.
- Coulet M, Eeckhoutte C, Larrieu G, Sutra JF, Alvinerie M, Mace K, *et al.* (2000). Evidence for cytochrome P4501A2-mediated protein covalent binding of thiabendazole and for its passive intestinal transport: use of human and rabbit derived cells. *Chem Biol Interact* **127**(2): 109-124.
- Craig A, Sidaway J, Holmes E, Orton T, Jackson D, Rowlinson R, *et al.* (2006). Systems toxicology: integrated genomic, proteomic and metabonomic analysis of methapyrilene induced hepatotoxicity in the rat. *J Proteome Res* **5**(7): 1586-1601.

Cunningham ML, Pippin LL, Anderson NL, Wenk ML (1995). The hepatocarcinogen methapyrilene but not the analog pyrilamine induces sustained hepatocellular replication and protein alterations in F344 rats in a 13-week feed study. *Toxicol Appl Pharmacol* **131**(2): 216-223.

Dansette PM, Amar C, Smith C, Pons C, Mansuy D (1990). Oxidative activation of the thiophene ring by hepatic enzymes. Hydroxylation and formation of electrophilic metabolites during metabolism of tienilic acid and its isomer by rat liver microsomes. *Biochem Pharmacol* **39**(5): 911-918.

Dansette PM, Amar C, Valadon P, Pons C, Beaune PH, Mansuy D (1991). Hydroxylation and formation of electrophilic metabolites of tienilic acid and its isomer by human liver microsomes. Catalysis by a cytochrome P450 IIC different from that responsible for mephenytoin hydroxylation. *Biochem Pharmacol* **41**(4): 553-560.

Dansette PM, Bertho G, Mansuy D (2005). First evidence that cytochrome P450 may catalyze both S-oxidation and epoxidation of thiophene derivatives. *Biochem Biophys Res Commun* **338**(1): 450-455.

Dansette PM, Libraire J, Bertho G, Mansuy D (2009). Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem Res Toxicol* **22**(2): 369-373.

Dicker E, Cederbaum AI (1986). Inhibition of the low-K<sub>m</sub> mitochondrial aldehyde dehydrogenase by diethyl maleate and phorone in vivo and in vitro. Implications for formaldehyde metabolism. *Biochem J* **240**(3): 821-827.

Donato MT, Lahoz A, Castell JV, Gomez-Lechon MJ (2008). Cell lines: a tool for in vitro drug metabolism studies. *Curr Drug Metab* **9**(1): 1-11.

Goldring CE, Kitteringham NR, Jenkins R, Lovatt CA, Randle LE, Abdullah A, *et al.* (2006). Development of a transactivator in hepatoma cells that allows expression of phase I, phase II, and chemical defense genes. *Am J Physiol Cell Physiol* **290**(1): C104-115.

Gomez-Lechon MJ, Donato MT, Castell JV, Jover R (2004). Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab* **5**(5): 443-462.

Gomez-Lechon MJ, Donato T, Ponsoda X, Castell JV (2003). Human hepatic cell cultures: in vitro and in vivo drug metabolism. *Altern Lab Anim* **31**(3): 257-265.

Graham E PhD Thesis, University of Liverpool, Liverpool, 2007.

Graham EE, Walsh RJ, Hirst CM, Maggs JL, Martin S, Wild MJ, *et al.* (2008). Identification of the thiophene ring of methapyrilene as a novel bioactivation-dependent hepatic toxicophore. *J Pharmacol Exp Ther* **326**(2): 657-671.

Greer ML, Barber J, Eakins J, Kenna JG (2010). Cell based approaches for evaluation of drug-induced liver injury. *Toxicology* **268**(3): 125-131.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, Guguen-Guillouzo C (2007). The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact* **168**(1): 66-73.

Hollmuller I, Stadlmann S, Graziadei I, Vogel W (2006). Clinico-histopathological characteristics of clopidogrel-induced hepatic injury: case report and review of literature. *Eur J Gastroenterol Hepatol* **18**(8): 931-934.

Homberg JC, Andre C, Abuaf N (1984). A new anti-liver-kidney microsome antibody (anti-LKM2) in tienilic acid-induced hepatitis. *Clin Exp Immunol* **55**(3): 561-570.

Joshi EM, Heasley BH, Chordia MD, Macdonald TL (2004). In vitro metabolism of 2-acetylbenzothiothiophene: relevance to zileuton hepatotoxicity. *Chem Res Toxicol* **17**(2): 137-143.

Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, *et al.* (2005). A comprehensive listing of bioactivation pathways of organic functional groups. *Curr Drug Metab* **6**(3): 161-225.

Kasai T, Otake Y, Kiriya S (1989). Changes in Ophthalmic Acid and Free Amino Acids in Rats Fed with an Excess Methionine Diet *Agric. Biol. Chem* **53**(2).

Kitteringham NR, Powell H, Clement YN, Dodd CC, Tettey JN, Pirmohamed M, *et al.* (2000). Hepatocellular response to chemical stress in CD-1 mice: induction of early genes and gamma-glutamylcysteine synthetase. *Hepatology* **32**(2): 321-333.

Larrauri A, Lopez P, Gomez-Lechon MJ, Castell JV (1987). A cytochemical stain for glutathione in rat hepatocytes cultured on plastic. *J Histochem Cytochem* **35**(2): 271-274.

Lee MS, Jung BH, Chung BC, Cho SH, Kim KY, Kwon OS, *et al.* (2009). Metabolomics study with gas chromatography-mass spectrometry for predicting valproic acid-induced hepatotoxicity and discovery of novel biomarkers in rat urine. *Int J Toxicol* **28**(5): 392-404.

Leeuwenburgh C, Ji LL (1998). Glutathione and glutathione ethyl ester supplementation of mice alter glutathione homeostasis during exercise. *J Nutr* **128**(12): 2420-2426.

Li F, Chordia MD, Woodling KA, Macdonald TL (2007). Irreversible alkylation of human serum albumin by zileuton metabolite 2-acetylbenzothiothiophene-S-oxide: a potential model for hepatotoxicity. *Chem Res Toxicol* **20**(12): 1854-1861.



Lijinsky W, Reuber MD, Blackwell BN (1980). Liver tumors induced in rats by oral administration of the antihistaminic methapyrilene hydrochloride. *Science* **209**(4458): 817-819.

Lim HK, Chen J, Cook K, Sensenhauser C, Silva J, Evans DC (2008). A generic method to detect electrophilic intermediates using isotopic pattern triggered data-dependent high-resolution accurate mass spectrometry. *Rapid Commun Mass Spectrom* **22**(8): 1295-1311.

Lindblom P, Rafter I, Copley C, Andersson U, Hedberg JJ, Berg AL, *et al.* (2007). Isoforms of alanine aminotransferases in human tissues and serum--differential tissue expression using novel antibodies. *Arch Biochem Biophys* **466**(1): 66-77.

Liu DQ, Hop CE, Beconi MG, Mao A, Chiu SH (2001). Use of on-line hydrogen/deuterium exchange to facilitate metabolite identification. *Rapid Commun Mass Spectrom* **15**(19): 1832-1839.

Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, *et al.* (1999). Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* **277**(1 Pt 1): G12-21.

Liu ZX, Govindarajan S, Kaplowitz N (2004). Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology* **127**(6): 1760-1774.

Lopez-Garcia MP, Dansette PM, Coloma J (2005). Kinetics of tienilic acid bioactivation and functional generation of drug-protein adducts in intact rat hepatocytes. *Biochem Pharmacol* **70**(12): 1870-1882.

Lopez-Garcia MP, Dansette PM, Mansuy D (1994). Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry* **33**(1): 166-175.

Mace K, Aguilar F, Wang JS, Vautravers P, Gomez-Lechon M, Gonzalez FJ, *et al.* (1997). Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* **18**(7): 1291-1297.

Man WJ, White IR, Bryant D, Bugelski P, Camilleri P, Cutler P, *et al.* (2002). Protein expression analysis of drug-mediated hepatotoxicity in the Sprague-Dawley rat. *Proteomics* **2**(11): 1577-1585.

Mansuy D, Dansette PM, Foures C, Jaouen M, Moinet G, Bayer N (1984). Metabolic hydroxylation of the thiophene ring: isolation of 5-hydroxy-tienilic acid as the major urinary metabolite of tienilic acid in man and rat. *Biochem Pharmacol* **33**(9): 1429-1435.

Martin JW, Chan K, Mabury SA, O'Brien PJ (2009). Bioactivation of fluorotelomer alcohols in isolated rat hepatocytes. *Chem Biol Interact* **177**(3): 196-203.

Medower C, Wen L, Johnson WW (2008). Cytochrome P450 oxidation of the thiophene-containing anticancer drug 3-[(quinolin-4-ylmethyl)-amino]-thiophene-2-carboxylic acid (4-trifluoromethoxy-phenyl)-amide to an electrophilic intermediate. *Chem Res Toxicol* **21**(8): 1570-1577.

Mercer AE, Regan SL, Hirst CM, Graham EE, Antoine DJ, Benson CA, *et al.* (2009). Functional and toxicological consequences of metabolic bioactivation of methapyrilene via thiophene S-oxidation: Induction of cell defence, apoptosis and hepatic necrosis. *Toxicol Appl Pharmacol* **239**(3): 297-305.

Mersch-Sundermann V, Knasmuller S, Wu XJ, Darroudi F, Kassie F (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology* **198**(1-3): 329-340.

Molden E, Asberg A, Christensen H (2000). CYP2D6 is involved in O-demethylation of diltiazem. An in vitro study with transfected human liver cells. *Eur J Clin Pharmacol* **56**(8): 575-579.

Nam H, Chung BC, Kim Y, Lee K, Lee D (2009). Combining tissue transcriptomics and urine metabolomics for breast cancer biomarker identification. *Bioinformatics* **25**(23): 3151-3157.

Nishiya T, Kato M, Suzuki T, Maru C, Kataoka H, Hattori C, *et al.* (2008a). Involvement of cytochrome P450-mediated metabolism in tienilic acid hepatotoxicity in rats. *Toxicol Lett* **183**(1-3): 81-89.

Nishiya T, Mori K, Hattori C, Kai K, Kataoka H, Masubuchi N, *et al.* (2008b). The crucial protective role of glutathione against tienilic acid hepatotoxicity in rats. *Toxicol Appl Pharmacol* **232**(2): 280-291.

Nishiya Y, Hagihara K, Ito T, Tajima M, Miura S, Kurihara A, *et al.* (2009a). Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab Dispos* **37**(3): 589-593.

Nishiya Y, Hagihara K, Kurihara A, Okudaira N, Farid NA, Okazaki O, *et al.* (2009b). Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**(11): 836-843.

O'Brien PJ, Chan K, Silber PM (2004). Human and animal hepatocytes in vitro with extrapolation in vivo. *Chem Biol Interact* **150**(1): 97-114.

O'Brien PJ, Siraki AG (2005). Accelerated cytotoxicity mechanism screening using drug metabolising enzyme modulators. *Curr Drug Metab* **6**(2): 101-109.

O'Donnell JP, Dalvie DK, Kalgutkar AS, Obach RS (2003). Mechanism-based inactivation of human recombinant P450 2C9 by the nonsteroidal anti-inflammatory drug suprofen. *Drug Metab Dispos* **31**(11): 1369-1377.

Ortiz de Montellano PR, Mathews JM (1981). Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. Isolation of an NN-bridged benzyne-protoporphyrin IX adduct. *Biochem J* **195**(3): 761-764.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**(3): 194-205.

Ponsoda X, Pareja E, Gomez-Lechon MJ, Fabra R, Carrasco E, Trullenque R, *et al.* (2001). Drug biotransformation by human hepatocytes. In vitro/in vivo metabolism by cells from the same donor. *J Hepatol* **34**(1): 19-25.

Puga A, Raychaudhuri B, Salata K, Zhang YH, Nebert DW (1990). Stable expression of mouse Cyp1a1 and human CYP1A2 cDNAs transfected into mouse hepatoma cells lacking detectable P450 enzyme activity. *DNA Cell Biol* **9**(6): 425-436.

Ratra GS, Cottrell S, Powell CJ (1998a). Effects of induction and inhibition of cytochromes P450 on the hepatotoxicity of methapyrilene. *Toxicol Sci* **46**(1): 185-196.

Ratra GS, Morgan WA, Mullervy J, Powell CJ, Wright MC (1998b). Methapyrilene hepatotoxicity is associated with oxidative stress, mitochondrial dysfunction and is prevented by the Ca<sup>2+</sup> channel blocker verapamil. *Toxicology* **130**(2-3): 79-93.

Ratra GS, Powell CJ, Park BK, Maggs JL, Cottrell S (2000). Methapyrilene hepatotoxicity is associated with increased hepatic glutathione, the formation of glucuronide conjugates, and enterohepatic recirculation. *Chem Biol Interact* **129**(3): 279-295.

Regan SL In vitro and in vivo models for the investigation of drug bioactivation and drug-induced liver injury. PhD, University of Liverpool, Liverpool, 2009.

Reznik-Schuller HM, Lijinsky W (1981). Morphology of early changes in liver carcinogenesis induced by methapyrilene. *Arch Toxicol* **49**(1): 79-83.

Rosner MH (2009). Urinary biomarkers for the detection of renal injury. *Adv Clin Chem* **49**: 73-97.

Saha S, New LS, Ho HK, Chui WK, Chan EC (2010a). Direct toxicity effects of sulfo-conjugated troglitazone on human hepatocytes. *Toxicol Lett* **195**(2-3): 135-141.

Saha S, New LS, Ho HK, Chui WK, Chan EC (2010b). Investigation of the role of the thiazolidinedione ring of troglitazone in inducing hepatotoxicity. *Toxicol Lett* **192**(2): 141-149.

Scaffidi P, Misteli T, Bianchi ME (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**(6894): 191-195.

Shimizu S, Atsumi R, Nakazawa T, Fujimaki Y, Sudo K, Okazaki O (2009). Metabolism of ticlopidine in rats: identification of the main biliary metabolite as a glutathione conjugate of ticlopidine S-oxide. *Drug Metab Dispos* **37**(9): 1904-1915.

Soga T, Baran R, Suematsu M, Ueno Y, Ikeda S, Sakurakawa T, *et al.* (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem* **281**(24): 16768-16776.

Steele RD, Weber H, Patterson JI (1984). Characterization of alpha-ketobutyrate metabolism in rat tissues: effects of dietary protein and fasting. *J Nutr* **114**(4): 701-710.

Tirmenstein MA, Hu CX, Gales TL, Maleeff BE, Narayanan PK, Kurali E, *et al.* (2002). Effects of troglitazone on HepG2 viability and mitochondrial function. *Toxicol Sci* **69**(1): 131-138.

Waterfield CJ, Mesquita M, Parnham P, Timbrell JA (1993a). Taurine protects against the cytotoxicity of hydrazine, 1,4-naphthoquinone and carbon tetrachloride in isolated rat hepatocytes. *Biochem Pharmacol* **46**(4): 589-595.

Waterfield CJ, Turton JA, Scales MD, Timbrell JA (1993b). Effect of various non-hepatotoxic compounds on urinary and liver taurine levels in rats. *Arch Toxicol* **67**(8): 538-546.

Waterfield CJ, Turton JA, Scales MD, Timbrell JA (1993c). Investigations into the effects of various hepatotoxic compounds on urinary and liver taurine levels in rats. *Arch Toxicol* **67**(4): 244-254.

Waterman CL, Kian-Kai C, Griffin JL (2010). Metabolomic strategies to study lipotoxicity in cardiovascular disease. *Biochim Biophys Acta* **1801**(3): 230-234.

Waters NJ, Waterfield CJ, Farrant RD, Holmes E, Nicholson JK (2006). Integrated metabolomic analysis of bromobenzene-induced hepatotoxicity: novel induction of 5-oxoprolinosis. *J Proteome Res* **5**(6): 1448-1459.

Watkins PB, Dube LM, Walton-Bowen K, Cameron CM, Kasten LE (2007). Clinical pattern of zileuton-associated liver injury: results of a 12-month study in patients with chronic asthma. *Drug Saf* **30**(9): 805-815.

Wen B, Ma L, Nelson SD, Zhu M (2008). High-throughput screening and characterization of reactive metabolites using polarity switching of hybrid triple quadrupole linear ion trap mass spectrometry. *Anal Chem* **80**(5): 1788-1799.

Williams DP, Antoine DJ, Butler PJ, Jones R, Randle L, Payne A, *et al.* (2007). The metabolism and toxicity of furosemide in the Wistar rat and CD-1 mouse: a chemical and biochemical definition of the toxicophore. *J Pharmacol Exp Ther* **322**(3): 1208-1220.

Ziegler R, Ho B, Castagnoli N, Jr. (1981). Trapping of metabolically generated electrophilic species with cyanide ion: metabolism of methapyrilene. *J Med Chem* **24**(10): 1133-1138.