Approaches to the Detection of Adducts Formed via the Covalent Binding of Reactive Metabolites to Proteins

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<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>AMS</td>
<td>Accelerator mass spectrometry</td>
</tr>
<tr>
<td>AQQI</td>
<td>Amodiaquine quinone imine</td>
</tr>
<tr>
<td>BG</td>
<td>Benoxaprofen glucuronide</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
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<tr>
<td>CHAPS</td>
<td>3-[3-cholamidopropyl]dimethylammonio]-propanesulfonic acid</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxy cinnamic acid</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DCE</td>
<td>1,1-dichloroethylene</td>
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<tr>
<td>DDI</td>
<td>Drug-drug interactions</td>
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<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin monooxygenases</td>
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<tr>
<td>FT</td>
<td>Fourier transform</td>
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<tr>
<td>GluFib</td>
<td>[Glu1]-Fibrinopeptide B</td>
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<tr>
<td>GGT</td>
<td>Gamma-glutamyltranspeptidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HLM</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>HM</td>
<td>High mass</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IADR</td>
<td>Idiosyncratic adverse drug reaction</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
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</table>
LC-MS/MS  Liquid chromatography/tandem mass spectrometry
LM  Low mass
LTQ Orbitrap  Linear ion trap Orbitrap
LSC  Liquid Scintillation counting
MALDI  Matrix-assisted laser desorption/ionisation
MALDI-MS  Matrix-assisted laser desorption/ionisation mass spectrometry
MAO  Monoamine oxidases
MDD  Major depressive disorder
MDI  Metabolism dependent inhibition
MPO  Myeloperoxidase
MRM  Multiple reaction monitoring
MS  Mass spectrometry
MS/MS  Tandem mass spectrometry
Mw  Molecular weight
m/z  Mass-to-charge ratio
B-NADP  β-nicotinamide adenine dinucleotide phosphate
NADPH  Reduced nicotinamide adenine dinucleotide phosphate
NBDAQI  N-bisdesethylamodiaquine quinone imine
NDAQI  N-desethylamodiaquine quinone imine
NOAEL  No adverse effect level
NAPQI  N-acetyl-p-benzoquinone imine
NMR  Nuclear magnetic resonance
PSD  Post source decay
Q  Quadrupole
QTof  Quadrupole time-of-flight
QWBA  Quantitative whole body autoradiography
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRM  Selected reaction monitoring
SSRI  Selective serotonin re-uptake inhibitors
TFA  Trifluoroacetic acid
Th  Thomson
TIC  Total ion chromatogram
Tof  Time-of-flight
Tris  tris(hydroxymethyl)methylamine
USA  United States of America
<table>
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<th>Acronym</th>
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<tbody>
<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatogram</td>
</tr>
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<td>UV</td>
<td>Ultraviolet</td>
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Abstract

Approaches to the Detection of Adducts formed via the Covalent Binding of Reactive Metabolites to Proteins

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences (School of Chemistry)

Metabolism of xenobiotic drug molecules can result in the formation of metabolites which are more chemically reactive than the parent drug from which they are derived. These reactive species have the potential to covalently modify biological macromolecules if they are not detoxified. The formation of drug-protein adducts carries a potential risk of clinical toxicities and idiosyncratic adverse drug reactions which can, in severe cases, result in hospitalisation and even death. Current methods for the evaluation of the risk for a drug to cause adverse drug reactions due to drug-protein binding rely on risk factors such as quantitative covalent binding value, structure, dose etc. The objective of this project was to develop methods for the detection of reactive metabolites directly bound to proteins, which could be used in future evaluations of the mechanisms of binding of candidates in drug development. Three compounds known to produce reactive metabolites, acetaminophen, SB-649869 and amodiaquine, were used as tool substrates. In vitro incubations with human liver microsomes and individual cytochrome P450 enzymes (as Supersomes™) were used to produce reactive metabolite species and binding with the incubation proteins evaluated. Analysis of the intact proteins, peptides generated via trypsin digestion of the incubation protein, and amino acids generated via digestion with pronase were evaluated using a combination of LC/MS and LC-MS/MS. Reactive metabolite trapping experiments with glutathione were used to provide information about the likely structure of the bound species and the specificity of binding, and were useful in the development of sensitive targeted precursor ion scanning and multiple reaction monitoring methods. [14C] radiolabelled acetaminophen and SB-649868 were used to assess the quantitative levels of binding (< 5% modification of protein in both cases). Radiodetection using accelerator mass spectrometry (AMS) was used to evaluate the stoichiometry of binding and aid the identification of adducted peptides through retention time comparison. Chemical and electrochemical methods were utilised to produce stable solutions of N-acetyl-p-benzoquinone imine (NAPQI) and amodiaquine quinone imine (AQQI), reactive metabolites of acetaminophen and amodiaquine, respectively, which were bound to selected proteins and used as chromatographic and mass spectrometric standards. These methods were used to successfully identify an acetaminophen-modified peptide (T56) of cytochrome P450 CYP2E1. No modified proteins were observed for the SB-649868 incubations, however, examination of the AMS chromatograms for the incubations with acetaminophen and SB-649868 revealed a difference in the stoichiometry of binding, with one modified peptide observed with acetaminophen, and several for the incubations with SB-649868. The detection and identification of drug-protein adducts remains extremely challenging due to the low levels of any adducts observed, which can be exacerbated by binding on multiple sites of a protein; however this project has demonstrated that sensitive and selective LC/MS methods can be successfully developed to identify drug-protein adducts.

Bianca Squillaci 16th April 2013
Declaration

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Preface

After graduating from UMIST with a MChem (Hons.) degree in Chemistry in 2001, I joined the DMPK department at GlaxoSmithKline, initially working in the quantitative bioanalysis group analysing samples from animal and human in vivo studies for the support of many different drug compounds in development. I then moved into the structural identification group within DMPK, analysing biological samples by LC/MS to elucidate the structure of any metabolites observed following dosing to animals and humans. In recent years, this has included the analysis of animal tissue such as liver and testes by MALDI imaging mass spectrometry to evaluate the tissue distribution of drug and metabolites.

The research contained in this thesis was initiated to aid our understanding of the risks associated with reactive metabolites and the mechanisms by which they may cause idiosyncratic drug reactions. As detailed in the Introduction, the current understanding of these mechanisms is limited and the use of the methods described in this research to identify how reactive metabolites adduct directly to proteins will help us understand the mechanism of these reactions. This information will be used to aid the understanding of the risks associated with drug candidates, and ultimately, improve the safety of our drugs for patients.

Previous publications:


1 Introduction

1.1 Drug metabolism

Drug metabolism is the biochemical or enzymatic conversion of a drug moiety into another, primarily to promote the elimination of the drug from the body. It has two important effects:

1. The drug is made more hydrophilic – this hastens its excretion by the kidneys because the less lipid-soluble metabolite is not readily reabsorbed in the renal tubes.
2. Metabolites excreted are generally less active than the parent drug.

Drug metabolism is normally divided into two phases: Phase I (or functionalisation reactions) and Phase II (or conjugative reactions). Phase I reactions generally transform the drug into more polar metabolites, either for direct, rapid, renal excretion, or to prepare the drug for a subsequent Phase II reaction by either producing or uncovering a chemically reactive functional group on which the Phase II reaction can occur. This can sometimes result in the formation of metabolites which are more toxic or carcinogenic than the parent drug, hence Phase II reactions are generally thought of as detoxification pathways. Their products account for the bulk of the inactive, excreted products of the drug in question. Phase I metabolism principally includes oxidation, reduction, hydrolysis and hydration, as well as other rarer miscellaneous reactions such as dimerisation, ring cyclisation and transamidation. A drug entering the body may undergo a combination of these reactions on several sites of its structure. Oxidation is the most common of these reactions and this is principally catalysed by a family of microsomal mixed-function oxidases known as the cytochrome P450 enzymes. Other enzymes capable of catalysing oxidation include flavin monooxygenases (FMOs), monoamine oxidases (MAOs) and heme-containing peroxidases such as myeloperoxidase (MPO).

Phase II metabolism involves the conjugation of a drug or its Phase I metabolite with endogenous compounds to produce a generally less active, more hydrophilic product for excretion by the kidneys or liver into the bile, urine and faeces. For example, as shown in Figure 1.1, rosiglitazone, a peroxisome proliferator-activated receptor gamma agonist marketed for the treatment of Type II diabetes, is principally metabolised via a combination of Phase I oxidation, demethylation and dealkylation reactions with subsequent Phase II conjugation reactions with endogenous glucuronide and sulfate moieties. Phase I and II reactions can involve a diverse group of enzymes and often require a cofactor such as the reduced form of nicotinamide adenine dinucleotide phosphate (known as NADPH) in order for reactions to proceed.
Figure 1.1: Metabolism of rosiglitazone in humans (1)
Common Phase II conjugation reactions include sulfation, methylation, acetylation and glucuronidation which generally occur on alcohols, phenols, amines, thiols and carboxylic acids. Glucuronidation is the most widespread and important form of conjugation for drug molecules due to the relative abundance of the cofactor for the reaction, UDP-glucuronic acid, and the ubiquitous nature of the enzymes responsible for this reaction, UDP-glucuronosyltransferases (2, 3). Glucuronides are often excreted in the bile and released into the gut where they can be broken down back to the parent compound by any β-glucuronidase present, and possibly reabsorbed. Glutathione conjugation is particularly important in drug metabolism as it is involved in the detoxification process of potentially toxic or reactive metabolites. Many drugs either are, or can be, metabolised by phase I reactions to strong electrophiles, for example epoxides, and these can react with glutathione to form non-toxic conjugates. Where endogenous levels of glutathione are high, the predominant mechanism of conjugation is via spontaneous reaction, however enzymes catalysing glutathione conjugation reactions such as glutathione-S-transferases (4, 5), which are mainly located in the cytosol of the liver, kidney, gut and other tissues, also have a role to play, particularly when endogenous levels of glutathione have been depleted. Glutathione conjugates can be excreted directly in the urine or bile but are often metabolised further to form glycyl cysteine, cysteine and mercapturic acid conjugates.

The resulting Phase II conjugate formed is almost always pharmacologically inactive and more hydrophilic that its precursor. However, there are some notable exceptions. Phase II glucuronide conjugation of carboxylic acid drugs or metabolites results in the formation of acyl glucuronides, which have demonstrated the tendency to rearrange and covalently modify proteins, with an associated risk of toxicity in patients (6-12). This toxicity risk is associated with both spontaneous hydrolysis of the acyl glucuronide back to the aglycone moiety (which can then be reabsorbed by the body, prolonging the drug half-life) and their ability to transacylate (during which the acyl group rearranges from its initial C1 position on the glucuronic acid, to the C2, C3 and C4 positions, as shown in Figure 1.2 (13). Anomerisation can also occur via ring-opening, thereby converting the β-anomers to the α-forms.
where $R =$ drug moiety

1-O-$\beta$-glucuronide

2-O-$\beta$-glucuronide

3-O-$\beta$-glucuronide

4-O-$\beta$-glucuronide

2-O-$\alpha$-glucuronide

3-O-$\alpha$-glucuronide

4-O-$\alpha$-glucuronide

**Figure 1.2:** The seven possible acyl glucuronide isomers formed via transacylation (14, 15).

where $R =$ drug moiety

**Figure 1.3:** Mechanism of acyl migration (14)

During the transacylation process (Figure 1.3), it is possible for the acyl glucuronide to react with a protein molecule, resulting in binding to the protein, with the subsequent loss of the glucuronide moiety (15). In addition, ring-opening of the glucuronide moiety during glycation forms a transient aldehyde which can be modified by a protein molecule, with the retention of the glucuronide (Figure 1.4).
Figure 1.4: Modification of acyl glucuronides by proteins (14)

1.2 Drug oxidation and the cytochrome P450 family of enzymes

The mixed-function oxidase cytochrome P450 system is a large, diverse family of heme-containing proteins found in the endoplasmic reticulum of many cells, most notably those of the liver, and also kidney, lung and intestine. They have been noted in all areas of life including mammals, birds, fish, insects, plants, fungi, and bacteria (16, 17). In humans, 18 P450 families, 44 subfamilies, and 57 P450 individual enzymes have been identified (18, 19). The primary role of the CYP450 enzymes is to catalyse oxidation reactions as part of the metabolism process, and they account for approximately 75% of drug metabolism reactions. Of the 57 human P450 enzymes, five (CYP1A2, CYP2C8/9, CYP2C19, CYP2D6 and CYP3A4/5) are involved in approximately 95% of these reactions (20). Most compounds are able to act as substrates for more than one P450 isoform because their structures fit the enzyme active sites, although they may be more selective towards one P450 than another.

One of the characteristics of the cytochrome P450 family of enzymes is the large number and wide variety of structural classes of their substrates, diversity of reactions and their selectivity. The levels of the P450 enzymes in the liver are affected by a variety of factors including dietary habits (21, 22), age (19, 23), ethnicity (19), environment (24), genetic variation (25), as well as disease (26). For example, Fisher et al. (26) report a decline in the microsomal protein
expression of CYP1A2, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 with the progression of non-alcoholic fatty liver disease. Differences in cytochrome P450 levels can be an important consideration during drug development since differences in first-pass metabolism and drug clearance due to cytochrome P450-driven metabolism can have a significant effect on bioavailability and drug exposure.

The oxidation of a drug requires the cytochrome P450 heme protein, molecular oxygen, cytochrome P450 reductase and NADPH. Cytochrome P450 reductase catalyses the following reaction:

\[
\text{Substrate} + \text{NADPH} + \text{O}_2 + \text{H}^+ \rightarrow \text{Substrate} - \text{O} + \text{NADP}^+ + \text{H}_2\text{O}
\]

Two electrons, which are required for the subsequent oxidation reaction, are generated. During the oxidation of a drug molecule by the P450 system, the cytochrome P450 undergoes cyclic oxidation/reduction, as shown in Figure 1.5.

The substrate binds to the active site of the enzyme which induces a change in conformation. An electron is transferred from NADPH via the cytochrome P450 reductase, reducing the ferric iron (Fe\(^{3+}\)) of the heme group to the ferrous state (Fe\(^{2+}\)). Molecular oxygen then binds to the heme iron and a second electron is transferred, reducing the dioxygen adduct to a negatively charged peroxo group which is rapidly protonated, releasing a water molecule and leaving a highly reactive ferric oxene species which extracts a hydrogen atom from the drug molecule. The free radical drug complex acquires the bound OH radical, and the hydroxylated drug molecule is released from the active site and the enzyme returns to its original state. While this example describes the oxidation of a drug molecule, the cytochrome P450s can catalyse a number of bioactivation reactions.
Figure 1.5: The mono-oxygenase P450 cycle (17)

1.3 Covalent binding and drug toxicity

Phase I metabolism of xenobiotic drug molecules can result in the formation of metabolites which are more chemically reactive than the parent drug from which they are derived. These reactive species have the potential to covalently modify biological macromolecules if they are not detoxified. The formation of drug-protein adducts carries a potential risk of clinical toxicities and adverse drug reactions, where the resulting toxicological response may be dependent on a number of factors, some of which are discussed further here.

1.3.1 Adverse reactions

Adverse drug reactions pose a significant health risk to the patient population as they contribute to patient morbidity and mortality and are one of the common causes for pharmaceutical product withdrawals and black box warning labels (27). They account for 6.5% of all hospital admissions in the United Kingdom (28) and occur in 10-20% of in-patients (29), resulting in an increased average length of hospital stay, at significant cost to healthcare providers (30, 31), and an estimated 100,000 fatalities annually in the United States (29).

The safety of a drug molecule is comprehensively assessed at all stages of the drug development process, first in in vitro systems and in silico models, in vivo in animal species and
finally in the clinic, firstly in healthy volunteers (Phase I), then patients (Phase II and III) and finally post-marketing (Phase IV). Safety margins are calculated based on the ratio of the administered dose in humans to the no adverse effect level (NOAEL) dose in animals. Once in the clinic, a combination of monitoring of biomarkers, both for safety and efficacy, and clinical observation are used to ensure that the drug is well tolerated. Adverse drug reactions such as nausea, vomiting, convulsions etc. are recorded, initially in animal studies and then in human trials and their severity has a direct impact on the continuation or termination of a drug programme.

Adverse drug reactions (ADRs) can be categorised into two main types - Type A which are predictable from the pharmacology of the drug and are generally identified in preclinical toxicological studies, and Type B which are unpredictable idiosyncratic reactions (32). A typical example of Type A ADRs is the hair loss and nausea associated with chemotherapy treatments. They are usually dose dependent and their effects can generally be reversed by reducing the administered dose or terminating the therapy. They can also be dependent on other factors such as physiological changes or abnormalities due to disease state, variation in pharmaceutical formulation or drug-drug interactions. It has been reported that the likelihood of an adverse drug reaction occurring is 50% if the patient is taking five or more drugs simultaneously, a particular danger to elderly patients or patients suffering from chronic diseases such as HIV/AIDS (33, 34).

Type B, or idiosyncratic adverse drug reactions (IADRs) cannot be predicted by animal models. These reactions are typically not related to the pharmacology of the drug, can occur at any dose within the therapeutic range and can vary wildly depending on the individual dosed. An added complication is that IADRs sometimes only appear after moderate or long periods of latency (35), i.e. between 1 week and 1 year after the drug is dosed, and even large scale Phase III clinical trials of up to several thousand patients may not detect IADRs successfully, a factor which can introduce a large degree of uncertainty into the drug development process. The properties of IADRs suggest involvement of the immune system but in most cases the mechanisms have not been established with any certainty (35).

1.3.2 Clinical implications

There are significant ethical and financial implications associated with incidences of IADRs in the clinic. In total, IADRs constitute around 20% of all ADRs (32) and are a leading cause of drug withdrawal from the market. Between 1975 and 2000, more than 10% of new drugs
registered by the Food and Drug Administration (FDA) in the US, had to be either withdrawn or had a black box warning applied to their sale (36). Their unpredictable nature and potential to cause very severe reactions poses a considerable risk primarily to patient safety, but secondly to the financial viability of drug development. The example of nefazodone (Serzone™), a treatment for depression developed by Bristol Myers Squibb illustrates this. Serzone™ was approved by the Food and Drug Administration (FDA) for use in 1994 (37) in patients suffering from major depressive disorder (MDD) as an alternative to treatments with selective serotonin re-uptake inhibitors (SSRIs) which were themselves associated with increased suicide risk. It was withdrawn from the market in November 2003 (38) due to incidences of severe liver injury and death, with an approximate incident level of 1 case of liver failure resulting in transplant or death in 250,000 – 300,000 patient years (39). These incidences of severe liver toxicity had not been detected in any of the Phase I-III clinical trials, which included large patient populations, with the most common side effects being somnolence, dry mouth, nausea, dizziness, constipation, asthenia, light-headedness, blurred vision, confusion, and abnormal vision (40). As described by Health Canada, while the incidences of liver toxicity were small, Serzone™ posed a greater risk than the alternative treatments for depression that were available (41).

Notwithstanding the significant risk to patient safety, with the average cost of bringing a drug molecule to market in the region of $800 million (42), the withdrawal of Serzone™ and other drugs in similar situations can have a catastrophic impact on a company’s viability. It is important to note that several drugs which exhibit incidences of IADRs are still on the market and are being prescribed by physicians, although often with restrictions as to their use and close monitoring of patients. For example, felbamate (Felbatol™), a treatment for epilepsy has been shown to cause drug-induced liver failure with 1 case of liver failure resulting in transplantation or death, per 12,500 patient years (43). Patient data on tolcapone (Tasmar™) an inhibitor of catechol-O-methyltransferase for the treatment of Parkinson’s disease has shown 1 case of death due to hepatic failure per 13,000 patient years worldwide (44). Both epilepsy and Parkinson’s are diseases with a dearth of effective therapies for patients and treatment with these drugs continue with additional monitoring as, in many cases, no effective alternative exists. Telithromycin (Ketek™), a ketolide antimicrobial antibiotic, was approved by the FDA in 2004 for the oral treatment of acute bacterial sinusitis, acute exacerbation of chronic bronchitis, and community-acquired pneumonia (45), with approximately 5 million prescriptions dispensed in the US between July 2004 and April 2006 (46). Three incidences of acute liver injury attributed to telithromycin were reported to the FDA post-approval in 2006 (47) with additional incidences being reported at a later date (48, 49). Following a review of 42
cases (50), the FDA, in December 2006, reduced the indications of use for telithromycin to community-acquired pneumonia only. The treatment of acute bacterial sinusitis and acute exacerbation of chronic bronchitis were removed from the list of indications because the balance of benefit and risk for these conditions no longer supported approval (51).

As demonstrated by these examples, the risk/benefit must be carefully considered for every new drug in development. While all new drugs must be evaluated rigorously for safety prior to marketing, the disease indication for which the drug is intended can have a significant impact on how much adverse reaction risk is tolerated by regulatory authorities. Factors such as the following need to be carefully considered. Are the adverse reactions likely to be severe, i.e. debilitating or cause loss of life? Can the risk be effectively monitored by physicians? Is the drug for an acute or chronic condition and will it require long-term dosing? Is the drug for an unmet clinical need e.g. HIV or cancer? What are the target patient populations and the risks associated with them? Will the drug offer a significant improvement in quality/length of life over current treatments? For example, β-lactam antibiotics such as penicillin and cephalosporin which can react directly with cellular and serum proteins (52) have been shown to produce serious adverse drug reactions such as anaphylaxis in approximately 1 in every 10,000 patients (53). In this case the risk/benefit is still heavily in favour of the drug remaining on the market, as the incidence of ADRs are relatively small and can usually be effectively treated, while the number of lives saved by their introduction is incalculably large.

Drugs can adversely affect almost any organ in the body; however, drug-induced IADRs are often manifested as liver toxicity. This is the most common cause of drug withdrawal (54), accounting for approximately 50% of cases of acute liver failure, and mimics other forms of acute and chronic liver disease (55). This is not surprising, as the liver is the primary site for the metabolism of many xenobiotics. There is evidence that tissue damage, arising from these adverse reactions is regiospecific to the site of bioactivation (56). For example, clozapine, a drug which is metabolised by neutrophils, can in some cases cause idiosyncratic agranulocytosis, a condition which is characterised by a severely reduced neutrophil count in the blood (57, 58). Troglitazone, a treatment for diabetes mellitus, was withdrawn from the market in the UK due to incidences of hepatotoxicity (59). Studies in vivo in rats and in vitro in human microsomes (60) showed troglitazone was metabolised by the liver to form a reactive quinone methide metabolite. Confusingly, not all incidences of covalent binding result in toxicity, which can make defining the mechanisms of toxicity extremely complicated. An example of this is the bromophenol metabolite of bromobenzene which binds to proteins within the liver and kidney without causing toxicity in either organ (61).
1.3.3 Mechanisms of drug toxicity

The mechanisms of the physiological processes leading to idiosyncratic adverse drug reactions are generally poorly understood and may be dependent on a wide range of factors including gender, genetic background, diet, co-administered drugs, drug structure, function of bound protein and the administered dose. Many of the clinical implications of IADRs, for example delayed on-set after exposure, rash, hypersensitivity and inflammatory responses, imply that the mechanism is immune-mediated, however this remains to be proven definitively as many of these responses can be explained through other mechanisms. An example of evidence supporting an immune-mediated reaction is drug-induced aplastic anemia (35). Aplastic anemia is thought to be caused by a virus and is usually treated by bone-marrow transplant and immunosuppression. Successful treatment with immunosuppressant drugs suggests that it may be immune-mediated in mechanism. The successful response of drug-induced aplastic anemia to immunosuppressant drugs implies that it is also immune-mediated. However some IADRs lack the characteristics of an immune-response so it is likely that there may be differing mechanisms at work, possibly dependent on the drug itself.

Studies on carcinogenic aminoazo dyes bound to proteins in rat livers by Miller and Miller in the 1940s (62) first suggested the idea that covalent binding of reactive chemical moieties to proteins within the body could elicit a toxic effect. This concept was then applied to the hepatotoxicity observed in acetaminophen overdoses (63, 64). Since then, several research groups have attempted to deduce the mechanisms of covalent binding, and its clinical implications, with varying success.

The foremost theory to explain these processes is the hapten hypothesis (65-68). A hapten is defined as a small molecule which elicits an immune response when attached to a large carrier such as a protein. Covalent modification of a bio-macromolecule by a reactive metabolite results in a “foreign” protein, which, not being recognised by the body’s immune system, elicits an immune-mediated response. It has also been proposed that adduction of so called “critical” proteins, i.e. proteins essential to specific cell function, is an important factor in organ toxicity due to adverse reactions. Although the hapten hypothesis has never been proven definitively, there is circumstantial evidence to support it. IgE-mediated adverse reactions associated with an allergic reaction to penicillin are thought to be due to antibodies raised against penicillin-modified proteins (69). Anti-drug antibodies have been observed from some idiosyncratic drug reactions e.g. tienilic acid, however, this is rare (70, 71).
A supplemental theory to the hapten hypothesis is the danger hypothesis. This hypothesis is based on the observation that patients with pre-existing conditions often have a higher incidence of IADRs. Matzinger (72) proposed that the immune system tolerates most antigens and that it is only when a second “danger” signal is present that an active immune response is produced (68, 73, 74). In the absence of some type of cell damage (i.e. cell damage such as necrosis, resulting from a pre-existing infection or disease state), the antigens raised by the immune system against the drug-protein adduct are unable to trigger an immune response and no toxicity is observed. This hypothesis fits in with data generated which suggest that patients with existing infections have higher incidences of IADRs (67).

A third model for drug hypersensitivity reactions was proposed by Pichler et al. (75) and is known as the p-i (or pharmacological interaction) hypothesis. It states that some drugs can directly bind to antigen-specific T-cell receptors, without the need for bioactivation to a reactive metabolite, and that immune system stimulation is dependent on the how well the structural features of the drug fit into the T-cell receptor.

1.4 Reducing the risk of adverse drug reactions

In reality, incidence of IARs remain unpredictable and our lack of understanding of the physiological processes involved suggest that a combination of the described mechanisms are probably involved, and that their various importance may be drug and patient specific.

Although there is no effective animal model that can identify drug candidates with the potential to elicit hypersensitivity reactions in humans, there is a responsibility for pharmaceutical companies to minimise this potential through structural modification of drug candidates. The FDA Guidance for Industry, Safety Testing of Drug Metabolites 2008 (76) states “Based on the nature of the chemical reactions involved, metabolites formed from Phase I reactions are more likely to be chemically reactive or pharmacologically active and, therefore, more likely to need safety evaluation. An active metabolite may bind to the therapeutic target receptors or other receptors, interact with other targets (e.g., enzymes, proteins), and cause unintended effects” and “Metabolites that form chemically reactive intermediates can be difficult to detect and measure because of their short half-lives”. However, they can form stable products (e.g., glutathione conjugates) that can be measured and, therefore, may eliminate the need for further evaluation. Phase II conjugation reactions generally render a compound more water soluble and pharmacologically inactive, thereby eliminating the need for further evaluation. However, if the conjugate forms a toxic compound such as an acyl
glucuronide, additional safety assessment may be needed.” The implication from the FDA guidance is that where reactive metabolites are detected in *in vitro* or *in vivo* systems, the Industry has a responsibility to evaluate their toxicity and to discharge the risk to patients as effectively as possible.

### 1.4.1 Structure

The structure of the potential drug candidate will give the first indication of the potential for reactive metabolites to be formed. For example, acyl groups (which can be metabolised to acyl-glucuronides), para-substituted phenols (which can form dihydrodiol species), and any group which can form glutathione conjugates would generate a structural alert. Kalgutkar *et al.* (77) published a comprehensive review of the bioactivation pathways of compounds known to have the potential to form reactive metabolites capable of binding to proteins. For example, diclofenac (shown in Figure 1.6) is oxidised by cytochrome P450 CYP3A4 at both the 4’ and 5 positions to give a hydroxy group *para* to the nitrogen on the benzene ring which can be dehydrogenated to benzoquinones imine moieties, which are susceptible to binding to protein (78-80).

![Figure 1.6: Cytochrome P450-mediated metabolism of diclofenac to its reactive quinone imine metabolites (79)](image-url)
Similar mechanisms of protein binding have also been established for acetaminophen (81, 82) and amodiaquine (83), which both have structures with nitrogen and oxygen atoms in \textit{para} positions on a benzene ring, enabling the formation of a reactive quinone imine species. While the bioactivation to reactive quinone imines is thought to be involved in the covalent binding of several drugs e.g. indomethacin (84), nefazodone (85) and tolcapone (86), several other reactive pathways exist. For example, furosemide (87) and imipramine (57) are thought to be metabolised to reactive epoxides which can bind to macromolecules; a nitroso-derivative of procainamide (88) and an iminium species of mianserin (89) have also been postulated as potentially reactive metabolites.

By establishing common structural features of drugs which exhibit covalent binding there is the potential for the structural liabilities to be avoided in future drug design efforts by blocking potentially reactive sites of biotransformation.

1.4.2 Dose

In addition to structure, other factors must be taken into account when evaluating the reactive metabolite risk associated with a drug candidate. Adverse drug reactions such as hepatotoxicity are more frequently associated with compounds administered at a high dose. If there is a choice between selecting a low dose compound over a higher dose compound to continue in drug development, the risk associated with IADR and toxicity should be much lower for the low dose compound (77, 90). For example, it has been reported that structurally similar drugs clozapine and olanzapine (Figure 1.7) can both be metabolised to reactive nitrenium ions \textit{in vivo}.

![Figure 1.7: The structural similarity of clozapine and olanzapine](image)

In the case of clozapine, it has been postulated (91, 92) that the nitrenium ion formed may bind to neutrophil proteins, causing clozapine-induced agranulocystosis, while olanzapine is
not associated with the same toxic effect (93). The difference in dose of the two drugs (clozapine is administered as several 100 mg/day while the dose for olanzapine is below 10 mg/day) may explain the difference in toxicity observed (67). There are no cases listed in the literature of drugs dose below 10 mg/day causing IADRs (77).

Several pharmaceutical companies have published in recent years (94-98) on the internal risk assessments they use to prevent idiosyncratic toxicity for drugs in their development portfolio, with the predicted administered dose level being one of, if not the major factor in the decision to progress or terminate drugs with a potential IADR risk. Nakayama et al. (94) used quantitative covalent binding data generated for 42 drugs in three test systems (in vitro human liver microsomes and hepatocytes, and rat liver in vivo) and plotted these data against the daily administered dose. The tested drugs were divided into four safety categories based on literature data (safe, warning (for drugs with an IADR risk warning associated with their use), black box warning and withdrawn). While covalent binding data on its own was not sufficient to separate the compounds into their safety categories, for the majority of compounds tested, the authors were able to show a direct correlation between the safety category assigned for the drug based on the literature and the safety category indicated by the covalent binding v dose plotted data, thus supporting the importance of dose in risk assessment for idiosyncratic toxicity. This finding was supported by Sakatis et al. (97). Their study of 223 marketed drugs (approximately 50% of which were hepatotoxic, with the remainder non-hepatotoxic) showed that when the dose information was combined with other risk factors for toxicity such as the quantitative covalent binding value, glutathione adduct formation and cytochrome P450 metabolism-dependent inhibition, these data were predictive of hepatotoxicity in 80-100% of cases, significantly higher than where dose information was not used (approximately 65%).

The second consideration is usage. Since in most cases, toxicity is only observed after several weeks or months of administration. Drug candidates which are used in an acute setting, i.e. for two weeks or less, present a much reduced risk of IADR in patients (77). For example, drug-induced lupus has been observed for 40 drugs currently in use (99) after chronic dosing. Incidences of drug-induced lupus were observed in 5-8% of patients treated for over 1 year with hydralazine (100), with symptoms such as skin rashes, fatigue and musculoskeletal complaints observed. Circumstantial evidence suggests that the mechanism of toxicity is believed to be oxidation of hydralazine to a reactive hydroxylamine by neutrophils (see Figure 1.8) which can either act as a hapten and elicit an autoimmune response or can be cytotoxic (100).
Saturation of a particular metabolic route is also a consideration linked to dose. Anecdotal evidence tells us that acetaminophen, one of the most commonly used over-the-counter drugs available, displays a low risk for toxicity at normal doses. However, chronic use or overdose can saturate the protective metabolic pathway of detoxification i.e. the glutathione conjugation of the reactive N-acetyl-p-benzoquinone imine (NAPQI) species, allowing non-conjugated NAPQI to irreversibly bind to liver proteins, causing toxicity. This can be also be a significant issue in patient populations where numerous drugs are co-administered. For example, where two or more drugs are co-administered and share the same metabolic route of detoxification, e.g. glutathione conjugation, this can lead to depletion of the endogenous levels of the conjugate, resulting in a higher risk of toxicity.

When considering dose, it is important to also consider individual organ burden and the pharmacokinetics of the drug in question. Inhaled drugs, which typically are administered in doses of less than 1 mg/day and as such are generally excluded from traditional covalent binding investigations, can have a relatively high exposure in the lung per mg of tissue, while the exposure to the liver and other systemic organs is relatively low. The physiochemical properties of the drug can also have an important impact on the risk of idiosyncratic toxicity; a drug dosed orally at a relatively high level may be poorly absorbed by the body and largely excreted unchanged, making the exposure of the liver and other organs to potentially toxic metabolites relatively low. However, drugs with low bioavailability tend to be lipophilic in nature, which can result in a high degree of nonspecific binding throughout the body and off-target toxicity (97).
1.4.3 Metabolism and detoxification

Consideration of in vivo routes of detoxification must also be taken into account. Selection of a candidate for progression into drug development often occurs at a stage when in vivo metabolism is limited, with information heavily reliant on in vitro incubations with liver preparations such as microsomes (which will perform Phase I metabolic routes exclusively) and hepatocytes (which have the potential to carry out both Phase I and II metabolic routes). However, it is not unusual for the metabolic routes observed and/or their relative abundance observed using in vitro incubations to be significantly different to those observed in vivo. This can be for several reasons; reactive metabolites which can be formed via Phase I metabolism may be immediately detoxified to inactive metabolites by Phase II detoxification such as glutathione conjugation, which may not be immediately apparent from in vitro data alone. Microsomes have no Phase II metabolic capability at all, therefore they can only give a partial picture of the metabolic biotransformation expected in vivo. While hepatocytes are capable of Phase II metabolism, the functionality of cryopreserved or even freshly prepared hepatocytes can be significantly different to the functionality observed in vivo (101). Differences in the levels of influx and efflux transporters in in vitro systems compared to in vivo (102) can result in the saturation of metabolic pathways or the further metabolism of metabolites that would normally have been cleared in an in vivo situation. Multiple metabolic routes must also be considered. A drug containing a potential structural alert may be more readily metabolised on a different moiety within the molecule to an innocuous metabolite than via a potentially reactive route which may cause toxicity. Therefore, when considering the potential for a new drug candidate to cause toxicity in the clinic, all of these factors must be taken into account and while in vitro metabolic information is extremely useful, particularly at the early stages of the drug development process, it must be treated with caution.

1.4.4 Metabolism dependent inhibition

Metabolism dependent inhibition (MDI) of cytochrome P450 enzymes can also be a useful indicator of potential IADRs. When a drug is metabolised by a cytochrome P450 (CYP) enzyme into a reactive species, modification of the CYP enzyme by the reactive species can occur before release from the active site, resulting in irreversible CYP inactivation. This can cause clinical drug-drug interaction (DDI) issues if the drug is intended to be co-administered with another therapeutic agent, leading to increases in drug exposure levels (103) (due to the inhibition of metabolism) which itself can have direct toxic effects. An example of this is the work of Jushchyshyn et al (104) on the metabolism dependent inhibition of the isoenzymes
cytochrome P450 2B6 by phencyclidine (PCP), who showed that a metabolite of PCP binds to the apoprotein of CYP2B6, causing inhibition of the enzyme itself. Cyanide trapping was then used to confirm the involvement of a reactive iminium intermediate (105). The potential for MDI is routinely evaluated using in vitro expressed CYP isoenzymes and known CYP probe substrates.

1.4.5 Extractability

If radiolabelled compound is available, an assessment of non-extractable radioactivity in vitro can provide useful information as to the potential of a drug molecule to form reactive metabolites (106). Non-extractable radioactivity can be assessed using a filtration assay (107); the test molecule is incubated with human liver microsomes in the presence and absence of NADPH cofactor and the sample filtered to capture protein material. The filters can then be counted by liquid scintillation counting (LSC). The amount of radioactivity which is detected on the filters corresponds to the amount of compound that is bound irreversibly to the protein. The greater the amount of radiolabelled material present on the filters, the greater the level of covalent binding. Confirmation that drug-related material has indeed bound to protein, if considered necessary, can be achieved by SDS-PAGE of microsomal preparations after incubation with drug, to demonstrate co-migration of radioactivity with protein. An indication of potential covalent binding in in vivo studies can also be estimated by measuring the amount of non-extractible radioactivity in samples from pre-clinical studies, for example in the rodent and non-rodent toxicological species. Tissue from rodents and plasma samples are extracted with an organic solvent such as acetonitrile and the amount of radioactivity remaining in the protein pellet is measured to give the % recovery. At GlaxoSmithKline (GSK), if the recovery of drug-related material is greater than 90%, the compound is generally considered to have no extractability issues in vivo.

1.4.6 Quantitative whole body autoradiography

Quantitative whole body autoradiography (QWBA) studies can also provide some further evidence for metabolic activation to reactive species. A number of animals (usually a rat or mouse, depending on the rodent species selected for toxicological assessment studies) are dosed with a pharmacologically relevant dose (usually the no adverse effect dosed derived from drug safety studies) of the radiolabelled test compound. The animals are then sacrificed at time intervals (usually up to 35 days), frozen, sliced and imaged using autoradiography. While these images are primarily used to evaluate drug distribution, prolonged retention of
drug-related material in organs of the body, particularly the liver, can be an indication of covalent binding of drug to proteins within the body.

Takakusa and co-workers (108) used QWBA techniques to assess [14C] drug distribution and tissue retention of several drugs known to cause IADRs in patients, and to correlate the data with the toxicity observed (Figure 1.9).

![QWBA slices of rats](image)

**Figure 1.9:** QWBA slices of rats taken at 72 and 168 h after administration of a single oral dose of [14C] labelled A, amodiaquine; B, clozapine and C, futamide; at 3 mg/kg (Reproduced from (108) with permission)

All three compounds showed significant retention of radiolabelled material in the liver up to 168hr, with amodiaquine and clozapine also showing retention of drug in the bone marrow, suggesting the possibility of covalent binding of these drugs to liver and bone marrow proteins. The QWBA data, together with information gathered from *in vitro* and *in vivo* covalent binding measurements implies the involvement of covalent binding with the cases of hepatotoxicity seen for all three compounds, and agranulocytosis observed for amodiaquine and clozapine.
1.4.7 Trapping Experiments

Trapping experiments can help to identify reactive intermediates formed from the incubation of a test compound with in vitro systems such as human liver microsomes. The trapping agents stabilise these intermediates to allow for subsequent analysis by liquid chromatography/mass spectrometry (LC/MS). For example, reduced glutathione (GSH) (93, 109), a soft nucleophile, will trap soft electrophilic compounds (which encompass approximately 80% of reactive metabolites, such as epoxides, quinone imines, Michael acceptors and quinone methides), while cyanide (93, 110, 111), a hard nucleophile, will trap hard electrophiles such as iminium ions and aldehydes, to form stable adducts. The terms “hard” and “soft” refer to the Pearson Acid Base Concept (112). Hard nucleophiles or bases tend to be small, highly electronegative, and exhibit low polarisability, while soft nucleophiles have the opposite characteristics. Hard nucleophiles tend to react with hard electrophiles, and the resultant bond tends to be ionic in nature, whereas covalent bonding is more likely to occur between soft nucleophiles and electrophiles. Where reactive species can be trapped with trapping agents such as glutathione or cyanide (Figure 1.10), these trapped species can then be identified using LC-MS/MS and nuclear magnetic resonance spectroscopy (NMR), therefore giving an indication of the site of bioactivation on the molecule.

![Figure 1.10: Trapping with hard and soft nucleophiles](image)

This method is currently being used with some success within GSK and other pharmaceutical companies as it is easy to perform and can be used to screen large numbers of compounds at a relatively early stage within the discovery process as it does not require the use of
radiolabelled compound. GSH serves as a natural trapping agent in the liver to bind to reactive metabolites prior to elimination. GSH (0.2 – 5 mM) is added to in vitro experiments and the expected conjugates searched for using either full scan MS or constant neutral loss scanning for 129 Da (a common glutamyl moiety loss) (111, 113). The former of these methods is preferable since a loss of 129 is not always observed, allowing some adducts to escape detection. Recent advances in high resolution instruments and post-acquisition processing software to incorporate mass defect filtering for high mass accuracy (114, 115) have improved both the sensitivity and selectivity of this technique (116). Once identified, MS/MS and NMR techniques can then be used to characterise the reactive metabolite species. Alternative trapping agents for reactive drug metabolites include N-acetylcysteine, semi-carbazide and γ-glutamylcysteinyllysine (117). The formation of a glutathione conjugate does not necessarily mean that a drug will cause IADRs but it can serve as a signal for the formation of reactive intermediates which may warrant further investigation. The disadvantage to using these trapping systems is that the structure of the actual protein bound species and the mechanism for its binding can only be postulated from the trapped species. The method also offers no information about the site of binding on the protein, the nature of the bound protein or any modifications of the protein structure due to binding with reactive species. It would therefore be advantageous to identify the bound metabolites when directly attached to the protein to try to answer these questions.

1.4.8 Animal models

While in vitro covalent binding measurements and ex vivo assessments of extractability can be useful in determining the likelihood of a drug candidate to elicit an adverse drug reaction in patients, these data can only go so far in helping investigators to understand the biochemical mechanisms taking place. While the ideal situation would be to develop clinical models in patients, the ethics of exposing patients to potentially life-threatening drug reactions and the low incidence of their occurrence prevent this. The next best situation would be to develop animal models (primarily in rodents) to mimic these reactions. However, this is problematic; the characteristics of human idiosyncratic toxicity are not always replicated in animals. Also where both animals and humans do both elicit an adverse reaction, differences in the immune systems and metabolism pathways of rats and humans can be such that the mechanisms are quite different. If the mechanisms of toxicity are assumed to be the same in humans and animals, then it would also be reasonable to assume that incidences of idiosyncratic toxicity would be equally rare in animals as they are in human. This means that very large numbers of animals would need to be dosed before idiosyncratic toxicity was observed, which would be an
ethical issue. Shenton et al. (118) have presented a review of the current models available to study idiosyncratic drug reactions in animals with varying success. For example, Shelton and co-workers have reported an animal model for the characterisation of nevirapine-induced skin rash in the rat (119).

1.4.9 Clinical biomarkers

The low incidence and non-predictable nature of idiosyncratic drug reactions in patients mean that it would be useful to have clinical biomarkers which could be used to predict the on-set of an adverse drug reaction, thus giving physicians an early warning signal to lower the dose level or, if possible, to remove the patient from treatment altogether. This has been achieved with some success with drug-induced idiosyncratic hepatotoxicity. Liver injury can be detected using a variety of markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, gamma-glutamyltransferase (GGT) and alkaline phosphatase (AP) which can be monitored in plasma (120, 121). However, while these markers are useful, measureable changes in these markers generally indicate significant liver injury. Therefore, there is a need for biomarkers which could predict hepatic injury before it occurs. For example, Gao et al. (122) report the development of in vitro protein biomarkers, BMS-PTX-265 and BMS-PTS-837, which they were able to correlate with the toxicity of toxic and non-toxic drugs in vitro. Measurement of these kinds of protein biomarkers could provide a potential early warning system for hepatotoxicity or other types of toxicity. The need for new and more sensitive clinical biomarkers is being addressed by the Innovative Medicines Initiative (IMI) Safer and Faster Evidence-based Translation (SAFE-T) consortium (123, 124). The consortium is a European Union sponsored, 5 year partnership between academic groups, advisory boards and industry, which aims to detect, share and cross-validate new clinical markers of drug-induced injury to the liver, kidney and vascular systems in man to improve the safety of new medicines in patients. A recent paper by members of the consortium highlighted the use of ALT as a biomarker for drug-induced liver injury (125).

1.5 Evaluating the mechanisms of covalent binding

Evans et al. (57) have comprehensively reviewed the current approaches being undertaken within the pharmaceutical industry (particularly at Merck & Co.) to identify and characterise drug related species covalently bound to proteins. The link between organ toxicity and the formation of drug-protein adducts in vivo has not been proven, however covalent binding remains an area of interest to the pharmaceutical industry since the potential for drug-protein
adducts to be cytotoxic is certainly plausible. Identification and characterisation of drug-protein adducts is seen to be important by regulators such as the FDA and if the structure of new drug candidates can be modified to reduce the formation of such adducts, this can potentially improve the safety of any drugs reaching the market. The cost of bringing a new drug candidate to market is considerable; therefore it is certainly advantageous to have any information about possible covalent binding issues as early in the drug development process as possible. This may be before any radiolabelled drug is available and lack of a radiolabelled compound can make identification of the protein-bound species and the mechanism of binding challenging.

The most common methods in the literature for identifying drug-protein covalent binding involve using in vitro systems such as isolated cytochrome P450 enzymes, human liver microsomes or isolated hepatocytes, to reveal the metabolic activation processes, although some work has been completed on in vivo animal models.

Several papers have been reported using isolated cytochrome P450 enzymes to activate the compound (126-131), forming a reactive intermediate which can bind to the P450 or other proteins. LC-MS/MS analysis is then used to detect and identify the covalent adducts. Bateman et al. (126) described the methodology used to identify covalent adducts to intact P450 3A4 using 6',7'-dihydroxybergamottin (DHB – mw 372.41) and a Merck compound L-754,394 (mw 653.81) using recombinant (RECO) P450 3A4 and 3A4 Supersomes™ (Supersomes™ are commercially available recombinant cDNA-expressed cytochrome P450 enzymes prepared from the baculovirus-infected insect cell systems). Both compounds were incubated with both RECO™ P450 3A4 and 3A4 Supersomes™ for 3 min after the addition of NADPH and the reaction was stopped by freezing. Inhibition of 3A4 activity was monitored by a fluorescence-based assay, monitoring the depletion of NADPH. Incubations were prepared in triplicate to assess the accuracy and reproducibility of the mass measurement.

Two-dimensional HPLC was used to separate the components in the RECO™ incubation mixture prior to analysis by MS. Both compounds had been selected as they previously had shown covalent binding of P450 3A4 using radiolabelled compound. Incubation of L-754,394 resulted in detection of the apoprotein and the apoprotein plus 684.9 ± 2.9 Da. This corresponds to the mass of the inhibitor plus two oxygen atoms. Using previous knowledge of the metabolism of the compound it was deduced that the compound had undergone a double oxidation, or oxidation plus hydrolysis. However, definitive proof of the mechanism required structural characterisation by MS/MS, which was not reported. LC/MS analysis of the
incubation with DHB resulted in a peak corresponding to the apoprotein plus 386.8 ± 2.1 Da, as well as a second adduct present at a mass shift of 387.9 ± 3.5 Da relative to the first adduct. These mass shifts were rationalised by the mass of DHB plus one oxygen atom, and by the mass of two oxygen atoms and two DHB molecules. This can be explained by activation of the furan ring of DHB to an epoxide which attaches to the protein via nucleophilic attack. The two mass shifts suggest that more than one ligand has bound to the P450 active site. The experiment was repeated using the more complex Supersome™ incubations and similar results were obtained.

The methodology described provides direct evidence for the metabolic activation and covalent binding of drug compounds to P450 3A4. However, it has some limitations. While the authors have postulated some mechanisms for the formation of covalently bound protein adducts, this requires previous knowledge of the metabolic routes of the compound of interest, as well as the previous use of radiolabelled compound to identify and quantify the covalent binding. This would make the methodology unsuitable for use in early drug discovery studies where radiolabelled compound would not be available. The authors also offer no information about the nature of the binding site on the protein which could be crucial to understanding the mechanism of activation and any potential physiological effects produced in the body.

Digestion of the protein with an enzyme such as trypsin, prior to analysis by LC-Ms/MS may be useful, with the potential to identify the amino-acid binding site and the structure of the apoprotein. LC-MS/MS could also be used to try and produce a definitive structure of the bound species.

Qui et al. (132) reported a similar method to investigate the covalent binding of the glucuronide conjugate of benoxaprofen, a non-steroidal anti-inflammatory drug containing a carboxylic acid function, to human serum albumin. The formation of acyl glucuronides is a major metabolic pathway for this class of compounds and they are known to be capable of reacting with protein nucleophiles to form adducts, either directly or following tautomerisation. Benoxaprofen glucuronide (BG) was prepared from incubations with sheep liver microsomes and purified by HPLC. BG was then incubated in the presence and absence of sodium cyanoborohydride with human serum albumin and sodium phosphate buffer at 37 °C for 96 hours. Control experiments were also performed under the same conditions using benoxaprofen itself. The samples were then digested with trypsin, and a portion reserved for on-line LC-MS/MS analysis, with the remainder subjected to reversed-phase HPLC using a Vydac C18 column and an aqueous trifluoroacetic acid / acetonitrile gradient. Elution was
monitored with a fluorescence detector and each peak detected was collected and analysed by MS analysis.

MALDI-MS analysis was used to determine the molecular weights of the tryptic peptides and compared with those observed for the control sample. Any peaks that did not match known or anticipated tryptic peptides were subjected to post source decay (PSD) analysis to obtain the peptide sequence and site of modification. MALDI-MS/MS analysis was also carried out on each HPLC fraction.

Several direct modifications of amino-acid residues were observed and sufficient data was generated to elucidate the structure of the peptide involved and the site of modification. Binding was shown to occur predominantly at lysine residues and at serine and arginine residues to a lesser extent. The experiment also showed that the covalent binding occurred predominately by different mechanisms in the presence and absence of sodium cyanoborohydride (which acts to stabilise reactive imine species, enabling their detection). This method was successful in identifying the bound protein and the site of modification. It also gave some information on the reactive species bound to the protein.

Hypothetically, the use of in vivo samples from an intact animal would provide the most useful information about covalent binding and its effects, and work has been carried out by several research groups investigating covalent binding using in vivo samples. Qui, Benet and Burlingame (133) used a combination of two-dimensional gel electrophoresis and mass spectrometry to identify covalent binding and hepatic protein targets in vivo, from mice dosed with a toxic level of acetaminophen, a widely used analgesic and antipyretic. Both radiolabelled and non-labelled acetaminophen was dosed to mice at a dose level of 350 mg/kg and the livers removed following cervical dislocation. The livers were then homogenised and the protein concentration determined. SDS-PAGE analysis of the mouse liver proteins was used to separate the proteins in the sample and was combined with in-gel digestion with trypsin. MALDI mass spectrometric analysis was then performed, coupled with database searching of appropriate spots on the gels, to identify 23 protein targets for acetaminophen reactive metabolites.

Jones et al. (134) used a combination of SDS-PAGE, LC-MS/MS and database searching using the SEQUEST and BLAST databases to determine protein adducts in rat bile samples, from rats dosed with 1,1-dichloroethylene (DCE), a groundwater contaminant and diclofenac, a widely prescribed non-steroidal anti-inflammatory drug. Previous work had been carried out to
characterise the metabolism of DCE which forms the reactive metabolites DCE oxide and chloroacetylchloride, which reacts with glutathione. Diclofenac forms benzoquinone imine intermediates which also react with glutathione and potentially with other protein nucleophiles, together with acyl glucuronides which can react with lysine residues.

Bile samples were taken from rats dosed with DCE and diclofenac and the proteins separated using one-dimensional SDS-PAGE, which also removed the bile salts present. Bands from each gel were subjected to in-gel tryptic digestion and the resultant digest samples were analysed by LC/MS/MS, together with control bile samples. 23 additional proteins were identified in the samples from rats dosed with DCE, compared to the untreated control bile samples, including DCE-modified peptides from serum albumin.

Analysis of diclofenac samples resulted in only 6 additional proteins being identified which were not present in the control bile samples; however SALSA searches of the MS/MS data failed to confirm the presence of any diclofenac-derived modifications.

The method described in this paper (134) provides direct in vivo evidence for the DCE metabolic routes proposed in the formation of covalent adducts. However, it does rely heavily on previous knowledge of the metabolic profile of DCE, from in vivo studies and in vitro incubations using hepatocytes and isolated P450 enzymes to postulate these mechanisms.

It would appear that in order to gain the most information about covalent binding, it is important to have good background knowledge of the compound in question, as well as a relatively simple system to work with. Most successes to date have been seen for groups studying covalent binding using in vitro systems such as incubations with human serum albumin, human liver microsomes and isolated P450 enzymes. Bailey and Dickinson (12) compared the use of in vitro rat hepatocyte and rat liver microsome systems with in vivo rat plasma samples, and found that there was some variability in the protein targets for covalent binding, depending on the model chosen. They attributed this to a partial loss of functionality of isolated hepatocytes and liver microsomes, compared with the in vivo system. Therefore it must be understood that drug-protein adducts observed in vivo may be markedly different to those observed in in vitro models. However there are several advantages to using in vitro systems, such as the relative simplicity of the samples, increased drug-adduct concentration and ease of preparation, which mean that using in vitro systems to investigate drug-protein covalent binding is often a good starting point.
The detection and identification of drug-protein adducts remains a challenging task for many reasons. The isolation of complex mixtures of proteins, especially from \textit{in vivo} samples but also from \textit{in vitro} incubations such as human liver microsomes, can be problematic. Generally the extent of modification of the target protein is low, making identification difficult, particularly where radiolabelled drug is unavailable. Lohmann and Karst (135, 136) have presented an alternative method for the evaluation of covalent protein binding of reactive metabolites. Electrochemical oxidation was used to produce large quantities of the reactive metabolites of three compounds, acetaminophen, amodiaquine and clozapine which were then incubated with a low molecular weight protein $\beta$-lactoglobulin A. LC/MS analysis was used to directly identify the covalent binding of each molecule to the protein by intact protein analysis. Digestion of the covalently bound protein with trypsin and subsequent LC/MS analysis provided structural information on the position of binding on the protein. The data clearly demonstrates the applicability of this electrochemical method for the evaluation of covalent binding of drugs to proteins.

Mitrea and co-workers (137) recently presented an alternative method for the assessment of covalent binding without the use of radiolabelled compound, using a strategy of complete digestion (using immobilized and in-solution pronase digestion and acid hydrolysis) of proteins from an \textit{in vitro} microsomal incubation with subsequent LC/MS analysis. For each compound (acetaminophen, fipexide and diclofenac), one cysteine adduct peak, derived from reactive metabolite covalent binding, was detected. For estradiol, four cysteine adducts were detected.

1.6 Mass spectrometry for protein analysis

Mass spectrometry (MS) refers to the separation of gas-phase ions according to their mass/charge ratio. It is a widely applicable technique, capable of providing a wealth of information regarding a particular molecule. The technique is extensively used in academia and industry for many applications, and is particularly useful for providing accurate mass, elemental composition and structural information of inorganic, organic and biological samples, as well as accurate quantitation of samples for analytical testing.

Mass spectrometry has become the technique of choice in recent years for many proteomic and biochemical applications and can provide several useful pieces of information. It can be used to determine the intact molecular mass of a protein to a high degree of accuracy, which can then be compared against either information from protein databases such as UniProt if the
sequence is unknown, or to the theoretical mass based on a known protein sequence, which can be used to confirm protein identification.

Enzymatic or chemical digestion (138) of a protein into smaller peptides, with subsequent LC-MS analysis, can provide additional information on the protein sequence. A combination of MS and protein database searching to determine an accurate mass of each peptide can be used to determine the identity and sequence of the original protein. Collision induced dissociation (CID) fragmentation of the peptides can yield specific sequence information as well as the site and type of any post-translational modifications.

In its simplest form, a mass spectrometer is composed of the elements shown in Figure 1.11.

![Flow diagram of the components of a mass spectrometer](image)

Figure 1.11: Flow diagram of the components of a mass spectrometer

A number of different ionisation modes and mass spectrometer types were used during the course of this project and these will be discussed in the context of their use for the identification of drug-protein adducts and related applications.

### 1.6.1 Electrospray ionisation

Electrospray ionisation (139, 140) is an important tool in the analysis of proteins and peptides by mass spectrometry, mainly due to its capacity to be coupled to reversed-phase chromatography, thereby allowing the chromatographic separation of proteins and peptides prior to their introduction into the MS. It can also produce multiply charged ions, allowing the analysis of high molecular weight molecules, such as intact proteins, using analysers with a limited mass range such as a quadrupole mass analyser. A schematic of an electrospray source is shown in Figure 1.12.

The analyte is introduced into the electrospray source in the liquid phase, typically at flow rates anywhere between nL and μL/min, depending on the type of source. The ‘electrospray’ is formed by the application of a strong electric field to the liquid in the capillary. This field induces a charge accumulation at the liquid surface located at the end of the capillary, the solution is drawn into a ‘Taylor’ cone and budding occurs to form highly charged droplets.
Repulsion within the droplet causes the ions to be pushed to the surface of the droplet and as the droplet passes from the atmospheric pressure region of the source to the high vacuum of the mass analyser, the solvent in the droplet evaporates, causing the droplet to shrink until the concentration of the charge becomes so great that, at the Rayleigh Limit, the coulombic repulsion overcomes the cohesive forces within the droplet and the droplet explodes, releasing the desolvated ions (141, 142).

There is some debate as to the final part of this process, the formation of ions from highly charged droplets, and two mechanisms have been suggested. The Charge Residue Model (142, 143), states that as solvent evaporates from the droplet, the charge density becomes so great the charge on the droplet overcomes the surface tension, causing it to split (fission) to form smaller droplets. This process is then repeated until a single solvated ion remains. The Ion Evaporation Model, suggested by Iribarne and Thomson (144), suggests that as the solvent evaporates, the force of coulombic repulsion is great enough to push ions out of the droplet. It has been postulated that the actual mechanism is a combination of these two processes, with smaller ions being produced by the Iribarne and Thomson model, and larger ions by the Charge Residue Model.

Figure 1.12: Schematic of electrospray ionisation

Nanospray, developed by Mann and co-workers (145, 146), is an extension of the electrospray ionization technique, but with a very significant reduction in flow rate and needle diameter. The corresponding reduction in dimensions of the Taylor Cone and the sputtered droplets formed at low flow rates produce the enhancement in sensitivity observed with nanospray. The efficiency of desorption of analyte ions from the droplet increases as the size of the droplet decreases because of the larger surface area of the droplet compared to its volume.
This results in a greater proportion of analyte being desorbed from the droplet, and transmitted from the spray needle to the entrance aperture of the mass spectrometer. Mann quotes this improvement in efficiency to be in the order of a 500-fold increase (145). It is often used in the mass spectrometric analysis of protein digests as the improvement in sensitivity of this magnitude means it is possible to detect signals in the attomole range for peptides.

Several instrument manufacturers have utilised a perpendicular (or Z-spray) electrospray source rather than the conventional linear trajectory source as shown in Figure 1.12. In a linear trajectory source, the ions passing through the skimmer are accompanied by neutral material which can accumulate and gradually block the skimmer hole, significantly reducing the signal. The perpendicular source overcomes this by using a lens to focus the trajectory of the ions into the skimmer lenses of the ion source, while the neutrals remain on a linear trajectory and impact harmlessly on the inside of the ion source block, thereby reducing the build up of deposits.

1.6.2 Matrix-assisted laser desorption/ionisation

Matrix-assisted laser desorption/ionisation (MALDI) (147) is an alternative ionisation technique which involves the co-crystallisation of the sample with a large excess of a matrix. The sample is then bombarded with ions from a laser pulse (usually ultraviolet (UV) or infra-red (IR)) as shown in Figure 1.13. The energy from the laser is preferentially absorbed by the matrix and then transferred via vibrational transfer to the sample molecules, and both the matrix and the sample are ejected from the surface and ionised. This indirect energy transfer protects the sample from pyrolysis and in-source fragmentation and eliminates the necessity of changing the wavelength of the laser to match the absorption frequency of each analyte. The process is independent of the absorption properties and the size of the analyte. This means molecules of large molecular weight, such as proteins, can be desorbed and ionised. The technique favours the formation of singly charged [M+H]+ ions, making it unsuitable for coupling to quadrupole mass analysers, although very large proteins may carry several charges (148).

Suitable matrices for MALDI ionisation must absorb in the UV or IR range (depending on the laser used), distribute evenly to ensure efficient energy transfer and be acidic to promote protonation of the sample. Typical matrices include nicotinic acid, 2,5-dihydroxybenzoic acid (DHB), sinapinic acid and α-cyano-4-hydroxycinnamic acid (CHCA). DHB and CHCA are commonly used in peptide analysis because both of these matrices produce relatively few ions
above m/z 400 and do not complicate the mass spectrum by forming adducts with peptide ions.

Figure 1.13: Schematic of MALDI ionisation

The pulsatile nature of the laser, and the subsequent formation of ions in pulses, mean MALDI is particularly suitable to be used in conjunction with a Time-of-Flight analyser (ToF). The high sensitivity and large mass range capability of the ToF complement the high sensitivity and high mass range achieved by the MALDI source.

1.6.3 Mass analysers

Once ions have been produced in the source, they are separated by the mass analyser according to their mass to charge ratio. Mass analysers include quadrupoles, time-of-flight, ion trap, Orbitrap and ion cyclotron resonance, however since ion cyclotron resonance mass analysers were not used as part of this project, they will not be discussed further.

1.6.3.1 Quadrupole mass analysers

The quadrupole is the most widely used mass analyzer due to its ease of use, mass range covered, good linearity for quantitative work, resolution and quality of mass spectra (149-151). It consists of four cylindrical, or ideally, hyperbolic, rods to which a combination of direct and alternating potentials are applied. An ion entering the quadrupole experiences an oscillating field defined by the potentials on the rods, causing the ion to accelerate. As the voltages
applied to the rods varies, at any given moment, only those ions with a certain m/z value will have a stable trajectory through the centre of the quadrupole. Any other ions will impact on the rods and be neutralised. In this way, the quadrupole acts as a mass filter. The rods can either be gradually ramped to transmit the full m/z range of ions produced or set to transmit a specific ion of m/z ratio. Quadrupole mass analysers have a typical mass range of 100 – 4000 Da, low resolving power (typically 1000) and low mass accuracy, making them ideal for use as focussing devices and mass selectors in tandem mass spectrometers such as the quadrupole-time-of-flight, but generally unsuitable in isolation for coupling to MALDI ionisation and for modern qualitative protein and peptide analysis.

1.6.3.2 Time-of-flight mass analysers

The principle of time of flight (ToF) mass analysers (152-154) is to separate ions according to the time it takes for each ion to cross a field-free region, which in turn is dependent on the m/z ratio of the ion.

![Diagram of a time-of-flight mass analyser with a reflectron ion mirror](image)

**Figure 1.14: Diagram of a time-of-flight mass analyser with a reflectron ion mirror**

Ions are accelerated out of the source under a potential gradient and into a drift region. They pass through this region and impinge upon a detector, as shown in Figure 1.14. Ions entering the field-free region theoretically have the same kinetic energy, which is defined by the
applied accelerating potential. The use of a reflectron ion mirror (155) improves the resolving power of the time-of-flight mass analyser by increasing the length of the flight tube and compensating for any small variations in kinetic energy for ions of the same m/z ratio. Theoretically, there is no upper mass limitation for the ToF instrument; all ions can be made to proceed from the source to the detector. In practice, there is a mass limitation as it becomes increasingly difficult to discriminate between times of arrival at the detector as the m/z value becomes large. Another limitation is that very large molecules are difficult to ionise. Utilising an ionisation technique which produces multiply charged ions, such as electrospray ionisation, extend the working range. Modern ToF analysers are high resolution, high mass accuracy instruments capable of obtaining 40,000 FWHM (full width half maximum) resolution and better than 2 ppm mass accuracy, making them ideal for the mass measurement of proteins and peptides.

1.6.3.3 Orbitrap mass analysers

The Orbitrap mass analyser (156-161) consists of a hollow cylindrically shaped electrode with a co-axial inner electrode. Ions are injected into the Orbitrap into the electrical field that exists between the two electrodes and are trapped since the electrostatic and centrifugal forces acting on the ions are balanced. Ions oscillate around the central electrode in rings of a specific mass-to-charge ratio and the path of the ion ring is a combination of rotation around the central spindle with oscillation along the spindle axis, resulting in an approximate spiral motion. The frequency of these oscillations is inversely proportional to the square root of the mass-to-charge ratio. Fourier transform (FT) detection is used to detect the image current of each ring of ions on receiver plates as a time-domain signal. This data is then transformed to produce the mass spectrum.

One of the advantages of the Orbitrap over other mass analysers such as the quadrupole and the ToF is its superior mass resolving power (in excess of 100,000 FWHM), high mass accuracy (usually < 2 ppm (parts per million) with internal calibration) and large dynamic range (typically greater than 5000, an order of magnitude greater than ToF instruments) (162, 163).

1.6.4 Tandem mass spectrometry

The use of tandem mass spectrometry can offer significant advantages in terms of increasing both the selectivity and sensitivity of mass spectrometric analyses, as well as generating structural information through fragmentation. Tandem mass spectrometry (164, 165) involves
the coupling of mass spectrometric analysers in series. These analysers can be separated either in space (known as “tandem-in-space”) or in time (known as “tandem-in-time”). For tandem-in-space mass spectrometers, mass analysers are physically separated by a collision cell. Triple quadrupoles are the most common type of tandem mass spectrometer, but other examples of tandem-in-space mass spectrometers include a time-of-flight mass analyser coupled with a quadrupole mass analyser, which is known as a QTof instrument. The coupling of low resolution mass analysers such as the quadrupole with high resolution mass analysers such as time-of-flight or ion trap mass analysers offers the ability to perform fragmentation experiments on ions while maintaining the advantages of high resolution and high mass accuracy as discussed previously.

The collision cell is often a quadrupole mass analyser which acts to focus and isolate the ions. Fragmentation of ions entering the collision cell can be promoted by colliding analyte ions with neutral, inert, gas molecules (such as argon) – a process known as collision induced dissociation (CID). Ions generated in the ion source pass into the first mass analyser MS1, where a survey or selection of precursor ions takes place. Fragmentation of the selected ions then takes place in the collision cell. The fragments generated then enter the second mass spectrometer MS2, where the analysis of the product ions takes place. The advantage of this kind of analysis is that the fragments observed by MS2 can only have been derived from the ion selected by MS1.

Tandem-in-time methodology can be achieved using a quadrupole ion trap mass spectrometer, where the sequential stages of mass selection, CID and mass analysis are performed within the same ion trap mass analyser. The precursor ion is selected and isolated in the trap, with all other ions being ejected. Fragmentation of the selected ion then occurs within the trap via resonant excitation i.e. a small oscillating voltage is applied to the trap, increasing the ion’s internal energy, causing it to eventually fragment. The resultant fragments can be analysed. The advantage to this type of methodology is that the resultant fragment ions can be subsequently isolated and fragmented further; therefore it is possible to perform MS^n experiments to generate additional structural information. In reality, MS^n, where n represents infinity, is not possible as the ion yield from each fragmentation cycle decreases.

The coupling of scanning mass analysers to form tandem mass spectrometers such as the triple quadrupole mass spectrometer has advantages in terms of sensitivity and selectivity. Four main scanning modes exist for tandem mass spectrometry (Figure 1.15), using a triple quadrupole as an example.
Product ion scanning involves using the first quadrupole to select a particular precursor ion. The selected ion is transmitted into the collision cell, where it is fragmented. The third quadrupole is then used to scan the surviving precursor ions and fragment ions. This scanning mode is generally used to gain fragmentation information on a particular component, without the interference of other ions, i.e. the fragment ions can only have come from the ion selected in quadrupole 1, and so is useful for structural determination. For precursor ion scanning, quadrupole 1 is set to scan all of the ions emerging from the source, thereby sequentially transmitting different ions into the collision cell. Quadrupole 3 is set to select a particular product ion. Therefore a signal is only obtained for all precursors which fragment to form a defined product ion. This is useful for selecting ions with a common structural feature, since only ions which share a common fragment ion will be observed. In constant neutral loss scanning both quadrupoles 1 and 3 scanned with a fixed offset between them. Therefore, only ions which fragment to give the defined neutral loss will be detected. Selected reaction monitoring (SRM) involves monitoring a specific product ion produced by fragmentation of a specific precursor ion. Quadrupole 1 and 3 are fixed to select a specific precursor and product ion, therefore a signal will only be obtained for the specific product ion formed from fragmentation of a specific precursor. The experiment is extremely selective and can be particularly useful for quantification since SRM is generally more sensitive than other MS modes, and can be effective for searching for a known component in a very complex sample.
With all the scanning modes, their utility depends on the amount of information known about the ion of interest. For example, the high selectivity and sensitivity of SRM can only be utilised when the m/z of the ion of interest is known and its fragmentation is well characterised.

1.6.5 Peptide fragmentation

Collision Induced Dissociation (CID) of protonated tryptic peptides can provide important identification sequencing information of proteins of interest. Low energy fragmentation predominantly occurs in predictable patterns along the amide backbone and can be represented by the nomenclature devised by Biemann (166) as shown in Figure 1.16.

![Figure 1.16: The nomenclature of peptide fragmentation](image)

Figure 1.16: The nomenclature of peptide fragmentation

The mobile proton model (167) represents a general model for peptide fragmentation in the gas phase. Several studies carried out using peptide structures composed of amino acids of varying basicity have shown that the basicity of the basic residue affects the collision energy required to induce dissociation. Therefore the dissociation energy is greatest for arginine containing peptides, which are the most basic and smallest for non-basic peptides such as aspartic or glutamic acid. b and y ions (Figure 1.16) are the most prominent in low energy peptide fragmentation since they are formed via cleavage of the peptide bond and are therefore more stable. However, high-mass b ions will often fragment further to form low-mass b ions and therefore it is the low-mass b ions which are generally observed in spectra. A mechanism for b and y ion formation was devised by Yalcin et al. (168, 169) and is shown in Figure 1.17.
The carbonyl is more basic than the amide site; therefore protonation of the carbonyl is more likely. The nearby carbonyl acts as a nucleophile to attack the electropositive carbon of the protonated carbonyl. The proton resides on the nitrogen in the above mechanism for convenience and although protonation on the nitrogen is less likely, it does significantly decrease the C(O)-N bond strength, suggesting facile cleavage of the amide bond. The mechanism therefore proceeds via a charge-initiated mechanism. b₁ ions cannot be formed if the peptide has a free amine at the N-terminus as there is no carbonyl group to form the oxazalone. The predictable nature of peptide fragmentation means it can be utilised to protein sequencing and searching against known protein databases.

1.6.6 Ion mobility mass spectrometry

Ion mobility mass spectrometry is a technique which combines the use of an ion mobility drift tube with conventional mass spectrometric techniques. Ions are separated based on their collision cross-sectional area as the ions pass through a drift tube with an applied electric field and containing a carrier gas, usually nitrogen, which opposes the direction of ion flow (170, 171). The collision cross-sectional area of an ion is dependent on its mass, charge, size and
shape, meaning that ion mobility is capable of separating two ions of the same mass to charge ratio but different shape. The greater the collision cross-sectional area of the ion, the more the drift gas impedes the ion, meaning it takes longer to traverse the drift tube (Figure 1.18). The energy the ions gain from collisions with the buffer gas is greater than the energy they gain from the applied electric field, meaning the ions have approximately the same energy as the drift gas molecules and diffusion process are dominant (170).

![Figure 1.18: Separation in an ion mobility spectrometer](image)

The coupling of ion mobility spectrometry with conventional mass spectrometry can have several advantages. Ion mobility can add an additional dimension of separation, particularly useful for separation ions of the same mass-to-charge but different shape. This has been utilised by the groups of Clemmer (172) and Ashcroft (173) who have both used the technique to investigate protein conformation. Ion mobility also has the potential to separate ions of interest from endogenous ions of the same mass-to-charge in a complex mixture and so may aid the identification of low abundant components such as drug-protein adducts in a protein digest.

### 1.6.7 Accelerator mass spectrometry

Accelerator mass spectrometry (AMS) is an extremely sensitive technique, first developed in the 1970s for the detection of low–abundant, long-lived isotopes, typically for the analysis of samples from geochronology and archaeological research (174, 175). The application of AMS for biomedical research was first postulated by Keilson and Waterhouse in 1978 (176). Knowledge of the metabolism and pharmacokinetics of new drugs in humans is a key aspect in understanding its pharmacological and toxicological effects in patients. The definitive study to
generate this type of information is known as the human ADME study, and involves the dosing of the test drug, labelled with a radio isotope such as $^{14}$C, to a small number of volunteers. Samples of urine, faeces and plasma are collected over a time course and are analysed to monitor the circulation and elimination of the drug and its metabolites. The levels of radioactivity used in these types of studies is limited by the regulations set out by the World Health Organisation (177) and the International Commission on Radiological Protection (178) and is typically in the region of 1-10 mSv, and broadly comparable to the radiation dose received by a patient during a brain or chest CT scan (1-18 mSv) (179). The high cost of these studies, mainly due to the cost of radiolabelled synthesis and increased clinical costs due to the handling of radioactive material, and the ethical implications of exposing healthy volunteers to levels of ionising radiation, mean that these studies are traditionally conducted late in the drug development process, around the time of the Phase II clinical trials. However, this carries a substantial financial risk as the data from a human ADME study may reveal significant issues with the drug of investigation e.g. high levels of metabolites which may be unique to humans and therefore have not undergone toxicity testing in preclinical species.

The most common method of analysis of samples from human ADME studies is liquid scintillation counting (LSC). Energy detected from the atomic decay of the radio isotope excites the liquid scintillant and is converted into photons of light which can then be detected by photomultiplier tubes. The number of photons detected is directly proportional to the number of radioactive decay events, and so is dependent on the number of isotopes present (180). Although LSC is widely used, it does suffer from an inherent lack of sensitivity when used for long-lived radioactive isotopes such as $^{14}$C. The radioactive half-life of $^{14}$C is 5730 years (181), meaning very few of the $^{14}$C atoms in a sample are decaying at any one time, and so a very large number of $^{14}$C atoms would need to be present in order for it to be likely that a radioactive decay event will occur in the time-frame of the analysis. Lappin and Garner estimate that 50 billion $^{14}$C atoms would need to be present to generate an average of 50 disintegrations per minute (dpm) (182), which is the typical limit of detection of LSC. Therefore, for very low doses of radioactivity, or for situations where the residual levels of radioactivity in a sample are estimated to be very low, LSC is unsuitable as a detection method.

AMS can significantly improve the limit of detection of radio isotopes in biological samples as it literally counts the number of isotope atoms present and can detect down to the zeptomole ($10^{-21}$) range (181). However, despite the obvious advantages in sensitivity, the preparation of samples for analysis AMS is a lengthy, complex and costly process and therefore it is generally
only used in the pharmaceutical industry when the low levels of radiocarbon present make it a necessity, for example when a radiocarbon dose is administered to humans.

The method for the preparation of samples for AMS analysis is described by Vogel (183) and colleagues at Lawrence Livermore National Laboratory in California (184). For a complex sample such as an in-vitro metabolism preparation or a biological sample such as plasma, urine or faeces, the samples are first separated using an appropriate HPLC method and fractionated to separate the components of interest. Each fraction is transferred to a quartz tube and reduced to dryness, combined with aqueous sodium benzoate and copper oxide and heated to 900°C to form CO₂. The CO₂ is the cryogenically transferred to tubes containing zinc powder, titanium hydride and a cobalt catalyst and heated to 500-550 °C to form graphite. The graphite is then pressed into aluminium cathodes for analysis by AMS.

A caesium-sputter ion source is used to produce negative ions from a graphitised sample under high vacuum (185). The high kinetic energy of the caesium ions knocks atoms out of the sample and then donates electrons to these ejected particles. The use of a negative ion source eliminates contamination due to ¹⁴N for ¹⁴C analysis. These negative ions are accelerated by a tandem Van der Graff accelerator and converted to positive ions through collisions with the electrons in a molecular dissociation foil. A magnetic quadrupole lens focuses the isotope of interest into a second mass spectrometer and any remaining interfering ions are removed based on their momentum per mass in the electric and magnetic fields of the Wien filter. The resultant ¹⁴C⁺ ions can then be detected.

As discussed by White and Brown (186), and Turteltaub and Dingley (187), AMS can be a useful tool in the detection and measurement of the covalent binding of reactive metabolites to proteins or DNA in biological samples. This has been achieved successfully for the detection of in vivo protein adducts of benzene in the liver and bone marrow of mice dosed with [¹⁴C] benzene at 5 μg/kg (188), in the attomole range.

While the superior sensitivity of AMS makes it an ideal technique for detecting the usually very low levels of covalent binding of [¹⁴C] labelled drugs to proteins, the complex sample preparation, costly analysis and lack of available instrumentation mean that AMS is not currently in widespread use for this kind of application. In addition, the disadvantage of AMS is that, unlike conventional mass spectrometry, it cannot provide any structural information on protein adducts themselves as it is purely a quantitative detection technique. To maximise the
benefits of AMS, coupling of AMS analysis with conventional analytical techniques such as MS and NMR is necessary.

1.7 Project objectives

It has been postulated that covalent binding of drug molecules to proteins \textit{in vivo} may be linked to organ toxicity effects, since there is a potential for drug-protein adducts to be cytotoxic. It is therefore important for the pharmaceutical industry to gain a greater understanding of both the reactive species and the binding mechanisms involved. With this greater understanding, comes the potential to help minimise, by structural modification, the ability of new drug candidates to bind to proteins within the body.

Principally, this project has three major aims:

1. To definitively identify the structure of the drug related reactive species covalently bound to the protein,
2. To deduce the identity of the protein to which the reactive species is bound,
3. To identify the specific site of binding.

It is hoped that this information will enable a probable mechanism for the covalent binding of drug-related material to proteins \textit{in vivo} to be deduced, with a view to using this information to reduce the potential of new drug candidates to covalently bind.
2 Experimental

2.1 Materials

Human liver microsomes (HLM, 20 mg/mL in 250 mM sucrose) were purchased from Gentest™, a BD Biosciences Company, Woburn, MA, USA, unless otherwise stated. Human liver microsomes were pooled from a mixed gender pool of 20 preparations and were validated for enzyme activity by Gentest™.

Human CYP3A4, P450 reductase and cytochrome b5 Supersomes™, human CYP2E1, P450 reductase and cytochrome b5 Supersomes™ and human CYP2C8, P450 reductase and cytochrome b5 Supersomes™ (all 12 mg/mL in 100mM potassium phosphate buffer, pH 7.4, 1 nmole/mL cytochrome P450, and produced using a baculovirus expression system) were purchased from Gentest™, a BD Biosciences Company, Woburn, MA, USA. The Supersomes™ were validated for enzyme activity by Gentest™ against testosterone hydroxylation for CYP3A4, chlorzoxazone hydroxylation for CYP2E1 and palitaxel 6(alpha)-hydroxylation for CYP2C8.

Dipotassium hydrogen orthophosphate (K₂HPO₄), potassium dihydrogen orthophosphate (KH₂PO₄), sodium bicarbonate (NaHCO₃), β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate (β-NADP), D-glucose 6-phosphate and glucose 6-phosphate dehydrogenase, sodium bicarbonate, reduced glutathione, ammonium bicarbonate, calcium chloride and ammonium acetate were all purchased from Sigma-Aldrich, Gillingham, Dorset, UK.

The test articles [14C] 4-acetamidophenol (specific activity 55 mCi/mmol) and non-radiolabelled 4-acetamidophenol (acetaminophen) were purchased from American Radiochemical, St Louis, MO, USA and Sigma-Aldrich, respectively. [14C] SB-649686 (specific activity 55 mCi/mmol) and non-radiolabelled SB-649868 were supplied by G.E. Healthcare UK Ltd., Amersham, UK and Product Development, GlaxoSmithKline, Stevenage, UK, respectively. Amodiaquine dihydrochloride dihydrate (4-([7-chloro-4-quinolinyl]amino)-2-[[diethylamino]methyl]phenol) was purchased from Sigma-Aldrich.

Packard SafeScint liquid scintillation fluid was purchased from PerkinElmer UK Ltd. (Beaconsfield, Buckinghamshire, UK). Whatman GF-B filters were purchased from Whatman Ltd. (Maidstone, Kent, UK).
Ultra pure water (HPLC grade, 18.2 MQ) was produced by a Maxima Ultra Pure Water system (Elga, Buckinghamshire, UK). Methanol, acetonitrile, trifluoroacetic acid (TFA) and formic acid (all HPLC grade) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Glacial acetic acid and glycerol were purchased from Sigma-Aldrich.

Silver nitrate solution (0.1M), potassium hydroxide pellets, cysteine and chloroform were all purchased from Sigma-Aldrich.

Trypsin (sequencing grade, modified from bovine pancreas) was purchased from Fisher Scientific. Pronase (Protease from Streptomyces grisis, Type XIV), and bovine serum albumin (lyophilized powder) purchased from Sigma-Aldrich. RapiGest SF™ surfactant was purchased from Waters Corporation (Milford, MA, USA).

NaCsI Mass Spectrometer calibration solution was purchased from Waters Corporation (Manchester, UK). Leucine Enkephalin (acetate salt, hydrate), myoglobin (from equine heart), enolase 1 (from baker’s yeast S. cerevisiae) and [Glu1]-Fibrinopeptide B were purchased from Sigma-Aldrich.

NuPAGE™ SDS-Page running buffer (x 20 concentrate), NuPAGE™ lithium dodecyl sulphate (LDS) sample buffer (x 4 concentration), NuPAGE™ dithiothreitol (DTT) reducing agent (0.5 M DTT, x 10 concentration), Nu-PAGE™ antioxidant mix, NuPAGE™ colloidal blue staining kit (Stains A and B), Novex Multimark™ marker protein and NuPAGE™ Novex pre-cast 10% Bis-Tris polyacrylamide gels were all purchased from Invitrogen Life Technologies Ltd. (Paisley, UK).

Methylated protein molecular weight marker (methyl 14C, 26.4 µCi/mg [976.8 kBq/mg]) was purchased from Perkin Elmer (Boston, MA, USA).

2.2 In vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

Microsomal reaction buffer 100 mM phosphate buffer pH 7.4 was prepared by adjusting the pH of a 100 mM solution of K2HPO4 (17.4 g dissolved in 1 L of ultra pure water) using 100 mM KH2PO4 (13.6 g dissolved in 1 L of ultra pure water) to pH 7.4.

2 % (w/v) sodium bicarbonate solution for dissolution of the cofactor was prepared by dissolving 5 g of NaHCO3 in 250 mL of ultra pure water.

The incubation cofactor was prepared immediately prior to use by dissolving approximately 2.22 mM β-NADP (1.7 mg/mL final concentration), 27.6 mM glucose-6-phosphate (7.8 mg/mL
final concentration) and 25 units of glucose-6-phosphate dehydrogenase per mL in 2% (w/v) sodium bicarbonate (aq.)

Human liver microsome incubations were performed in a total volume of 0.5 mL, containing 25 µL of human liver microsomes (1 mg/mL) and 100 µL cofactor and were made up to volume with 100 mM phosphate buffer pH 7.4. The samples were pre-incubated for 5 minutes in a shaking water bath at 37°C and the reaction initiated by the addition of the test article (5 µL of a 1 mM solution in ethanol, equivalent to a 10 µM final concentration). Incubations with CYP3A4 and CYP2E1 Supersomes™ were conducted using the same procedure, with the exception that 50 µL of Supersome™ were used.

In addition, control incubations without cofactor (i.e. with the replacement of 100 µL cofactor with µL 2% (v/v) sodium bicarbonate in water were performed alongside the test incubations.

Incubations were performed at 37°C for 0, 1, 2 and 4 hours after the addition of the test article, and the reactions terminated by centrifugation of the incubation for 10 minutes at 10,000 x g. Supernatants were removed and analysed separately by LC /MS to assess the metabolic turnover of the test articles, and the protein pellets were retained for further analysis.

2.3 Quantitative assessment of covalent binding to protein

All reagents used were as for Section 2.2. with the exception that a 90% (v/v) methanol in water was prepared as an extraction and wash solvent.

The incubations were carried out as detailed in Sections 2.2. with the exception that the reactions were terminated by the addition of 4 mL of 90% (v/v) methanol in water.

Following termination of the reactions, each incubation was filtered using a Millipore 1225 Vacuum Filtration manifold connected to a Millipore Vacuum Pressure Pump (both Millipore, Billerica, MA, USA), and loaded with Whatman GF-B filter papers soaked in 100 mM phosphate buffer pH 7.4. Each filter paper wash was washed five times by the addition of 4 mL of 90% (v/v) methanol in water and the vacuum applied after each addition. Each filter was then transferred to a scintillation vial and 10 mL of SafeScint 1:1 liquid scintillation fluid (LabLogic Ltd., Sheffield, UK) added. The filters were soaked overnight and each filter assayed using a Beckman Liquid Scintillation Counter (Beckman Coulter UK Ltd., High Wycombe, UK). Radioactivity was quantified using a liquid scintillation counter [PerkinElmer LAS (UK) Ltd.] using a 5 minute counting method (1% precision) with automatic quench correction, using an
external standard method (189). Samples were analysed together with blank samples containing liquid scintillation fluid to provide a background count rate which was subtracted from each sample count rate before calculation of individual results.

The total binding was calculated by first converting the disintegrations per minute (dpm) per sample value using the dpm/pmol for each test compound. The cofactor dependent binding (pmol/mg/hr) was calculated using the following equation:

\[
\text{cofactor dependant binding} = (\text{cofactor binding at } x \text{ hours} - \text{cofactor binding 0 hours}) - (\text{no cofactor binding at } x \text{ hours} - \text{no cofactor binding 0 hours})
\]

The cofactor independent binding (pmol/mg/hr) was calculated by:

\[
\text{cofactor independent binding} = \text{no cofactor binding at } x \text{ hours} - \text{no cofactor binding 0 hours}
\]

Finally, the total binding (pmol/mg/hr) was calculated by the addition of the cofactor dependent and cofactor independent values.

2.4 Digestion of incubation protein with trypsin, Lys-C and Asp-N

All reagents used for the incubations were as described in Section 2.2. In addition, aqueous 50 mM ammonium bicarbonate was prepared by dissolving 1.98 g in 500 mL of ultra pure water. 0.1% (w/v) 100 mM dithiothreitol (DTT) was prepared by dissolving 15.4 mg of DTT in 1 mL of 50 mM ammonium bicarbonate. 100 mM iodoacetamide (IAA) was prepared by dissolving 18.5 mg of IAA in 1 mL of 50 mM ammonium bicarbonate.

All incubations were carried out as detailed in Sections 2.2. After centrifugation and removal of the supernatant, 500 µL of 100 mM phosphate buffer, pH 7.4 was added to each protein pellet and vortex mixed to disturb the pellet. The sample was then centrifuged for 10 minutes at 10,000 x g and the supernatant removed. This was repeated a further two times to ensure the complete removal of non-covalently bound drug-related material.

After washing, the pellet was re-suspended in 100 µL of aqueous 50 mM ammonium bicarbonate and vortex mixed. 5 µL of 100 mM DTT in 50 mM ammonium bicarbonate was added and each sample incubated on a heating block for 30 minutes at 60°C, to reduce disulfide bridges in the protein. The sample was allowed to cool and 10 µL of the alkylation reagent 100 mM IAA in 50 mM ammonium bicarbonate added, vortex mixed and incubated in the dark at room temperature, for 20 minutes, to alkylate any free thiol groups to prevent the
re-forming of disulfide bridges. Digestion with trypsin was performed by the addition of 5 µL of a 0.2 µg/µL solution of trypsin in 50 mM ammonium bicarbonate, digestion with Lys-C was performed by the addition of 5 µL of a 0.2 µg/µL solution of Lys-C in 50 mM ammonium bicarbonate and digestion with Asp-N was performed by the addition of 5 µL of a 0.2 µg/µL solution of Asp-N in 50 mM ammonium bicarbonate. All samples were incubated overnight on a heating block at 37°C.

After incubation, samples were reduced to dryness under N₂ and reconstituted in 100 µL 50:50 acetonitrile: water for LC-MS analysis.

### 2.5 Digestion of incubation protein with trypsin using the RapiGest SF™ reagent

All reagents used for the incubations were as described in Experimental Section 2.2. In addition, 0.1% (w/v) RapiGest SF™ solution in was prepared by dissolving 1 mg of RapiGest SF™ in 1 mL of aqueous 50 mM ammonium bicarbonate.

All incubations were carried out as detailed in Experimental Section 2.2. After centrifugation and removal of the supernatant, 500 µL of 100 mM phosphate buffer, pH 7.4 was added to each protein pellet and vortex mixed to disturb the pellet. The sample was then centrifuged for 10 minutes at 10,000 x g and the supernatant removed. This was repeated a further two times to ensure the complete removal of non-covalently bound drug-related material.

After washing, the pellet was re-suspended in 100 µL of 0.1% RapiGest SF™ solution and vortex mixed. 5 µL of 100 mM DTT in ammonium bicarbonate was added and each sample incubated on a heating block for 30 minutes at 60°C, to reduce disulfide bridges in the protein. The sample was allowed to cool and 10 µL of the alkylation reagent 100 mM IAA in 50 mM ammonium bicarbonate added, vortex mixed and incubated in the dark, at room temperature, for 20 minutes, to alkylate any free thiol groups to prevent the re-forming of disulfide bridges. Digestion with trypsin was performed by the addition of 5 µL of a 0.2 µg/µL solution of trypsin in 50 mM ammonium bicarbonate, with samples incubated overnight on a heating block at 37°C.

1 µL of concentrated TFA was added to each sample to terminate the digestion, and the samples incubated at 37°C for 45 minutes to precipitate the RapiGest SF™ reagent. The samples were centrifuged for 10 minutes at 10,000 x g and the supernatant transferred to HPLC vials for LC-MS analysis.
2.6 Glutathione trapping of reactive metabolites of acetaminophen and SB-649868

All reagents used were as for Experimental Section 2.2. with the exception of a 100 mM solution of reduced glutathione (30.7 mg/mL in ultra pure water) which was freshly prepared on the day of use.

All incubations were carried out as described in Experimental Section 2.2. with the exception that 50 µL of the 100 mM reduced glutathione solution (to give a final glutathione concentration of 10 mM) was added to the incubation, prior to the addition of the test article.

Large volume preparations of the 4 hour in vitro incubation of human liver microsomes, in the presence of glutathione and the incubation cofactor were prepared for acetaminophen using the protocol described in Experimental Section 2.5, with the exception that the concentration of acetaminophen was increased to 10 mM (equivalent to a 100 µM final concentration). Incubations were performed at 37°C for 4 hours after the addition of the test article, and the reactions terminated by centrifugation of the incubation for 10 minutes at 10,000 x g. The supernatants from 20 incubations (each individual volume of 500 µL) were combined to give a final volume of 10 mL.

2.7 Preparative high performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR) analysis of the glutathione-trapped reactive metabolites of acetaminophen

Preparative HPLC analysis was performed by Rita Tailor and NMR analysis was performed by Andrew Roberts (both GlaxoSmithKline, Ware). The preparation of a large volume 4 hour in vitro incubation of acetaminophen with human liver microsomes, in the presence of glutathione is described in Experimental Section 2.6.

A chromatographic separation was performed on an Agilent 1100 Preparative HPLC system consisting of an autosampler, injection pump, two binary HPLC pumps, a fraction collector and a diode array detector (all Agilent 1100, Agilent Technologies Ltd., Wokingham, UK), controlled by ChemStation software Rev B. 03.02 (Agilent Technologies Ltd.), and coupled to a Waters ZQ single quadrupole mass spectrometer controlled using MassLynx v4.1 software (both Waters Ltd., Manchester, UK). Chromatographic separation was performed on a Waters X-Bridge prep C18 5µm, 10 x 250 mm column, maintained at 30°C using a Jasco (Jasco UK, Dunmow, UK) column oven. All 10 mL of the sample was injected onto the column. A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (Solvent B) at a flow rate of 4 mL/min. The gradient is described in Table 2.1.
Table 2.1 Description of the gradient employed for the preparative chromatographic separation of acetaminophen and its glutathione-trapped metabolites

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>90</td>
<td>10</td>
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<tr>
<td>31</td>
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<td>95</td>
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<tr>
<td>34</td>
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<td>95</td>
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<tr>
<td>36</td>
<td>100</td>
<td>0</td>
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<tr>
<td>40</td>
<td>100</td>
<td>0</td>
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</table>

MS detection was used to detect fractions containing drug-related material. These fractions were reduced to dryness under N₂ and reconstituted in an appropriate NMR solvent (50:50 v/v acetonitrile: deuterium oxide) for ¹H NMR analysis.

¹H NMR experiments were conducted using an Avance 600 spectrometer (Bruker UK Ltd. Coventry, UK) equipped with an inverse 5-mm TCI Cryo-Probe (²H/¹³C/¹⁵N) operating at 600.40 MHz, and controlled using Topspin 2.1 software (both Bruker UK Ltd.). ¹H NMR spectra were acquired using a standard NOESY-PRESAT pulse sequence with spoil gradients for solvent suppression with time-shared double pre-saturation of the water and acetonitrile frequencies. 128 transients were acquired into 49,152 data points over a spectral width of 12,019 Hz (20 ppm) with an inter-scan delay of 3 s, giving a total pulse repetition time of 5 s. A standard of acetaminophen (1 mg/mL, in 50:50 (v/v) acetonitrile: deuterium oxide) was also analysed by ¹H NMR using the method described, for comparative purposes.

2.8 Synthesis of N-acetyl-p-benzoquinone imine (NAPQI) using silver oxide

Fresh silver oxide was prepared by adding 10 mL of 100 mM silver nitrate solution (purchased as a 100mM solution from Sigma-Aldrich) to a solution of 100 mM potassium hydroxide (prepared by dissolving 100 mg in 18 mL of water) on ice. The mixture was left for approximately 15 minutes and a precipitate of silver oxide allowed to form. This precipitate was filtered, washed with acetone and allowed to dry in air.

\[ 2\text{AgNO}_3 + 2\text{KOH} \rightarrow \text{Ag}_2\text{O} + 2\text{KNO}_3 + \text{H}_2\text{O} \]
Once dry, the silver oxide powder was added to 10 mL of a 1 mg/mL solution of acetaminophen in chloroform and the mixture stirred for 1 hour until the colourless solution turned a pale yellow colour. This solution was filtered, evaporated to dryness under nitrogen and then reconstituted in 6.6 mL of acetonitrile to give a final concentration of 10 mM NAPQI in acetonitrile.

2.9 Incubation of chemically generated NAPQI with cysteine and glutathione

10 µL aliquots of a freshly prepared 10 mM NAPQI solution (Experimental Section 2.8) were added to 1 mL of a 10 mM solution of cysteine in water, and 1 mL of a 10 mM solution of glutathione in water, respectively (100-fold molar excess). Each solution was incubated in a shaking heated water bath at 37°C for 2 hours. The reaction mixtures were then transferred to glass vials for LC/MS analysis.

2.10 Evaluation of the thermal stability of the chemically generated cysteine and glutathione adducts of acetaminophen

Cysteine and glutathione adducts of acetaminophen (approximately 1 mL of each) were generated as described in Experimental Section 2.9. For both the cysteine and glutathione adducts, each solution was vortex mixed and divided into two aliquots (each of approximately 500 µL). One aliquot from each preparation was incubated at 37°C and 50°C overnight in a shaking water bath to evaluate the thermal stability of the acetaminophen-adducts formed. After incubation, each aliquot was transferred to glass vials for LC/MS analysis using the method described in Experimental Section 2.12.

2.11 Incubation of chemically generated NAPQI with cytochrome P450 CYP2E1 Supersomes™ and digestion with trypsin

A 5 µL aliquot of a freshly prepared 10 mM NAPQI solution (Experimental Section 2.8) was added to 500 µL of CYP2E1 Supersomes™ (1 pmol/ µL of protein) and the volume made up to 1 mL with 100 mM potassium phosphate buffer (pH 7.4). The solution was incubated in a shaking heated water bath at 37°C for 2 hours. After centrifugation at 10,000 x g for 10 minutes, and removal of the supernatant, the protein pellet was washed with 100 mM phosphate buffer pH 7.4 and digested with trypsin using the method described in Experimental section 2.5.
2.12 LC/MS analysis of the metabolites formed after *in vitro* incubation of acetaminophen with human liver microsomes and cytochrome P450 CYP2E1 Supersomes™, and the glutathione and cysteine conjugates of acetonitrile formed via the chemical synthesis of NAPQI and non-enzymatic incubation with glutathione and cysteine, respectively

Following centrifugation upon completion of the *in vitro* incubation of acetaminophen with human liver microsomes and cytochrome P450 Supersomes™ (Experimental Section 2.2), the supernatant were retained and transferred to HPLC vials for LC/MS analysis to evaluate any metabolites formed. Cysteine and glutathione adducts of acetaminophen (approximately 1 mL of each) were generated via the incubation of chemically generated NAPQI with an excess of cysteine and glutathione, as described in Experimental Section 2.9.

LC/MS analysis was performed on a QToF Premier mass spectrometer coupled to an Acquity UPLC system (both Waters Ltd.) consisting of a binary solvent manager, sample manager and UV detector. MassLynx v4.1 software from Waters was used to control both the MS and Acquity systems. External mass calibration of the mass spectrometer was performed over an m/z range of 50 – 1000 using a solution of NaCsI (purchased from Waters Ltd.). The instrument was tuned to give the best possible performance using a 10 µg/mL solution of acetaminophen in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.15 mL/min 1:1 0.1% (v/v) formic acid in water: acetonitrile, using the integrated syringe pump. An external lock mass (1 µg/mL leucine enkephalin in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using an dedicated HPLC pump (Shimadzu, Manchester, UK) during each analyses, for automatic mass correction.

Chromatographic separation was performed on a Waters Acquity BEH C18 1.7 µm, 2.1 x 100 mm column, at 40°C. The autosampler was set at 4°C. The injection volume was 10 µL (partial loop with needle overfill). A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (Solvent B) at a flow rate of 0.3 mL/min. The solvent flow from the UPLC system was split 1:1 (MS:waste) post-column using a PEEK T-piece. The gradient is described in Table 2.2.

Where radiolabelled [14C] acetaminophen was used, the solvent flow from the UPLC system was split 1:1 (MS: fraction collector) post-column using a PEEK T-piece. Fractions were collected using a HTX-PAL fraction collector (CTC-Analystics AG, Zwingen, Switzerland) into four 96-well Luma Plate™ -96 plates, containing yttrium silicate solid scintillant (PerkinElmer LAS UK Ltd.), at a speed of 2.3 s per well. Once collected, the plates were allowed to dry in air and then sealed
with Top Seal A (PerkinElmer LAS UK Ltd.) clear adhesive sealing tape, prior to off-line radiodetection. Off-line radiodetection (190) was performed by scintillation counting using a Packard TopCount NXT counter (PerkinElmer LAS UK Ltd.) using a 5 s count time per well. The scintillation counting data was imported into Laura v3 Software (LabLogic Systems Ltd.) using the LSC import function and processed to generate a reconstructed radiochromatogram for each analysis.

Table 2.2 Description of the gradient employed for the chromatographic separation of acetaminophen and its metabolites and glutathione and cysteine conjugates

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>99</td>
<td>1</td>
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<tr>
<td>1</td>
<td>99</td>
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<td>9.5</td>
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<tr>
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<td>99</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>99</td>
<td>1</td>
</tr>
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ESI positive ion MS data was collected over the mass range m/z 50 – 1000, using the following conditions: capillary voltage, 2 kV; sampling cone, 35 arbitrary units; extraction cone, 4.5 arbitrary units; ion guide, 2.5 arbitrary units; source temperature, 150 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 400 L/hour; low mass (LM) and high mass (HM) resolution, 4.7 and 15.0 arbitrary units, respectively, to give a resolution of approximately 8000 at m/z 400. Data was collected using an MS² experiment composed of two functions each of scan time 0.2 s, inter-scan time 0.02 s, where function 1 used a collision energy offset of 4 arbitrary units (low energy) and function 2 utilised a collision energy offset ramp of 20 – 30 arbitrary (high energy). The third function was used to collect data on the external lock mass for automatic mass correction. Data was collected for 0.5 s at an interval of 10 s, and averaged over 10 scans. Additional MS/MS experiments were conducted on ions of interest which were selected after interrogation of the MS² data described above. These MS/MS experiments were conducted using the MS conditions above with the exception that the LM resolution was changed to 15 arbitrary units (on the Waters QTof Premier, this equates to an approximate mass ion selection window of 0.2 Th). The m/z was selected to 1 decimal place in each method, with the fragmentation energy employed.
determined by a collision energy offset ramp of 20 – 30 arbitrary units. Data was collected with a scan time of 0.2 s with an inter-scan delay of 0.02 s.

### 2.13 LC/MS of intact cytochrome P450 CYP2E1 Supersome™ protein

CYP2E1 Supersome™ (5 µL) was injected directly onto a reversed-phase column for LC/MS analysis as described below. In addition, 100 µL of CYP2E1 Supersome™ was centrifuged at 10,000 x g for 10 minutes, the supernatant removed and the protein pellet resolubilised in 100 µL 100 mM potassium phosphate buffer (pH 7.4) prior to analysis by LC/MS. 20 pmol/µL solutions of myoglobin and enolase were also prepared in water for use in method development.

LC/MS was conducted on a QTof Premier mass spectrometer coupled to an Acquity UPLC system as described in Experimental Section 2.12. The instrument was tuned to give the best possible performance using a 20 pmol/µL solution of myoglobin in 10% (v/v) acetonitrile in water, infused into a solvent flow of 0.1 mL/min 80:20 0.1% (v/v) formic acid in water: 0.1% (v/v) formic acid in acetonitrile, using the integrated syringe pump.

Chromatographic separation was performed on a Waters Acquity BEH C4 1.7 µm, 2.1 x 100 mm column, at 40°C. The autosampler was set at 4°C. The injection volume was 5 µL (partial loop with needle overfill). A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (Solvent B) at a flow rate of 0.2 mL/min. The solvent flow from the UPLC system was split 1:1 (MS:waste) post-column using a PEEK T-piece. The gradient is described in Table 2.3.

#### Table 2.3 Description of the gradient employed for the chromatographic separation of intact proteins

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
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<td>22.1</td>
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<td>25</td>
<td>95</td>
<td>5</td>
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</table>
ESI positive ion MS data was collected without automatic mass measurement over the mass range m/z 400 – 2500, using the following conditions: capillary voltage, 2.5 kV; sampling cone, 25 arbitrary units; extraction cone, 4 arbitrary units; ion guide, 2.5 arbitrary units; source temperature, 150 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 300 L/hour; low mass (LM) and high mass (HM) resolution, both 15.0 arbitrary units, respectively. Data was collected using an MS experiment composed of one scan function with scan time 1 s, inter-scan time 0.1 s and collision energy offset of 4 arbitrary units.

2.14 In-solution trypsin digestion of the protein pellet retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

All reagents used for the incubations were as described in Section 2.5.

All incubations were carried out as detailed in Section 2.2. After centrifugation and removal of the supernatant, 500 µL of 100 mM phosphate buffer, pH 7.4 (unless otherwise stated) was added to each protein pellet and vortex mixed to disturb the pellet. The sample was then centrifuged for 10 minutes at 10,000 x g and the supernatant removed. This was repeated a further two times to ensure the complete removal of non-covalently bound drug-related material.

After washing, the pellet was re-suspended in 100 µL of 0.1% RapiGest SF™ solution and vortex mixed. 5 µL of 100 mM DTT in50 mM ammonium bicarbonate was added and each sample incubated on a heating block for 30 minutes at 60°C. The sample was allowed to cool and 10 µL of the alkylation reagent 100 mM IAA in 50 mM ammonium bicarbonate added, vortex mixed and incubated in the dark, at room temperature, for 20 minutes. Digestion with trypsin was performed by the addition of 5 µL of a 0.2 µg/µL solution of trypsin in 50 mM ammonium bicarbonate and incubated overnight on a heating block at 37°C. 1 µL of concentrated TFA was added to each sample to terminate the digestion, and the samples incubated at 37°C for 45 minutes precipitate the RapiGest SF™ reagent. The samples were centrifuged for 10 minutes at 10,000 x g and the supernatant transferred to HPLC vials for LC/MS analysis.

2.15 LC/MS analysis of the trypsin digestion of protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

All reagents used for the incubations were as described in Experimental Section 2.5. All incubations were carried out as detailed in Experimental Sections 2.2. Trypsin digestion was
carried out as described in Experimental Section 2.14. LC-MS analysis was performed on a QTof Premier mass spectrometer coupled to an Acquity UPLC system (both Waters, Manchester, UK), as described in Section 2.12.

External mass calibration of the mass spectrometer was performed over an m/z range of 50 – 2000 using a solution of NaCsl. The instrument was tuned to give the best possible performance for peptide analysis using a 20 pmol/µL solution of [Glu₁]-Fibrinopeptide B (purchased from Sigma-Aldrich) in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.10 mL/min 1:1 0.1% (v/v) formic acid in water: 0.1% (v/v) formic acid in acetonitrile, using the integrated syringe pump.

Chromatographic separation was performed on a Waters Acquity BEH C18 1.7 µm, 2.1 x 100 mm column, at 60°C. The autosampler was set at 4°C. The injection volume was 10 µL (partial loop with needle overfill). A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (Solvent B) at a flow rate of 0.2 mL/min (post-column split 1:1 MS: waste). The gradient is described in Table 2.4.

Table 2.4 Description of the gradient employed for the chromatographic separation of peptides produced by the trypsin digestion of the protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

<table>
<thead>
<tr>
<th>Time (minute)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
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<tr>
<td>54</td>
<td>5</td>
<td>95</td>
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<tr>
<td>56</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>56.1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>60</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

ESI positive ion MS data was collected without automatic mass measurement, in an MS² experiment composed of two functions. Function 1 collected data over the mass range m/z 300 – 1990, using a scan speed of 0.5 s, inter-scan speed of 0.05 s and collision energy offset of 4 arbitrary units (low energy). Function 2 collected data over the mass range m/z 100 - 1990 using a scan speed of 0.5 s, inter scan speed of 0.05 s and utilising a collision energy offset ramp of 20 – 40 arbitrary units (high energy).
The MS settings were as follows: capillary voltage, 2.5 kV; sampling cone, 30 arbitrary units; extraction cone, 4.5 arbitrary units; ion guide, 2.5 arbitrary units; source temperature, 140 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 400 L/hour; LM and HM resolution, 4.7 and 15.0 arbitrary units respectively.

Where appropriate, additional MS/MS experiments were conducted on ions of interest which were selected after interrogation of the MS² data described above. These MS/MS experiments were conducted using the MS conditions above with the exception that the LM resolution was changed to 15 arbitrary units (on the Waters QTof Premier, this equates to an approximate mass ion selection window of 0.2 Da). The m/z was selected to 1 decimal place in each method, with the fragmentation energy employed determined by a collision energy offset ramp of 20 – 40 arbitrary units. Data was collected with a scan time of 0.5 s with an inter-scan delay of 0.05 s.

2.16 Qualitative assessment of drug-protein covalent binding by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

All reagents used for the incubations were as described in Section 2.2.

The SDS-PAGE running buffer was prepared by diluting 50 mL of the stock buffer with 950 mL of ultra pure water to give a final composition of 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 50 mM Tris-base, 0.1% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.7. The SDS-PAGE LDS sample buffer was adding 1 mL to 0.4 mL of 10X reducing agent (0.5 M DTT) and 2.6 mL of ultra pure water to give a final concentration of 10% glycerol, 141 mM Tris-base, 106 mM Tris-HCl, 2% LDS, 50 mM DTT, 0.51 mM EDTA, 0.22 mM G250 Blue, 0.175 mM phenol red, pH 8.5. 300 mL of gel fixing solution was prepared by adding 150 mL of methanol and 30 mL of glacial acetic acid to 120 mL of ultra pure water. The gel staining solution was prepared by mixing 60 mL of stain A, 15 mL of stain B and 60 mL of 100% methanol to 165 mL of deionised water. The composition of both stains A and B are Invitrogen proprietary information. A 10% (w/v) glycerol solution was prepared by weighing 100 g of glycerol and dissolving in 1 L of ultra pure water.

All incubations were carried out as detailed in Section 2.2. Each protein pellet was re-suspended in 100 µL of the diluted LDS sample buffer and vortex mixed thoroughly to ensure dissolution. Each sample was then heated at 70°C on a heating block for 10 minutes and allowed to cool.
An X-Cell Surelock Midi-Cell SDS-PAGE system (Invitrogen, Paisley, UK) was used to carry the gel electrophoresis. The NuPAGE gels were first washed with ultra pure water several times to remove the storage buffer and placed in the Surelock apparatus. The upper and lower chambers were then filled with approximately 600 mL of SDS-PAGE running buffer, with 500 µL of antioxidant mix (the composition of the antioxidant mix is proprietary information) added to the upper chamber buffer.

20 µL of each sample or 10 µL of marker standard was carefully loaded into the individual wells using a 100 µL square-tipped syringe (Hamilton, Bonaduz, Switzerland), the power pack connected and each gel run for 60 min at 200 V. Each gel was then incubated for 10 minutes in 200 mL of the gel fixing solution on an orbital shaker. The gels were then transferred to the gel staining solution (minus stain B) and allowed to incubate for 15 minutes on an orbital shaker. 15 mL of stain B was added and the gel incubated for 3 hours. After incubation with the gel staining solution, the gels were rinsed with ultra pure water and left to incubate in water, on an orbital shaker, overnight. The gels were then transferred to 200 mL of 10% (w/v) glycerol solution for 10 minutes, and dried on a gel dryer (Thermo Scientific, Waltham, MA, USA) for 45 minutes at 60°C, then 45 minutes at 30°C.

2.17 In-gel trypsin digestion of protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

All reagents used for the incubations were as described in Section 2.2. 0.1 M ammonium bicarbonate was prepared by dissolving 3.95g in 500 mL of ultra pure water. A 50 mM ammonium bicarbonate, 5 mM calcium chloride solution was prepared by diluting a portion of this 0.1 M solution 1:1 a 10 mM solution of calcium chloride (1.1 mg/mL in ultra pure water). 0.1% (v/v) formic acid in water was prepared by adding 1 mL of formic acid to 999 mL of ultra pure water.

All incubations were carried out as detailed in Section 2.2. The SDS-PAGE procedure was carried out as detailed in Section 2.16, with the exception that the procedure was halted prior to incubation in 10% (w/v) glycerol solution.

The in-gel digestion of protein bands with trypsin was carried out according to a modification of the method described by Schevchenko et al. (191). Protein bands of interest were excised from the gel using a clean scalpel. Each band was chopped into cubes of approximately 1 mm³ and the pieces transferred to an eppendorf tube. The gel pieces were washed with ultra pure water (150 µL) for 5 minutes on an orbital shaker and the liquid removed with a pipette. 50 µL
of acetonitrile was added; the pieces allowed to shrink for 15 minutes and dried under N₂ for 15 minutes. The gel pieces were then swollen on a heating block in 50 µL 1.5 mg/mL DTT in 0.1 M ammonium bicarbonate for 50 minutes at 56°C to reduce the protein. The residual liquid was removed and acetonitrile added (50 µL) to shrink the gel and the pieces dried under N₂. After drying, the gel pieces were swollen in 50 µL 10 mg/mL iodoacetamide in 0.1 M ammonium bicarbonate for 20 minutes at room temperature in the dark to derivatise the cysteine residues in the protein. The residual liquid was removed and the gel pieces washed with 200 µL 0.1M ammonium bicarbonate for 15 minutes on an orbital shaker. The residual liquid was removed and acetonitrile added (50 µL) for 10 minutes to shrink the gel pieces. Finally the sample was dried under N₂ for 30 minutes.

To initiate digestion of the protein, the dry gel pieces were swollen in 20 µL of a 20 µg/mL solution of trypsin in 50 mM ammonium bicarbonate, 5 mM calcium chloride for 45 minutes on ice. Unabsorbed liquid was removed, 50 µL of 50 mM ammonium bicarbonate, 5 mM calcium chloride added and the sample digested on a heating block overnight at 37°C.

After digestion, 50 µL of acetonitrile was added for 10 minutes to shrink the gel pieces. The liquid was removed using a pipette and transferred to a suitable HPLC vial. The liquid was reduced to dryness under N₂ and reconstituted in 0.1% (v/v) formic acid in water for LC/MS analysis.

2.18 LC analysis and off-line radiodetection of the trypsin digestion of protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 CYP2E1 Supersomes™ with [¹⁴C] acetaminophen

All reagents used for the incubations were as described in Section 2.2. All incubations were carried out as detailed in Section 2.2. Trypsin digestion was carried out as described in Experimental Section 2.14. LC analysis was performed on an Acquity UPLC system as described in Section 2.15, with the exception that the solvent effluent from the HPLC system was collected into fractions using an HTX-PAL fraction collector (CTC-Analytics AG) into four 96-well Luma Plate™-96 plates, containing yttrium silicate solid scintillant (PerkinElmer LAS UK Ltd.), at a speed of 9.4 s per well. Once collected, the plates were allowed to dry in air and then sealed with Top Seal A (PerkinElmer LAS UK Ltd.) clear adhesive sealing tape, prior to off-line radiodetection. Off-line radiodetection was performed by scintillation counting using a Packard TopCount NXT counter (PerkinElmer LAS UK Ltd.) using a 20 s count time per well. The scintillation counting data was imported into Laura v3 Software (LabLogic Systems Ltd.) using
the LSC import function and processed to generate a reconstructed radiochromatogram for each analysis.

2.19 Accelerator mass spectrometry (AMS) analysis of the trypsin digestion of protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with [14C] labelled test compounds

Sodium benzoate used as a carbon carrier; copper (II) wire (ACS) used in the combustion phase; zinc (100 Mesh, 99.8% purity) and titanium (II) hydride (325 Mesh, 98% purity) used in the reduction phase of graphitisation and cobalt (100 Mesh, 99.9% purity) used as a catalyst for the formation and deposition of graphite, were all purchased from Sigma-Aldrich. ANU sugar (certificated value = 1.5061 Times Modern) was purchased from Quaternary Dating Research Centre, Australian National University (Canberra, Australia) for use as in instrument normalisation and as a process control standard. Fused quartz test tubes, fused quartz tapered drawn point tubes used in the combustion process, and borosilicate glass test tubes and borosilicate culture tubes used in the reduction and graphitisation process, were all purchased from York Glassware Services Ltd. (York, UK). Aluminium cathodes were purchased from National Electrostatic Corp. (Middleton, WI, USA). A synthetic graphite AMS control (200 Mesh, 99.9999% purity) was purchased from Alfa Aesar (Johnson Matthey PLC, Royston, Herts. UK) for instrument background determination.

An Autodose Powdernium OTM system (Synx Technologies SA, Geneva, Switzerland) was used for the automated dispensing of reagents into glassware.

All reagents used for the incubations were as described in Section 2.2. All incubations were carried out as detailed in Section 2.2. Trypsin digestion was carried out as described in Experimental Section 2.14. Due to the highly specialised nature of sample preparation and analysis, the sample was prepared and analysed by AMS experts (Adrian Pereira and Claire MacIntyre for sample preparation, and Steven Corless for sample analysis.)

The experimental procedure for sample preparation and analysis for AMS is fully described by Young et al. (192). Briefly, the borosilicate glass tubes and copper wire were pre-baked for 2-4 hours at 500°C in a Carbolite RWF11/23 furnace (Keson Products, Chelmsford, UK) prior to analysis to remove organic contaminants. The radioactivity present in 10 µL aliquots of each sample was measured by LSC (Beckman Coulter UK) after mixing with SafeScint (LabLogic Ltd.) liquid scintillant. Aliquots of the trypsin digests were diluted to 0.1% (v/v) formic acid in water to give a total radioactivity measurement of 500 dpm/mL (a 180-fold dilution of the [14C] SB-
649868 incubation, 18-fold dilution of the $[^{14}\text{C}]$ acetaminophen incubation). 5 µL aliquots of each sample were combined with 40 µL of sodium benzoate of carbon carrier (a 90 mg/mL solution in water). Each sample was chromatographically separated using the method described in Experimental 2.15, and fractions collected at 25 s intervals between 5 and 45 minutes of the chromatographic run-time into quartz tubes using HTX-PAL fraction collector (CTC-Analytics AG).

Each fraction was dried using a SpeedVac SPD2010 (Thermo Electron Corp., Basingstoke, UK), combined with copper oxide (approximately 50 mg), sealed into evacuated tapered quartz tubes under vacuum (Turbovac PT50, Leybold Vacuum Ltd., London, UK) using an oxy-propane torch (Junior Jet 7, Glass Precision Engineering Ltd., Leighton Buzzard, UK) and heated at 900°C for 2 hours in a Carbolite RWF11/23 furnace (Keison Products). The CO$_2$ formed was cryogenically transferred into evacuated tubes containing zinc powder, titanium (II) hydride and cobalt catalyst, and the tube sealed. This reduction tube was heated to 500°C for 4 hours, followed by 550°C for 6 hours in a Carbolite RWF11/23 furnace (Keison Products) to complete the graphitisation process. Carbon as graphite, deposited on the cobalt, was pressed into aluminium cathodes using a pallet press (Parr Instrument Co., Moline, USA) and analysed by AMS.

Analysis by AMS was conducted on a 250 kV single-stage accelerator mass spectrometer (National Electrostatics Corp., Middleton, USA) at GlaxoSmithKline, Ware. An ion beam containing $^{12}\text{C}$, $^{13}\text{C}$, and $^{14}\text{C}$ ions, as well as other ions, was generated by accelerating a Cs$^+$ ion beam onto the graphite surface. Ions were accelerated into the magnet with the magnet set to inject $^{12}\text{C}$ (500 µs), $^{13}\text{C}$ (2100 µs) and $^{14}\text{C}$ (0.1 s) sequentially at 40 keV. The negative ions were accelerated through the instrument into the stripper where argon gas stripped electrons from the ions resulting in positively charged carbon ions ($^{12,13,14}\text{C}^+$ to C$^{+4}$). $^{12}\text{C}^+$ and $^{13}\text{C}^+$ were measured using Faraday cups, whereas data was collected on $^{14}\text{C}^+$ using a solid-state silicon surface barrier. The burn-in and sample analysis times for each graphite were 600 cycles (60.4 s) and 100 cycles (100.7 s), respectively, and three measurements were collected per graphite.

Control samples, including Australian National University (ANU) sugar (ca 6.5 mgs, n=6) and dried aliquots (20 µL, n=6) of the 90 mg/mL solution of sodium benzoate were processed with the run. Instrument standards of pooled ANU graphite and synthetic graphite were used to normalise the data and check suitability of instrumental background, respectively. The level of carbon present in the sodium benzoate solution was calculated on the basis of the molecular weight of the solid material which was assumed to be 100% pure (i.e. 58% carbon). The data
from the AMS and the carbon content of the sodium benzoate solution were combined to provide radiocarbon levels for each sample.

2.20 In-solution digestion with Pronase (Protease from Streptomyces griseus, Type XIV) of protein pellets retained following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

All reagents used for the incubations were as described in Experimental Section 2.2. All incubations were carried out as detailed in Section 2.2. After centrifugation and removal of the supernatant, the pellets were washed to remove any non-covalently bound drug-related material.

After washing, the pellets were re-suspended in 100 μL of 100 mM phosphate buffer pH 7.4 and vortex mixed to ensure complete dissolution. 10 μL of a 20 mg/mL solution of pronase (Protease from Streptomyces griseus, Type XIV) in water was added to each sample and the mixtures left to digest for 24 hours on a heating block at 50°C. Removal of pronase after digestion was completed by quenching each mixture with 500 μL of ice-cold acetonitrile followed by centrifugation at 10,000 x g for 20 minutes. The supernatants were transferred to glass vials, reduced to dryness under N₂ and reconstituted in 100 μL in 10% (v/v) acetonitrile in water.

2.21 Derivatisation of pronase digests for LC/MS analysis using 6-aminoquinolyl N-hydroxysuccinimidy carbamate

Digestion of protein using pronase is detailed in Experimental Section 2.20. Amino acids were derivatised using 6-aminoquinolyl N-hydroxysuccinimidy carbamate, purchased from Waters Ltd. (as AccQ.Tag™ Ultra reagent, 10 mM in acetonitrile). 10 μL of the amino acid mixture was reacted with 20 μL in borate buffer (AccQ.Tag™ Ultra borate buffer from Waters Ltd., total reaction volume 100 μL), vortex mixed, and left to stand for 1 minute. The reaction mixture was then heated at 55°C for 10 minutes and allowed to cool prior to analysis by LC/MS.

2.22 Precursor ion scanning mode LC/MS analysis of amino acids following in vitro incubation of human liver microsomes and cytochrome P450 Supersomes™ with test compounds

LC/MS analysis was carried out on derivatised amino acids performed on an AB Sciex Triple Quad 5500 (AS Sciex, Framingham, MA, USA) mass spectrometer, controlled by Analyst v1.5 software (AS Sciex) coupled to a Waters Acquity UPLC system (controlled by MassLynx v4.1
Chromatographic separation was performed on a Waters Acquity AccQ.Tag Ultra C18 1.7 µm, 2.1 x 100 mm column, at 60°C. The autosampler was set at 4°C. The injection volume was 10 µL (partial loop with needle overfill). A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (Solvent B) at a flow rate of 0.4 mL/min. The gradient is described in Table 2.5.

Table 2.5 Description of the gradient employed for the chromatographic separation of derivatised amino acids

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
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<td>0.5</td>
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<td>15.6</td>
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<td>5</td>
</tr>
<tr>
<td>16.5</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

ESI positive ion data was collected in precursor ion scanning mode, based on the assumption of reactive metabolites of acetaminophen and SB-649868 binding to cysteine residues, as suggested by the glutathione trapping data. Precursors of the fragment ion m/z 171.1 were monitored.

The MS settings were as follows: ionspray voltage, 4700 V; temperature, 650°C; ion source gas pressures, 30 and 45 L/hour; collision gas pressure, 8 L/hour; curtain gas, 35 L/hour; declustering potential, 42 V; exit potential, 6 V; collision energy offset, 40 arbitrary units, collision exit potential, 13 V. Data was collected at unit resolution in profile mode, at a scan rate of 1000 Da/s

2.23 MS6 LC/MS analysis of derivatised amino acids

All reagents and procedures used for the incubation, digestion and derivatisation were as detailed in Experimental Sections 2.2, 2.20 and 2.21. LC/MS analysis was performed on a QTof Premier mass spectrometer coupled to an Acquity UPLC system (both Waters, Manchester, UK), as described in Section 2.12. External mass calibration of the mass spectrometer was
conducted as described in Section 2.12. An external lock mass (1 µg/mL leucine enkephaline in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using an dedicated HPLC pump (Shimadzu, Manchester, UK) during each analyses, for automatic mass correction. Chromatographic separation was performed on an Acquity UPLC system using the method described in Experimental Section 2.22.

ESI positive ion MS data was collected in an MS² experiment composed of two functions. MS Function 1 collected data over the range m/z 100 – 1000, using a scan speed of 0.5 s, inter-scan speed of 0.05 s and collision energy offset of 4 arbitrary units (low energy). Function 2 collected data over the range m/z 100 - 1000 using a scan speed of 0.5 s, inter-scan speed of 0.05 s and utilising a collision energy offset ramp of 20 – 30 arbitrary units (high energy). A third function was used to collect data on the external lock mass for automatic mass correction using leucine enkephaline. Data was collected for 0.5 s at an interval of 10 s, and averaged over 10 scans.

The MS settings were as follows: capillary voltage, 2.5 kV; sampling cone, 30 arbitrary units; extraction cone, 4.5 arbitrary units; ion guide, 2.5 arbitrary units; source temperature, 140 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 400 L/hour; LM and HM resolution, 4.7 and 15.0 arbitrary units respectively.

2.24 Multiple reaction monitoring (MRM) mode LC/MS analysis of derivatised amino acids

LC/MS analysis was carried out on derivatised amino acids (as described in Experimental Section 2.20 and 2.21) and performed on an AB Sciex Triple Quad 5500 mass spectrometer coupled to a Waters Acquity UPLC system as described in Experimental Section 2.22. Chromatographic separation was performed on an Acquity UPLC system using the method described in Experimental Section 2.22.

ESI positive ion data was collected in multiple reaction monitoring (MRM) mode, based on the assumption of reactive metabolites of acetaminophen and SB-649868 binding to cysteine residues, as suggested by the glutathione trapping data.

For acetaminophen, the transition m/z 441.2 → m/z 171.1 was monitored over a dwell time of 200 ms. For SB-649868, transitions m/z 785.2 → m/z 171.1 and m/z 767.1 → m/z 171 (-H₂O), each over a dwell time of 200 ms.
2.25 MRM mode LC/MS analysis of peptides generated by digestion with trypsin of the protein pellet retained following non-enzymatic incubation of chemically generated NAPQI with CYP2E1 Supersomes™ and, and following in vitro incubation of human liver microsomes and cytochrome P450 CYP2E1 Supersomes™ with acetaminophen

All reagents and procedures used for the incubations were as detailed in Section 2.2. Trypsin digestion was carried out as described in Experimental Section 2.14. LC/MS analysis was performed on an AB Sciex Triple Quad 5500 mass spectrometer coupled to an Acquity UPLC system, using the chromatographic separation method described in Experimental Section 2.22.

ESI positive ion data was collected in MRM mode. MRM transitions were selected based on in silico digestion of the cytochrome P450 CYP2E1 protein (based on its sequence as describe in the UniProt database (human cytochrome P450 CYP2E1, P05181 (193)), and using BioLynx software (Waters Ltd., Manchester, UK) with trypsin selected as the protease and two missed cleavage sites allowed.

Cysteine containing peptides were selected and mass modification calculated based on the addition of acetaminophen (+149.1 Da) and carbamidomethylation (+57 Da), based on the information generated from glutathione trapping experiments. Details of the transitions monitored for the incubations with acetaminophen are detailed in Table 2.6.

The MS settings were optimised for peptide analysis using a 20 pmol/µL solution of [Glu]$^3$-Fibrinopeptide B in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.10 mL/min 1:1 0.1% (v/v) formic acid in water: 0.1% (v/v) formic acid in acetonitrile, using a standalone syringe pump (Shimadzu, Manchester, UK). The MS settings were as follows: ionspray voltage, 4700 V; temperature, 650°C; gas pressures, 30 and 40 arbitrary units; collision gas pressure, 8 arbitrary units; declustering potential, 42 V; exit potential, 6 V; collision offset, 47 arbitrary units, collision exit potential, 13 V.
Table 2.6  Expected m/z and MRM transitions monitored during the MRM mode LC/MS analysis of peptides generated by digestion with trypsin of the protein pellet retained following non-enzymatic incubation of chemically generated NAPQI with CYP2E1 Supersomes™, and following in vitro incubation of human liver microsomes and cytochrome P450 CYP2E1 Supersomes™ with acetaminophen

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence of peptides of CYP2E1 (derived from in silico digestion)</th>
<th>Mw</th>
<th>Mw + acetonaminophen</th>
<th>Mw + iodoacetamide</th>
<th>m/z expected or Q1 transition (m/z)</th>
<th>Q3 transition (m/z)</th>
<th>Dwell time (ms)</th>
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<td>T56</td>
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<td>283.82</td>
<td>213.12</td>
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<td>T56-57</td>
<td>LCVIPRS</td>
<td>786.44</td>
<td>935.49</td>
<td>468.75</td>
<td>312.83</td>
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<td>T52</td>
<td>VCAGEGLAR</td>
<td>874.43</td>
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<td>342.16</td>
<td>256.87</td>
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<td>T55-56</td>
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<td>380.87</td>
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<td>T51-52</td>
<td>RVCAGEGLAR</td>
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<td>T55-57</td>
<td>YKLCVIPRS</td>
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<td>1407.66</td>
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<td>779.36</td>
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<td>799.04</td>
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<td>5648.57</td>
<td>-</td>
<td>1883.86</td>
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2.26 LC/MS analysis of peptides generated from trypsin digestion of the protein pellet retained following in vitro incubation of test compounds with human liver microsomes and cytochrome P450 Supersomes™, using an Acquity UPLC system coupled to a Waters Synapt G2S mass spectrometer

All reagents and procedures used for the incubations were as detailed in Section 2.2. Trypsin digestion was carried out as described in Experimental Section 2.14. LC/MS analysis was performed on a Synapt G2S mass spectrometer coupled to an Acquity UPLC system (both Waters Ltd.). MassLynx version v4.1 software from Waters Ltd. was used to control both the MS and Acquity systems. External mass calibration of the mass spectrometer was performed over an m/z range of 50 – 2000 using a solution of NaCl infused into the MS source using the integrated pump. An external lock mass (1 µg/mL leucine enkephaline in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using the integrated pump during each analysis, for automatic mass correction. The MS settings were optimised for peptide analysis using a 2 pmol/µL solution of [Glu¹]-Fibrinopeptide B in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.10 mL/min 1:1 0.1% (v/v) formic acid in water: 0.1% (v/v) formic acid in acetonitrile, using the integrated pump. Chromatographic separation was performed on an Acquity UPLC system as described in Experimental Section 2.15.

ESI positive ion MS data was collected in high resolution mode (approximately 35,000) with the ion mobility data collection and enhance dynamic range switched on. The MS² experiment was composed of two functions, with automatic mass correction included. MS Function 1 collected data over the range m/z 100 – 2000, using a scan speed of 0.5 s, inter-scan speed of 0.05 s and collision energy offset in the transfer cell of 2 arbitrary units (low energy). Function 2 collected data as over the range m/z 100 - 2000 using a scan speed of 0.5 s, inter-scan speed of 0.05 s and using collision energy offset in the transfer cell of 20 – 30 arbitrary units (high energy). A third function was used to collect data on the external lock mass for automatic mass measurement using leucine enkephaline. Data was collected for 0.2 s at an interval of 10 s, and averaged over 10 scans.

The MS settings were as follows: capillary voltage, 2.5 kV; sampling cone 25 arbitrary units; source offset, 80 arbitrary units; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow rate, 10 L/hour; desolvation gas flow rate, 600 L/hour; LM and HM resolution, 4.7 and 15 arbitrary units respectively; trap collision energy offset, 4 arbitrary units; helium cell
gas flow, 180 mL/min; IMS gas flow, 90 mL/min; IMS wave velocity, 2000 m/s; IMS wave height, 40 V.

2.27 Identification of the metabolites of [14C] SB-649868 after incubation with human liver microsomes and cytochrome P450 CYP3A4 Supersomes™ by LC/MS

All reagents and procedures used for the incubations were as detailed in Section 2.2. Following centrifugation upon completion of the incubation, the supernatant were retained and transferred to glass vials for LC/MS analysis.

LC/MS analysis was performed on a QTof Premier mass spectrometer coupled to an Acquity UPLC system as described in Experimental Section 2.12. External mass calibration of the mass spectrometer was performed over an m/z range of 50 – 1000 using a solution of NaCls. The instrument was tuned to give the best possible performance using a 10 µg/mL solution of a standard of SB-649868 in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.15 mL/min 1:1 0.1% (v/v) formic acid in water: acetonitrile, using the integrated syringe pump. An external lock mass (1 µg/mL leucine enkephaline in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using an dedicated HPLC pump (Shimadzu) during each analyses, for automatic mass correction.

Chromatographic separation was performed on a Waters Acquity BEH C18 1.7 µm, 2.1 x 100 mm column, at 40°C. The autosampler was set at 4°C. The injection volume was 10 µL (partial loop with needle overfill). A gradient system was employed, using 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (Solvent B) at a flow rate of 0.3 mL/min. The gradient is described in Table 2.7. The solvent flow from the UPLC system was split 1:1 (MS: fraction collector) post-column using a PEEK T-piece. Fractions were collected and off-line radiodetection was performed by TopCount scintillation counting conducted as described in Experimental Section 2.12.
Table 2.7  Description of the gradient employed for the chromatographic separation of the metabolites of $[^{14}C]$ SB-649868 formed after incubation with human liver microsomes and cytochrome P450 CYP3A4 Supersomes™

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
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<td>5</td>
<td>95</td>
</tr>
<tr>
<td>13.1</td>
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<td>5</td>
</tr>
<tr>
<td>15</td>
<td>95</td>
<td>5</td>
</tr>
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</table>

ESI positive ion MS data was collected over the mass range m/z 50 – 1000, using the following conditions: capillary voltage, 2 kV; sampling cone, 30 arbitrary units; extraction cone, 4.5 arbitrary units; ion guide, 2.0 arbitrary units; source temperature, 150 °C; desolvation temperature, 400 °C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 400 L/hour; LM and HM resolution, 4.7 and 15.0 arbitrary units, respectively, to give a resolution of approximately 8000 at m/z 400. Data was collected using an MS² experiment composed of two functions each of scan time 0.2 s, inter-scan time 0.02 s, where function 1 used a collision energy offset of 4 arbitrary units (low energy) and function 2 utilised a collision energy offset ramp of 20 – 40 arbitrary units (high energy). The third function was used to collect data on the external lock mass for automatic mass correction. Data was collected for 0.5 s at an interval of 10 s, and averaged over 10 scans. Additional MS/MS experiments were conducted on ions of interest which were selected after interrogation of the MS² data described above. These MS/MS experiments were conducted using the MS conditions above with the exception that the LM resolution was changed to 15 arbitrary units (on the Waters QToF Premier, this equates to an approximate mass ion selection window of 0.2 Da). The m/z was selected to 1 decimal place in each method, with the fragmentation energy employed determined by a collision energy offset ramp of 20 – 40 arbitrary units. Data was collected with a scan time of 0.2 s with an inter-scan delay of 0.02 s.
2.28 MALDI-MS analysis of peptides generated from in-gel trypsin digestion of the protein pellet following \textit{in vitro} incubation of human liver microsomes and cytochrome P450 CYP3A4 Supersomes™ with SB-649868

All reagents and procedures used for the incubations were as detailed in Section 2.2. The experimental procedure for the SDS-PAGE separation and in-gel digestion of proteins was as described in Experimental Sections 2.16 and 2.17, respectively, with the exception that after incubation overnight with trypsin, no acetonitrile was added to shrink the gel pieces, as required for LC/MS analysis.

1 µL of the residual liquid from each gel piece after digestion with trypsin was each mixed thoroughly in a small eppendorf tube with 1 µL of a 10 mg/mL solution of α-cyano-4-hydroxyccinnamic acid (194) (purchased from Bruker UK Ltd., Coventry, UK) in 50:50 acetonitrile: water with 0.2% (v/v) trifluoroacetic acid (purchased from Sigma-Aldrich). 1 µL of this was then spotted carefully in triplicate onto a 384 position stainless steel MALDI plate (Bruker UK Ltd.) for analysis using the Bruker UltraFleXtreme III MALDI Tof/Tof instrument (Bruker Daltonik GmbH, Bremen, Germany). The instrument was operated using Compass v1.3 software (Bruker Daltonik GmbH), with the laser power varied according to the spot analysed. The data was interrogated manually using FlexAnalysis v3.0 (Bruker Daltonik GmbH).

2.29 MRM mode LC/MS analysis of peptides generated by digestion with trypsin of the protein pellet retained following \textit{in vitro} incubation of human liver microsomes and cytochrome P450 CYP3A4 Supersomes™ with SB-649868

All reagents and procedures used for the incubations were as detailed in Section 2.2. Trypsin digestion was carried out as described in Experimental Section 2.14. LC/MS analysis was performed on an AB Sciex Triple Quad 5500 mass spectrometer coupled to an Acquity UPLC system as described in Experimental Section 2.22. Chromatographic separation was performed as described in Experimental Section 2.22.

ESI positive ion data was collected in MRM mode. MRM transitions were selected based on \textit{in silico} digestion of the cytochrome P450 CYP3A4 protein (based on its sequence as describe in the UniProt database (human cytochrome P450 CYP3A4, P08684 (195)), and using BioLynx software (Waters, Manchester, UK) with trypsin selected as the protease and two missed cleavage sites allowed. The MS settings used are described in Experimental Section 2.25.

Cysteine containing peptides were selected and mass modification calculated based on the addition of carbamidomethylation (+57 Da), SB-649868 (+493.1 Da) and loss of water from SB-
649868 (+475.1 Da), based on the information generated from glutathione trapping experiments. Details of the transitions monitored for the incubations with SB-649868 are detailed in Tables 2.8 and 2.9.
Table 2.8

Expected m/z and MRM transitions monitored during the MRM mode LC/MS analysis of peptides generated from digestion of the protein
pellet retained following in vitro incubation of human liver microsomes and cytochrome P450 CYP3A4 Supersomes™ with SB‐649868

Peptide

Sequence of peptides of CYP3A4
(derived from in silico digestion)

Mw

Mw +
SB‐649868

T39
T39‐40
T50
T38‐39
T38‐40
T9
T53
T4
T4‐5
T50‐51
T37‐39
T8‐9
T39‐41
T4‐6
T52‐53
T50‐52
T53‐54
T9‐10
T49‐50
T52‐54
T51‐53
T48‐50
T8‐10
T49‐51
T3‐4
T3‐5
T2‐4
T9‐11
T23
T53‐55
T7‐9
T22‐23
T21‐23
T23‐24
T23‐25
T22‐24

VCK
VCKK
NCIGMR
LERVCK
LERVCKK
ECYSVFTNR
VLQNFSFKPCK
GFCMFDMECHK
GFCMFDMECHKK
NCIGMRFALMNMK
LFPIAMRLERVCK
TVLVKECYSVFTNR
VCKKDVEINGMFIPK
GFCMFDMECHKKYGK
LALIRVLQNFSFKPCK
NCIGMRFALMNMKLALIR
VLQNFSFKPCKETQIPLK
ECYSVFTNRRPFGPVGFMK
DNIDPYIYTPFGSGPRNCIGMR
LALIRVLQNFSFKPCKETQIPLK
FALMNMKLALIRVLQNFSFKPCK
NKDNIDPYIYTPFGSGPRNCIGMR
TVLVKECYSVFTNRRPFGPVGFMK
DNIDPYIYTPFGSGPRNCIGMRFALMNMK
LGIPGPTPLPFLGNILSYHKGFCMFDMECHK
LGIPGPTPLPFLGNILSYHKGFCMFDMECHKK
KLGIPGPTPLPFLGNILSYHKGFCMFDMECHK
ECYSVFTNRRPFGPVGFMKSAISIAEDEEWK
FDFLDPFFLSITVFPFLIPILEVLNICVFPR
VLQNFSFKPCKETQIPLKLSLGGLLQPEKPVVLK
VWGFYDGQQPVLAITDPDMIKTVLVKECYSVFTNR
LLRFDFLDPFFLSITVFPFLIPILEVLNICVFPR
KLLRFDFLDPFFLSITVFPFLIPILEVLNICVFPR
FDFLDPFFLSITVFPFLIPILEVLNICVFPREVTNFLR
FDFLDPFFLSITVFPFLIPILEVLNICVFPREVTNFLRK
LLRFDFLDPFFLSITVFPFLIPILEVLNICVFPREVTNFLRK

348.18
476.28
692.3
746.4
874.5
1117.49
1309.69
1346.49
1474.59
1527.72
1574.88
1657.85
1719.91
1822.77
1876.08
2094.11
2119.15
2234.08
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2685.54
2711.48
2727.29
2774.44
3320.56
3461.68
3592.34
3592.34
3595.08
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4915.95

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969.43
1185.46
1239.55
1367.65
1610.64
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1839.64
1967.74
2020.87
2068.03
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2612.30
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3220.44
3267.59
3813.71
3954.83
4085.49
4085.49
4088.23
4166.61
4286.77
4527.82
4549.12
4677.29
5026.59
5154.77
5409.10

Mw +
iodoacetamide

1896.64
2024.74

2372.92

4011.83
4142.49
4142.49

98

m/z expected or Q1transition (m/z)
421.67
485.72
593.73
620.78
684.83
806.32
902.42
949.32
1013.37
1011.44
1035.02
1076.50
1107.53
1187.46
1185.62
1294.63
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2339.65
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2578.39
2705.55

281.44
324.14
396.15
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456.88
537.88
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633.21
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1804.03

211.33
243.36
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682.81
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817.90
954.43
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1036.62
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1353.28

169.27
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636.74
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654.52
763.74
791.97
818.10
818.10
818.65
834.32
858.35
906.56
910.82
936.46
1006.32
1031.95
1082.82

Q3 transition
(m/z)

Dwell time
(ms)

147
147
175
147
147
175
147
147
147
147
147
175
147
147
147
175
147
147
175
147
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175
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175
147
147

20
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<th>Mw + iodoacetamide</th>
<th>m/z expected or Q1 transition (m/z)</th>
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<td>T53</td>
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<td>NKNDIPYPHTPGSGPRNCIGMR</td>
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<td>1332.27</td>
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<td>LGIPGPPPPLIGNLHGYHKGFCMFDMFMECHKK</td>
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<td>1165.82</td>
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<td>2569.38</td>
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<td>2696.55</td>
<td>1798.03</td>
<td>1348.77</td>
<td>1079.218</td>
</tr>
</tbody>
</table>
2.30 Generation of electrochemical oxidation products of amodiaquine and MS analysis

A solution containing 0.1 mM amodiaquine in 80:20 (v/v) 20 mM ammonium acetate in water: acetonitrile was pumped with a flow rate of 50 µL/min through the Coulochem III guard cell (ESA Biosciences Inc., Chelmsford, MA, USA) using the LTQ Orbitrap (Thermo Scientific, Waltham, MA, USA) integrated syringe pump. The potential on the Coulochem III guard cell was controlled by a Coulochem III potentiostat (ESA Biosciences). The potential on the cell was increased stepwise (50 mV steps) to 700mV and to 1250 mV and the resultant oxidised metabolites detected by direct infusion of the effluent from the electrochemical cell into the LTQ Orbitrap (Thermo Scientific) electrospray source.

During direct infusion, the MS was controlled and data collected using Tune Plus v2.4 software (Thermo Scientific). Instrument tuning was conducted using a 10 µL/mL solution of a standard of amodiaquine in 50% (v/v) acetonitrile in water to ensure the best possible performance of the MS. The LTQ Orbitrap (Thermo Scientific) was set up as follows: ISpray voltage, 5 kV; spray current, 2.5 µA; sheath gas flow rate, 8 arbitrary units; aux and sweep gas flow rate, 0 arbitrary units; capillary voltage 35 V; capillary temperature, 275°C; and tube lens, 110V. MS and MS/MS experiments were performed on the electrochemical products to generate structural information. Experiments were conducted in both the Ion trap and Orbitrap (for high resolution data) using the normal mass range and scan rate; 2 microscans were selected, with a maximum inject time of 200 ms. Normalised collision energy values ranging from 20-45 arbitrary units, with an activation time of 30 ms were used. Individual ions of interest were isolated prior to fragmentation with an isolation window of 1 Th.

2.31 Glutathione conjugation of the electrochemical oxidation products of amodiaquine and subsequent LC/MS analysis

A solution containing 0.5 mM amodiaquine and 0.1 mM reduced glutathione in 80:20 (v/v) 20 mM ammonium acetate in water: acetonitrile was pumped with a flow rate of 50 µL/min through the Coulochem III guard cell (ESA) using the LTQ Orbitrap (Thermo Scientific) integrated syringe pump. The potential on the Coulochem III guard cell was controlled by a Coulochem III potentiostat (ESA Biosciences). The potential on the cell was increased to 1000mV and 1250 mV and the resultant oxidised metabolites and glutathione conjugates detected using the LTQ Orbitrap (Thermo Scientific). The MS settings used are described in Experimental Section 2.30.
5 mL of the effluent from the electrochemical cell operating at 1000 mV was collected in a vial and submitted for \(^1\)H NMR analysis.

### 2.32 Preparative HPLC and \(^1\)H NMR analysis of the glutathione conjugated electrochemical oxidation products of amodiaquine

The following protocol was conducted by Rita Tailor (preparative HPLC) and Steve Thomas (\(^1\)H NMR analysis). The 5 mL sample containing the glutathione conjugate of electrochemically generated amodiaquine quinone imine (AQQI) was reduced to dryness under \(N_2\) and reconstituted in 5 mL of water.

A chromatographic separation was performed on an Agilent 1100 Preparative HPLC system coupled to a Waters ZQ single quadrupole mass spectrometer as described in Experimental Section 2.7. Chromatographic separation was performed on a Waters X-Bridge prep phenyl 5µm, 10 x 250 mm column, maintained at 30°C using a Jasco column oven. All 5 mL of the sample was injected onto the column. A gradient system was employed, using 20 mM ammonium acetate in water (unadjusted pH) (solvent A) and acetonitrile (Solvent B) at a flow rate of 4 mL/min. The gradient is described in Table 2.10.

#### Table 2.10 Description of the gradient employed for the preparative chromatographic separation of the glutathione trapped electrochemical products of amodiaquine

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>95</td>
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<tr>
<td>45</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

Online MS detection was used to detect fractions containing drug-related material. These fractions were reduced to dryness under \(N_2\) and reconstituted in an appropriate NMR solvent (50:50 v/v acetonitrile: deuterium oxide) for \(^1\)H NMR analysis.

\(^1\)H NMR experiments were conducted as described in Experimental Section 2.7. A standard of amodiaquine (1 mg/mL, in 50:50 (v/v) acetonitrile: deuterium oxide) was also analysed by \(^1\)H NMR using the method described, for comparative purposes.
2.33 Incubation of electrochemically generated AQQI with bovine serum albumin (BSA) and trypsin digestion of the drug-protein adduct formed

A solution containing 0.5 mM concentration of amodiaquine in 80:20 (v/v) 20 mM aqueous ammonium acetate in water: acetonitrile was pumped with a flow rate of 50 µL/min through the Coulochem III guard cell (ESA) using the LTQ Orbitrap (Thermo Scientific) integrated syringe pump. The potential on the cell was increased to 800mV and maintained. 1 mL of effluent from the electrochemical cell was collected in to a vial containing 1 mL of a 0.1 mM solution of bovine serum albumin (BSA) in 8M urea (so that the drug related material was in 5-fold molar excess). The sample was incubated at 37°C in a heated water bath for 30 minutes. After the incubation time, a portion of the sample was digested with trypsin, while the remainder was analysed by LC/MS.

100 µL each of samples containing the synthesised drug-protein adduct and un-reacted BSA were reduced to dryness under N₂ and reconstituted in 100 µL of 0.1M RapiGest™ solution in aqueous 50 mM ammonium bicarbonate. 6 µL of 100 mM DTT in 50 mM ammonium bicarbonate was added and the samples incubated at 95°C on a heating block for 5 minutes. The samples were then allowed to cool and 12 µL of the alkylation reagent (100 mM IAA in 50 mM ammonium bicarbonate) added and vortex mixed. The samples were incubated in the dark, at room temperature, for 20 min. Digestion with trypsin was performed by the addition of 3 µL of a 0.2 µg/µL solution of trypsin in 50 mM ammonium bicarbonate to each sample, with incubation overnight at 37°C on a heating block. 1 µL of concentrated TFA was added to each sample to terminate the digestion, and the samples incubated at 37°C for 45 minutes to precipitate the RapiGest SF™ reagent. The samples were centrifuged for 10 minutes at 10,000 x g and the supernatant transferred to glass vials for LC/MS analysis.

2.34 LC/MS analysis of the intact amodiaquine-BSA adduct

A chromatographic separation was performed on the amodiaquine-BSA drug-protein adduct sample (produced in Experimental Section 2.33) to remove excess reactive metabolite and urea prior to MS analysis. The LC/MS system consisted of a Waters Acquity UPLC system and Waters QTof Premier as described in section 2.13.

The chromatographic separation was performed on a Waters Acquity BEH300 C4 1.7 µm, 2.1 x 100 mm column at 60°C. The injection volume was 5 µL. A gradient system was employed with 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 0.2 mL/min. The gradient is described in Table 2.11.
Table 2.11 Description of the gradient employed for separation of the intact BSA-amodiaquine protein adduct

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>95</td>
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<td>31</td>
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<td>31.01</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

ESI positive ion MS data was collected over the mass range m/z 350 – 2000 for a scan time of 0.5 seconds with an inter-scan time of 0.05 seconds, using the following MS conditions: capillary voltage, 3 kV; sampling cone, 30 arbitrary units; extraction cone, 4.0 arbitrary units; ion guide, 2.5 arbitrary units, source temperature, 150°C; desolvation temperature, 400°C; cone gas flow rate, 50 L/hour; desolvation gas flow rate, 400 L/hour; LM and HM resolution 15.0 arbitrary units; ion energy, 1.0 arbitrary units; collision energy offset, 4.0 arbitrary units. The mass spectra were deconvoluted using MaxEnt 1 software from Waters (Manchester, UK). The deconvolution parameters were: mass range, 60 – 70 kDa, Uniform Gaussian width at half height, 0.5 Da, minimum intensity ratios: left, 33% and right, 33%.

2.35 LC/MS analysis of the trypsin digest of the amodiaquine-BSA adduct

A chromatographic separation was performed on the digest sample to isolate the peptides generated following trypsin digestion of the amodiaquine-BSA adduct (described in Experimental Section 2.33) using the LC/MS system as described in Experimental Section 2.15. The chromatographic separation was performed on a Waters Acquity BEH C18 1.7 μm, 2.1 x 100 mm column at 60°C. The injection volume was 5 μL. A gradient was employed with 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 0.2 mL/min. The gradient used is described in Table 2.12.
Table 2.12  Description of the gradient employed for separation of the peptides generated from a trypsin digestion following incubation of BSA with electrochemically generated amodiaquine quinone imine (AQQI)

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>45</td>
<td>50</td>
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<td>5</td>
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<tr>
<td>55</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

ESI positive ion MS data was collected in an MS$^6$ experiment composed of two functions. Function 1 collected data over the mass range m/z 100 – 1990, using a scan speed of 1 s, inter-scan speed of 0.01 s and collision energy offset of 4 arbitrary units (low energy). Function 2 collected data over the range m/z 100 - 1990 using a scan speed of 1 s, inter-scan speed of 0.1 s and utilising a collision energy offset 35 arbitrary units (high energy). A third function was used to collect data on the external lock mass for automatic mass correction using leucine enkephaline. Data was collected for 0.5 s at an interval of 10 s, and averaged over 10 scans.

MS/MS experiments were conducted on the modified peptide at m/z 929.8 and the unmodified peptide at m/z 812.2, using a collision offset value of 35 arbitrary units, 0.5 scan time, with an inter-scan time of 0.05 seconds, over a mass range of m/z 50 – 2500.

2.36 Incubation of electrochemically generated AQQI with cytochrome P450 CYP2C8 Supersomes™ with subsequent digestion with trypsin and LC/MS analysis using an Acquity UPLC system coupled to a Waters Synapt G2S mass spectrometer

A solution containing 0.5 mM concentration of amodiaquine in 80:20 (v/v) 20 mM aqueous ammonium acetate in water: acetonitrile was pumped with a flow rate of 50 µL/min through the Coulomchem III guard cell (ESA) using the LTQ Orbitrap (Thermo Scientific) integrated syringe pump. The potential on the cell was increased to 800mV to produce predominately AQQI, the voltage maintained and the effluent collected into a glass vial. 100 µL of this effluent was combined with 100 µL of CYP2C8 Supersomes™ and the volume made up to 500 µL by the addition of 100mM phosphate buffer pH 7.4. The sample was then incubated at 37°C in a heated water bath for 60 minutes. After the incubation time, the sample was centrifuged for
10 minutes at 10,000 x g. The supernatant was removed, the pellet washed with 100 mM phosphate buffer pH 7.4 to remove any unbound drug material, vortex mixed and centrifuged for 10 minutes at 10,000 x g. This wash step was repeated a further two times, and the protein pellet retained for digestion.

The pellet was reconstituted in 100 µL of 0.1M RapiGest™ solution in 50 mM ammonium bicarbonate in water. 6 µL of 100 mM DTT in 50 mM ammonium bicarbonate was added and samples incubated at 95°C on a heating block for 5 minutes. The sample was then allowed to cool and 12 µL of the alkylation reagent (100 mM IAA in 50 mM ammonium bicarbonate) added and vortex mixed. The sample was incubated in the dark, at room temperature, for 20 minutes. Digestion with trypsin was performed by the addition of 5 µL of a 0.2 µg/µL solution of trypsin in 50 mM ammonium bicarbonate and incubation overnight at 37°C in a shaking water bath. The digestion was terminated and the RapiGest™ reagent removed by the addition of 1 µL of formic acid to the sample and incubation at 37°C in a shaking water bath for 45 minutes. The RapiGest™ precipitate was removed by centrifugation for 10 minutes at 10,000 x g and the supernatant retained for LC/MS analysis.

A chromatographic separation and MS analysis was performed on the digest sample to identify the any modified peptides. LC/MS analysis was performed on a Synapt G2S mass spectrometer coupled to an Acquity UPLC system (both Waters Ltd.), as described in Experimental Section 2.26. LC/MS analysis was performed on a Synapt G2S mass spectrometer coupled to an Acquity UPLC system (both Waters Ltd.). MassLynx version 4.1 software from Waters Ltd. was used to control both the MS and Acquity systems. External mass calibration of the mass spectrometer was performed over an m/z range of 50 – 2000 using a solution of NaCsI infused into the MS source using the integrated pump. An external lock mass (1 µg/mL leucine enkephaline in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using the integrated pump during each analyses, for automatic mass correction. The MS settings were optimised for peptide analysis using a 2 pmol/µL solution of [Glu]-fibrinopeptide B in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.10 mL/min 1:1 0.1% (v/v) formic acid in water: 0.1% (v/v) formic acid in acetonitrile, using the integrated pump. Chromatographic separation was performed on an Acquity UPLC system as described in Experimental Section 2.15.

ESI positive ion MS data was collected in high resolution mode (approximately 35,000) with the ion mobility data collection and enhance dynamic range switched on. The MS² experiment was
composed of two functions, with automatic mass correction included. MS Function 1 collected data over the range m/z 100 – 2000, using a scan speed of 0.5 s, inter-scan speed of 0.05 s and collision energy offset in the transfer cell of 2 arbitrary units (low energy). Function 2 collected data as over the range m/z 100 - 2000 using a scan speed of 0.5 s, inter-scan speed of 0.05 s and using collision energy offset in the transfer cell of 20 – 30 arbitrary units (high energy). A third function was used to collect data on the external lock mass for automatic mass measurement using leucine enkephaline. Data was collected for 0.2 s at an interval of 10 s, and averaged over 10 scans.

The MS settings were as follows: capillary voltage, 2.5 kV; sampling cone 25 arbitrary units; source offset, 80 arbitrary units; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow rate, 10 L/hour; desolvation gas flow rate, 600 L/hour; LM and HM resolution, 4.7 and 15 arbitrary units respectively; trap collision energy offset, 4 arbitrary units; helium cell gas flow, 180 mL/min; IMS gas flow, 90 mL/min; IMS wave velocity, 2000 m/s; IMS wave height, 40 V.
Development of methods for the detection of covalent protein-adducts in *in vitro* incubations using acetaminophen as a tool substrate

Acetaminophen (*N*-acetyl-∗p*-aminophenol (APAP), also known as paracetamol) is one of the most commonly used over-the-counter drugs in the world. A widely used analgesic and antipyretic, it is generally used for the treatment of mild-moderate pain such as headache and arthritis, and for fever reduction, and is the active ingredient in most cold and flu remedies. Research (196-198) suggests that the analgesic, antipyretic and mild anti-inflammatory qualities of acetaminophen are due to inhibition of the cyclooxygenase-2 (COX-2) pathway of prostaglandin synthesis. Prostaglandins are generated by COX-1 and COX-2 enzymes as part of the oxidation process of arachidonic acid and are central to the physiological processes for inflammation, fever and pain.

At normal therapeutic doses, acetaminophen is generally considered to be extremely safe and very well tolerated with minimal side effects. However, at larger doses it can be severely hepatotoxic, causing acute centrilobular hepatic necrosis (199-201) in humans. The most common cause of acute liver failure in both the US and UK is acetaminophen toxicity (202-204), either through intentional or unintentional overdose, and it accounts for approximately 50,000 hospital visits and nearly 500 deaths in the US annually (205).

Acetaminophen is rapidly absorbed and distributed after oral administration, with the maximum concentration occurring approximately 1 hour after dosing, and an elimination half-life of between 2 and 3 hours (206). At normal therapeutic doses, it is extensively metabolised and eliminated almost exclusively in the urine, with direct conjugation of the parent compound by sulphotransferase and UDP-glucuronyltransferase to the sulphate and glucuronide conjugates respectively, together representing elimination of approximately 80% of the dose (206). A minority of the dose is metabolised by P450 oxidation to form a reactive quinone imine metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) which can then be detoxified by the enzyme glutathione S-transferase to form glutathione, *N*-acetylcysteine and cysteine conjugates (207). Additional hydroxylation routes also occur, with a methylated 3-hydroxyacetaminophen noted in human urine (208). After a toxic dose, excessive formation of the NAPQI metabolite depletes levels of glutathione in the liver, allowing free NAPQI to covalently bind to proteins (209-211). The primary treatment for acetaminophen overdose is the administration of *N*-acetylcysteine (NAC), which can be effective if administered within 12-24 hours of ingestion. The NAC administered can be used by the body to replenish depleted levels of glutathione and provide sulphur containing amino acids such as cysteine to promote
protein synthesis for cellular repair (212). A schematic depicting acetaminophen and its metabolism in humans is shown in Figure 3.1.

Figure 3.1: Schematic of the metabolism of acetaminophen in human (206, 208)

The work of several laboratories has identified CYP2E1 together with 1A1, 3A4 and 2D6 as the cytochrome P450 enzymes responsible for the metabolism of acetaminophen to its reactive metabolite NAPQI (82, 213). The work of Lee et al. (214) confirmed the involvement of CYP2E1
in acetaminophen toxicity by using cyp2e1 knock-out mice. Both knock-out and wild type mice were administered up to 400 mg/kg of acetaminophen and the effects, based on animal survival and measurement of enzymes and serum components diagnostic for liver and kidney toxicity, compared. The knock-out animals were significantly more resistant to acetaminophen toxicity than the wild type animals. The cyp2e1 knock-out mice survived doses of 400 mg/kg, whereas over 50% of wild type animals died. In addition, levels of aspartate aminotransferase and alanine aminotransferase, considered biomarkers of liver cell death, were elevated in the wild type animals but remained at control levels in the knock-out mice. This liver damage was confirmed by the use of liver histology. At higher doses of 600 mg/kg, toxicity was observed in both the knock-out and wild type groups indicating that other P450 enzymes may mediate toxicity at higher doses.

CYP2E1 has been extensively studied due to its involvement in the oxidation and bioactivation of low-molecular weight substrates such as benzene, toluene and aniline, and its role in carcinogenesis (207, 215, 216). It is inducible by ethanol and is involved in its metabolism, which can significantly increase the toxicity of acetaminophen and other low molecular weight xenobiotics when taken with alcohol or given to those suffering the effects of alcohol-induced liver disease (214, 216). It is also known to be involved in the metabolism of endogenous fatty acids such as arachidonic acid and epoxyeicosatrienoic acid (217, 218). It is particularly active in the liver where it represents approximately 7% of liver P450 proteins (219) but is also noted in the lung, oesophagus and small intestine (220). The crystal structure of CYP2E1 was recently determined by Porubsky et al. (221), revealing CYP2E1 to have the smallest active site identified so far of all the human cytochrome P450 enzymes, which correlates well with the low molecular weight of its substrates, but would be too small to accommodate fatty acids. Porubsky identified the rotation of the side chain of Phe<sup>478</sup> in the active site, which would then allow metabolism of these longer chain moieties (221).

The widespread availability and the relatively high incidence of drug-induced hepatotoxicity mean that the different mechanisms of toxicity of acetaminophen have been extensively studied and reviewed (210, 222). Numerous proteins bound to acetaminophen have been identified by several laboratories (133, 223-234) using a combination of techniques including immunochemical assays, western blotting assays and MALDI-MS, a summary of which is shown in Figure 3.2. The proteins identified were very varied and included microsomal, cytosolic, ribosomal and mitochondrial proteins of sizes ranging from 20 kDa to 100 kDa, all with different functions and utilities, and included glutathione-s-transferase in rats (234) and N-10-formyltetrahydrofolate dehydrogenase (225) and aldehyde dehydrogenase (232) in mice.
Figure 3.2: A summary of the acetaminophen-adducted proteins identified in the literature (reproduced from (210) with permission).

<table>
<thead>
<tr>
<th>Mass</th>
<th>Fraction</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>Microsome</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>50</td>
<td>Mitochondria</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>54</td>
<td>Mitochondria</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>55-58</td>
<td>Cytosol</td>
<td>Selenium (acetaminophen) binding protein</td>
</tr>
<tr>
<td>74-75</td>
<td>Nucleus</td>
<td>Lam-1-A</td>
</tr>
<tr>
<td>100</td>
<td>Cytosol</td>
<td>N-10 Formyl THF dehydrogenase</td>
</tr>
<tr>
<td>22</td>
<td>Cytosol, mitochondria</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>28</td>
<td>Mitochondria</td>
<td>Housekeeping protein</td>
</tr>
<tr>
<td>29</td>
<td>Cytosol, microsomes</td>
<td>Thiolase 5-3-methyltransferase</td>
</tr>
<tr>
<td>16</td>
<td>Cytosol</td>
<td>Aryl sulfotransferase</td>
</tr>
<tr>
<td>32</td>
<td>Not known</td>
<td>Pyrophosphates</td>
</tr>
<tr>
<td>20</td>
<td>Cytoskeleton</td>
<td>Trichosatun-5 A</td>
</tr>
<tr>
<td>22</td>
<td>Cytosol, mitochondria</td>
<td>GSH peroxidase</td>
</tr>
<tr>
<td>52</td>
<td>Cytosol</td>
<td>Selenium binding protein</td>
</tr>
<tr>
<td>57</td>
<td>Cytosol</td>
<td>Acetaminophen binding protein</td>
</tr>
<tr>
<td>28</td>
<td>Cytosol</td>
<td>Proteasome subunit C8</td>
</tr>
<tr>
<td>45</td>
<td>Cytosol</td>
<td>Methionine S-adenosyl transferase</td>
</tr>
<tr>
<td>46</td>
<td>Ribosomes</td>
<td>Protein synthesis initiation factor 4A</td>
</tr>
<tr>
<td>56</td>
<td>Mitochondria</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>54</td>
<td>Cytosol</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>59</td>
<td>Mitochondria</td>
<td>ATP synthetase α-subunit</td>
</tr>
<tr>
<td>29</td>
<td>Cytosol</td>
<td>Carbonic anhydrase III</td>
</tr>
<tr>
<td>35</td>
<td>Peroxisomes</td>
<td>Urease activase</td>
</tr>
<tr>
<td>36</td>
<td>Mitochondria, peroxisome</td>
<td>2-4-Demethyl-CoA reductase</td>
</tr>
<tr>
<td>22</td>
<td>Cytosol, (macrophages)</td>
<td>Ornithin-specific factor 3</td>
</tr>
<tr>
<td>23</td>
<td>Cytosol, mitochondria</td>
<td>Glutathione transferase σ</td>
</tr>
<tr>
<td>40</td>
<td>Cytosol</td>
<td>Serine dehydrogenase precursor</td>
</tr>
<tr>
<td>32</td>
<td>Cytosol</td>
<td>Glycine 2-oxaloacrylate</td>
</tr>
<tr>
<td>32</td>
<td>Cytosol</td>
<td>3-Hydroxyanthranilic 3,4-dioxygenase</td>
</tr>
</tbody>
</table>

THF, tetrahydrofuran.
* Cohen et al. (1997).
* Qin et al. (1998).
A mechanism for the binding of proteins to acetaminophen has been shown to be through the cysteine residues of the protein and is depicted in Figure 3.3.

![Figure 3.3: Proposed mechanisms for the binding of NAPQI to cysteine residues in proteins](image)

The binding of acetaminophen to proteins outside of the endoplasmic reticulum where NAPQI is formed has been noted by Chen et al. (235) who suggest the reversible formation of a partially-stable *ipso*-adduct intermediate with glutathione (an *ipso*-adduct being defined as a moiety attached to an aromatic carbon where there is already a substituent (236)), which has sufficient stability to be able to move from its site of formation e.g. the CYP2E1 enzyme, to other cellular compartments, and then subsequently loses its glutathione conjugation, thereby releasing free NAPQI which is available to adduct to proteins.

A recent paper by Davern et al. (205) shows the utility of detecting protein-adducts of acetaminophen for use in clinical diagnostics. Protein-adducts formed in hepatocytes can be released into serum (224, 228). These protein-adducts were detected in the serum of patients with acute liver failure using high performance liquid chromatography with electrochemical detection (237) to provide a clinical method to confirm or exclude acetaminophen ingestion as the cause of severe liver injury in patients. This facilitated the rapid administration of N-acetylcysteine, the current treatment for acetaminophen overdose, where necessary.

While numerous studies have been conducted, the mechanism of toxicity of acetaminophen is still poorly understood and may involve other factors in addition to covalent binding, such as oxidative stress (238, 239), macrophage activation in the liver (240) and mitochondrial dysfunction (241). However, the wealth of information in the literature around acetaminophen and how its reactive metabolite NAPQI covalently binds to proteins within the body, mean that acetaminophen is an excellent tool compound with which to develop methods for detecting and characterising covalent adducts of other compounds of interest.
The work detailed in this chapter describes the optimisation of sample incubation, preparation and analysis techniques with a view to developing methods suitable for application to compounds within the GSK portfolio where covalent binding is suspected to be an issue, and uses the incubation of acetaminophen as a tool substrate. Isolated CYP2E1 Supersomes™ were selected as an in vitro metabolising system to produce the reactive metabolite NAPQI. The advantage of using CYP2E1 Supersomes™ is that they are relatively simple systems of known protein concentration. Supersomes™ are individual recombinant CYP enzymes produced by a baculovirus expression vector system in BTI-Tn-5B1-4 insect cells (242). Recombinant Supersomes™ are commercially available for many metabolising enzymes including most of the cytochrome P450s, flavin-containing mono-oxygenases, UDP-glucuronosyl transferases (UGTs), monoamine oxidases (MAOs) and N-acetyl transferases (NATs), and for a range of species, including human, rat and dog (243). The individual CYP Supersomes™ also contain NADPH cytochrome P450 reductase and cytochrome b₅, which act as redox partners (244). The use of simple Supersome™ systems was intended to minimise the complexity of the sample. Human liver microsomes (HLM) were also utilised to provide a comparison with the metabolism and covalent binding observed with individual CYP2E1. Acetaminophen labelled with [¹⁴C] was also employed to quantify the levels of covalent binding observed and to aid the identification of protein adducts in the sample, using radio-detection.

3.1 Sample preparation and optimisation

3.1.1 Sample incubation

As discussed above, initial analyses were conducted using simple Supersome™ systems to reduce the complexity of the sample for future protein analysis. In vitro Supersome™ incubations are used within GSK and the wider pharmaceutical industry, to evaluate the individual cytochrome P450 enzymes responsible for the metabolism of drug candidates. The pharmacokinetic properties of a drug that is primarily metabolised by CYP3A4 may be adversely affected by co-administration with a drug that acts as an inhibitor of that enzyme, such as ketoconazole (245-248), therefore, this information is useful in terms of assessing the risk of drug-drug interactions for patients taking multiple drug regimens. At GSK, methods have been long established to perform these experiments, which are equivalent to those suggested by the manufacturer (244, 249) and in the wider literature (250), and these form the basis of the incubation methods used throughout this thesis. The experimental details used are described in Experimental Section 2.2.
The incubation mixture is comprised of a number of essential components. The Supersomes™ contain cytochrome P450 reductase and cytochrome b₅ together with the cytochrome P450 enzyme itself, both of which play important roles in the transfer of electrons from NADPH to the P450 enzyme itself (as described in Introduction Section 2.2) to enable oxidation of the substrate to take place. NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) is added to the incubation in the form of an NADPH regenerating system and provides the energy for the reaction. This is achieved by transfer of an electron from NADPH through its reaction with cytochrome P450 reductase. The NADPH regenerating system is comprised of glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase in a sodium bicarbonate buffer. NADP⁺ is reduced to NADPH via the reaction of glucose-6-phosphate via glucose-6-phosphate dehydrogenase as shown below (251) in Figure 3.4.

![Glucose-6-phosphate to 6-phosphoglucono-6-lactone reaction](image)

**Figure 3.4: The NADPH regenerating system**

The advantage of using a regenerating system, rather than adding the required amount of NADPH directly means there is always a sufficient amount of cofactor in the incubation to allow the reaction to proceed efficiently (252). Finally, the incubation takes place in an incubation buffer of 100 mM potassium phosphate buffer (pH 7.4) to maintain the reaction at physiological pH, and in a gently shaking water bath to maintain the reaction at approximately the physiological temperature of 37 °C. The incubation time of *in vitro* experiments using metabolizing systems such as Supersomes™ is typically between 30 to 60 minutes. However, the effect of increasing the incubation time was investigated by increasing the incubation time to 2 and 4 hours; these results are described in Section 3.1.2.

**3.1.2 Quantitative assessment of the covalent binding of [14C] acetaminophen in CYP2E1 Supersomes™ and human liver microsomes**

A qualitative assessment of the degree of covalent binding observed in an *in vitro* incubation between [14C] acetaminophen, and CYP2E1 or human liver microsomes was conducted using the experimental procedures described in Experimental Section 2.3. These data are shown in Tables 3.1 and 3.2.
Table 3.1: Quantitative assessment of covalent binding of $[^{14}C]$ acetaminophen and CYP2E1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time point (hours)</th>
<th>dpm/sample$^1$</th>
<th>pmol/sample$^2$</th>
<th>pmol/mg of protein$^3$</th>
<th>Cofactor dependent/independent binding (pmol/mg)$^4$</th>
<th>Total covalent binding to protein$^5$ (pmol/mg)</th>
<th>Approximate % protein modification$^6$</th>
<th>Approximate % radioactivity bound$^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1 + $[^{14}C]$ acetaminophen + NADPH cofactor</td>
<td>0</td>
<td>10597</td>
<td>408</td>
<td>679</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>17352</td>
<td>667</td>
<td>1112</td>
<td>199</td>
<td>433</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14110</td>
<td>543</td>
<td>904</td>
<td>99</td>
<td>225</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16777</td>
<td>645</td>
<td>1075</td>
<td>172</td>
<td>396</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CYP2E1 + $[^{14}C]$ acetaminophen - NADPH cofactor</td>
<td>0</td>
<td>8807</td>
<td>339</td>
<td>565</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12453</td>
<td>479</td>
<td>798</td>
<td>234</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10773</td>
<td>414</td>
<td>691</td>
<td>126</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12295</td>
<td>473</td>
<td>788</td>
<td>224</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CYP2E1 No drug control</td>
<td>0</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{14}C]$ acetaminophen stock solution</td>
<td>NA</td>
<td>1343408</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1: dpm/sample value generated by LSC of the filter papers post-analysis.
2: dpm/sample converted by dividing by radioactivity present in incubation e.g. 22 dpm/pmol
3: pmol/mg protein converted by dividing by amount of protein present in incubation e.g. 0.6 mg
4: Cofactor dependent binding = (cofactor binding at x hours – cofactor binding at 0 hours) – (cofactor independent binding at x hours – cofactor independent binding at 0 hours). Cofactor independent binding = (cofactor independent binding at x hours – cofactor independent binding at 0 hours).
5: The total covalent binding value represents the combination of the cofactor dependent and cofactor independent values.
6: Approximate % protein modification is based on the Mw of CYP2E1 as 56800 Da (Uniprot, accessed 11 August 2012)
7: Approximate % radioactivity bound is based on the comparison of radioactivity bound with the equivalent volume of stock solution added to the incubation i.e. 5 µL.
Table 3.2: Quantitative assessment of covalent binding of $[^{14}C]$ acetaminophen and human liver microsomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time point (hours)</th>
<th>dpm/sample$^1$</th>
<th>pmol/sample$^2$</th>
<th>pmol/mg of protein$^3$</th>
<th>Cofactor dependent/independent binding (pmol/mg)$^4$</th>
<th>Total covalent binding to protein$^5$ (pmol/mg)</th>
<th>Approximate % protein modification$^6$</th>
<th>Approximate % radioactivity bound$^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM + $[^{14}C]$ acetaminophen + NADPH cofactor</td>
<td>0</td>
<td>12724</td>
<td>489</td>
<td>979</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>1</td>
<td>25514</td>
<td>981</td>
<td>1963</td>
<td>779</td>
<td>984</td>
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<td>272</td>
<td>677</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21510</td>
<td>827</td>
<td>1655</td>
<td>253</td>
<td>676</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>HLM + $[^{14}C]$ acetaminophen - NADPH cofactor</td>
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<td>9197</td>
<td>354</td>
<td>707</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>205</td>
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<td>405</td>
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<td>1130</td>
<td>423</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$[^{14}C]$ acetaminophen stock solution</td>
<td>NA</td>
<td>1343408</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1: dpm/sample value generated by LSC of the filter papers post-analysis.
2: dpm/sample converted by dividing by radioactivity present in incubation e.g. 22 dpm/pmol
3: pmol/mg protein converted by dividing by amount of protein present in incubation e.g. 0.5 mg
4: Cofactor dependent binding = (cofactor binding at x hours − cofactor binding at 0 hours) − (cofactor independent binding at x hours − cofactor independent binding at 0 hours). Cofactor independent binding = (cofactor independent binding at x hours − cofactor independent binding at 0 hours).
5: The total covalent binding value represents the combination of the cofactor dependent and cofactor independent values.
6: Approximate % protein modification is based on the assumption that the numerous proteins in human liver microsomes have an average Mw of 50 kDa
7: Approximate % radioactivity bound is based on the comparison of radioactivity bound with the equivalent volume of stock solution added to the incubation i.e. 5 µL.
As shown in the data in Tables 3.1 and 3.2, covalent binding of \([^{14}C]\) acetaminophen to protein was observed in both the CYP2E1 and human liver microsome incubations. The binding in human liver microsomes was approximately 2-fold higher than in CYP2E1. This could be due to a number of factors. While the formation of NAPQI has been reported to be primarily due to the cytochrome P450 enzyme CYP2E1, other enzymes in the liver may also play a part, either in its formation or its stability. As discussed earlier in this chapter, several laboratories have suggested the involvement of CYPs 1A1, 3A4 and 2D6, as well as 2E1 in the formation of NAPQI (82, 213). In the in vitro incubation of CYP2E1 Supersomes™, these additional cytochrome P450 enzymes were absent, therefore it is reasonable to assume that the metabolism of acetaminophen to NAPQI may not have been as efficient, or may not have resulted in an equivalent yield of NAPQI as the equivalent human liver microsome incubation. A second consideration is the source of both the CYP2E1 Supersomes™ and the human liver microsomes. As discussed, Supersomes™ are produced by over-expression of individual recombinant CYP enzymes in a baculovirus expression vector system, whereas human liver microsomes are derived from donor livers from which the microsomal fraction has been isolated by differential centrifugation (253). It could therefore be argued that human liver microsomes are more representative of the metabolism processes occurring in vivo in humans.

The data suggest that the amount of covalent binding does not seem to be significantly affected by the incubation time. Since an NADPH regenerating system was used, there should have been sufficient NADPH available to sustain the metabolic turnover of the enzymes throughout the incubation period. The lack of an increase in binding with incubation time may therefore suggest that the metabolising capability of the in vitro systems is diminished as the incubation time increases.

For both the CYP2E1 Supersomes™ and human liver microsome data, the levels of binding estimate the percentage of protein modified by covalent binding as being < 5%. This is most likely due to poor metabolic turnover of acetaminophen to NAPQI, meaning only a small proportion of the drug is metabolised to the reactive species and is therefore available to bind to protein. The low modification of protein indicates that the detection of any covalent adducts at the protein/peptide level will be technically challenging, since the vast majority of protein will be unmodified. In addition, this value represents the total degree of modification of protein, therefore, in reality, if the protein is modified at more than one residue, the amount of each individual position of modification will be even less. However, the use of in vitro systems still provides the best chance of identifying covalent modification of protein because relative levels of metabolism can be much higher in in vitro systems such as human
liver microsomes, than observed *in vivo* (254). Also, depending on the *in vitro* system used, reactive phase I metabolites which may be detoxified *in vivo* by Phase II metabolism routes such as glutathione conjugation, may be free to modify proteins in the *in vitro* incubation. While the levels of covalent binding may be artificially large compared to what may be observed *in vivo*, they still provide the best chance of exploring the mechanisms occurring.

### 3.1.3 Method development

#### 3.1.3.1 Digestion of cytochrome P450 Supersomes™

The digestion of cytochrome P450 Supersomes™ was primarily based on protease digestion methods well documented in the literature (255, 256). Trypsin was selected as the protease as, due to the natural abundance of lysine and arginine residues, it tends to produce peptides of between 5 and 20 residues long which is the optimal mass range for efficient sequencing by collision induced dissociation fragmentation, and the peptides produced have a basic residue at the carboxyl end of the peptide which aids efficient ionisation by electrospray in positive ion mode (257). However, additional proteases such as Lys-C and Asp-N (Figure 3.5) were also used to examine the effect on the % protein coverage observed in the LC/MS analysis. The experimental procedures for these digestions are described in Experimental Section 2.4.

![Figure 3.5: Total ion chromatogram (TIC) of the (A) trypsin, (B) Lys-C and, (C) Asp-N digestions of CYP3A4 Supersomes™](image)

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A comparison of the % protein coverage was made by processing the data using BioPharmaLynx, which compares the peptides observed in the LC/MS analysis with the peptide masses expected based on an *in silico* digestion of the protein with that protease. The software allows the user to specify the sequence of the protein or proteins which should be present in the sample, the protease used for the digestion, the number of missed cleavages expected, expected modifications to the protein e.g. carbamidomethylation from alkylation with iodoacetamide, and the residue where the modification is expected to occur. Custom modifications e.g. the addition of acetaminophen, can also be added. Modifications can either be fixed, i.e. the mass of the peptide is expected to be modified, or can be variable. In order to process data using the BioPharmaLynx software, peptide LC/MS data must be collected in MS^E mode i.e. data consisting of two distinct MS functions with high and low collision energies. The software is capable of applying a suitable external calibrant to aid mass correction during deconvolution to improve mass accuracy. For this experiment, data was collected without mass correction and was subsequently deconvoluted by the software. Peptide assignments are based on the m/z observed in the low collision energy MS^E function compared to the expected m/z values expected for the peptides based on *in silico* digestion, using the parameters and tolerance (+/- ppm) specified. Any fragment ions observed in the high collision energy MS^E function data is used to confirm the software peptide assignment.

The protein coverage maps from the BioPharmaLynx output for the trypsin, Lys-C and Asp-N digestions are shown in Appendix 2, A21-A2.3, respectively. The protein coverage was low in all cases; 23% for trypsin, 13% for Lys-C and 5% for Asp-N. This low coverage observed poses a problem for future analyses to try to identify peptide-drug adducts since the majority of the protein sequence is not represented by the peptides identified. There may be several reasons for the low protein coverage observed. Cytochrome P450 enzymes are membrane-bound lipophilic proteins, therefore, the aqueous conditions of the digestion may not allow for the protease to adequately access the protein in order for digestion to occur effectively. The use of a surfactant agent was subsequently evaluated with trypsin to improve the solubility of the protein within the digestion incubation.

The RapiGest™ SF (sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate ([I]) (258-260) reagent from Waters (Milford Haven, MA, USA) is an anionic, mild acid-labile surfactant that solubilises and unfolds the substrate protein, making it more amenable to proteolysis, as the cleavage sites are more accessible. The reagent does not inhibit the activity of trypsin, or modify the protein in any way, and it is also compatible with MS analysis. Upon treatment with acid, the reagent is cleaved into two components - a small
ionic moiety and an insoluble, hydrophobic group, which can be removed by centrifugation, thereby removing the surfactant which can interfere with mass spectrometric analysis. The RapiGest™ SF reagent was prepared at a concentration of 0.1 % (w/v) in 50 mM ammonium bicarbonate as described in Experimental Section 2.5. The data shows a significant increase in the protein coverage observed, when the RapiGest™ SF reagent is used. Protein coverage was improved from approximately 23%, to approximately 51% as shown in Figure 3.6. This improvement in protein coverage is due to improved solubilisation of the protein, making it more amenable to digestion with trypsin in the aqueous digestion environment.

<table>
<thead>
<tr>
<th>MALLAVFLV</th>
<th>LLYLYGTHSH</th>
<th>GLFKKLGIPG</th>
<th>PTPLPFLGNI</th>
<th>LSYHKGFCMF</th>
<th>DMECHKKYGK</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWGFYDGQQP</td>
<td>VLAITDPDMI</td>
<td>KTVLVCEYS</td>
<td>VFTNRRFPG</td>
<td>VGFMKSAISI</td>
<td>AEDEEWKRLR</td>
</tr>
<tr>
<td>SLLSPFTSG</td>
<td>KLKEMVPIIA</td>
<td>QYGDVLVRNL</td>
<td>RREAETGKPV</td>
<td>TLKDVFGAYS</td>
<td>MDVITSTSFG</td>
</tr>
<tr>
<td>VNIDSNNPQ</td>
<td>DPFVENTKKL</td>
<td>LRDFDLDPFF</td>
<td>LSITVFPLL</td>
<td>PILEVLNICV</td>
<td>FPRTVNFLR</td>
</tr>
<tr>
<td>KSVKRMKESR</td>
<td>LEDTQKHRVD</td>
<td>FQLMIDSQN</td>
<td>SKETESHKAL</td>
<td>SDLELVAQSI</td>
<td>IFIFAGYETT</td>
</tr>
<tr>
<td>SSVLSFIMYE</td>
<td>LATHPDVQQK</td>
<td>LQEIDAVLP</td>
<td>NKAAPTYDTV</td>
<td>LQMELYDMVV</td>
<td>NETLRLFPIA</td>
</tr>
<tr>
<td>MRLERVCKKD</td>
<td>VEINGMFIPK</td>
<td>GVVMIPSYA</td>
<td>LHRDPKYWTE</td>
<td>PEKFLPERFS</td>
<td>KKNKDNIDPY</td>
</tr>
<tr>
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<td>NMKLALIRVL</td>
<td>QNFSFKPCKE</td>
<td>TQJPLKLSLG</td>
<td>GLLQPEKPVV</td>
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<tr>
<td>LKVESRDGT</td>
<td>SGA</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 3.6:** Protein coverage by LC/MS observed following digestion of CYP3A4 with trypsin using RapiGest reagent. The amino acids highlighted in red indicate those peptides observed in the analysis.

### 3.1.3.2 Optimisation of LC/MS analysis

The LC method employed was based largely on those currently found in the literature for peptide mapping and protein identification work (261-263), utilising a reversed-phase column and gradient system to elute peptides over the course of the analysis directly into the electrospray source of the mass spectrometer. The mobile phases are typically of low pH (0.1% (v/v) formic acid has been used throughout this thesis) to ensure protonation of the peptides for positive ion mass spectrometry. Formic acid was used as the acid modifier in the mobile phase rather than TFA (trifluoroacetic acid) as TFA has been noted for its suppression of mass spectrometry signal (264). Chakraborty and Berger (261) report a 9-fold decrease in suppression effect in the LC/MS analysis by using formic acid rather than TFA, although they found it made a small increase in peptide peak width.

A 60 minute gradient was used to provide adequate separation of the digest peptides to allow mass spectra of a quality adequate to provide structural information on the protein and any modifications to the protein to be generated, while still keeping the analysis time as short as
possible. The gradient was not extended beyond this time since early method development work indicated that there was no significant improvement in peptide chromatographic resolution or spectral quality with increasing gradient length.

### 3.1.3.3 Reproducibility of injection

The reproducibility of the LC method was investigated using an enolase 1 (Saccharomyces cerevisiae) MassPrep™ standard purchased from Waters (Manchester, UK), prepared at a concentration of 1 pmol/µL in 0.1% (v/v) formic acid in water.

Replicate injections of the same sample (5 µL) were injected on the column and MS² data recorded. The total ion current (TIC) chromatograms for each replicate injection have been overlaid and are shown in Figure 3.7. In addition, the data was processed by BioPharmaLynx to evaluate the consistency of the peptides observed in each analysis. A screen shot of the output from BioPharmaLynx is shown in Figure 3.8.

![TIC overlaid data from replicate injections of enolase 1 MassPrep™ standard](image)

**Figure 3.7:** TIC for the repeated injection of an enolase 1 (Saccharomyces cerevisiae) MassPrep™ digestion standard from Waters

As shown in the overlaid TIC data in Figure 3.7, the mass spectrometric analyses were consistent. Using a visual comparison, there were no significant differences in the number of peaks observed in the TIC, in the width of the peaks, or in the retention times observed. In addition, the BioPharmaLynx processed data from each replicate injection showed a very good correlation of peptides in each replicate injection (Figures 3.8 and 3.9). While there were some
minor differences in the peptides identified in each run (shown by the blue and yellow regions in the protein coverage map) the vast majority of the peptides were identified in each of the replicate injections (shown by the green regions in the protein coverage map), and there was no significant difference in % protein coverage between the samples (all samples had an approximate protein coverage of 80%).

Based on the data from replicate injections of the enolase 1 MassPrep™ standard the LC method was considered to be robust and reproducible.

Figure 3.8: Screen shot for the BioPharmaLynx comparison of replicate injections of an enolase 1 (Saccharomyces cerevisiae) MassPrep™ digestion standard from Waters, showing the comparison of protein coverage between two injections. The data file from one injection has been specified in the software as belonging to the “control”, while the data from a second injection has been specified as belonging to the “analyte”. The data from these two data files has been processed by BioPharmaLynx and assigned to the expected peptides observed for the protein as specified in the software (in this case enolase 1), and is represented here as the percentage coverage of the protein sequence. The areas shown in green represent regions of the protein observed in both the control and analyte analyses. Areas in blue represent peptides only observed in the control sample, while areas shown in yellow represent peptides observed in the analyte sample only. The data displayed here shows a high degree of correlation between the control and analyte data files, indicating a high degree of reproducibility of injection of the same sample.
Figure 3.9: Screen shot for the BioPharmaLynx comparison of replicate injections of an enolase 1 (*Saccharomyces cerevisiae*) MassPrep™ digestion standard from Waters, showing the mirror image comparison plots of the chromatographic peaks detected. This view shows extracted ion chromatograms for the peptides observed, represented across the time scale of the analysis. As shown in Figure 3.8, one injection has been assigned as the control sample (shown in green at the top of the mirror image) with a second injection assigned as the analyte sample (shown in blue at the bottom of the mirror image). Since the two images are virtually identical, the plot shows that the same ions were present in both analyses, and the variability in retention time of these components was minimal.

### 3.1.3.4 Evaluation of BioPharmaLynx

As discussed in Section 3.1.3.1, BioPharmaLynx is a software program available from Waters which can be used to interrogate, interpret and assign protein and peptide LC/MS data acquired on Waters mass spectrometers (265). For LC/MS data of digested peptides, the software is able to assign the peptides detected to any given proteins entered into the software method, based on their *in silico* digestion with selected proteases, and to calculate the percentage protein coverage achieved in the experiment. The software is also capable of comparing peptide LC/MS data sets to detect unique or modified peptides in samples, compared to an assigned control. This is achieved either by selecting a known or customised modification in the method. The modification is then applied to the *in silico* digestion data, compared against the experimental data and assigned in the software. Alternatively a simple comparison of two or more data sets is conducted, with unique peaks subsequently
highlighted without assignment. For intact protein LC/MS analysis, the software is capable of deconvolution of the protein data and comparing two or more data sets to assign any differences or modifications. BioPharmaLynx has been used to process peptide LC/MS data only throughout this thesis.

The detection of the differences between two data sets was crucial to the success of this project, therefore, this feature was evaluated in the BioPharmaLynx software using a MassPrep™ enolase 1 digestion standard, spiked with varying molar proportions (equimolar – 100-fold lower concentration) of [Glu¹]-Fibrinopeptide B (GluFib). A representative screen shot of a spiked digestion standard processed with BioPharmaLynx is shown in Figure 3.10.

![Figure 3.10: BioPharmaLynx screen shot showing the mirror image comparison plots of (A) the enolase digestion standard (assigned as the control sample and shown at the top of the image) and, (B) the enolase digestion standard with GluFib spiked at a concentration of 0.5 pmol/µL (assigned as the analyte sample and shown at the bottom of the image). On comparison of the two data sets, a single difference was highlighted by BioPharmaLynx in the analyte sample (shown in red), which corresponds to the addition of GluFib, spiked into the enolase digestion standard. The identity and retention time of the GluFib ion was confirmed by manual interrogation of the data.](image)

At all concentrations (down to 0.01 pmol/µL, 100-fold lower molar concentration than enolase) a distinct peak representing GluFib was observed in the BioPharmaLynx output (displayed in Figure 3.10), showing the suitability of BioPharmaLynx to detect low level differences between two closely related samples.
3.2 Metabolites of acetaminophen detected following incubation of acetaminophen with CYP2E1 and human liver microsomes (HLM)

The supernatants from the incubations of \([^{14}C]\) acetaminophen with CYP2E1 Supersomes™ and human liver microsomes were collected to assess the metabolic turnover of the compound by the enzyme. The supernatants were analysed by LC/MS with off-line radiodetection, as described in Experimental Section 2.12.

![Reconstructed radiochromatogram showing a single peak present in the supernatant from the incubation of \([^{14}C]\) acetaminophen with CYP2E1 Supersomes™.](image)

Interrogation of the LC/MS data generated showed that this radio peak corresponded to unchanged acetaminophen.

For the incubation with Supersomes™, unchanged acetaminophen was the only drug-related component detected in the supernatant (Figure 3.11). This is not altogether surprising because any acetaminophen that is metabolised to the reactive NAPQI species by the CYP2E1 enzyme would either bind to available proteins, as demonstrated by the levels of covalent binding shown in Table 3.1, or would be amenable to reduction back to acetaminophen in the aqueous conditions of either the incubation or chromatographic system (266), or would be further hydrolysed to a 1,4-benzoquinone species (267). While a peak corresponding to the correct nominal mass for the 1,4-benzoquinone species (m/z 166) was detected in the incubation, the exact m/z of the ion did not correspond to the empirical formula expected for a 1,4-
benzoquinone i.e. C₈H₈NO₃. In addition, the peak at m/z 166 was also detected in the 0 hour, no cofactor and no drug controls, suggesting that it is an artefact of the incubation itself.

In incubations of isolated recombinant CYP2E1 enzyme and acetaminophen, Chen et al. (82) reported the formation of both NAPQI and a 3-hydroxy metabolite, however this hydroxy metabolite was not observed in the incubations reported here. Comparing the experimental methods used, there is no obvious reason for this disparity; the compositions of the incubations are largely similar, using comparable quantities of enzymes and acetaminophen substrate. However, the detection methods differ, with the Nelson group using HPLC with electrochemical detection to identify the metabolites observed by retention time comparison with authentic standards of the 3-hydroxy metabolite and the glutathione conjugate of acetaminophen. It is likely that the electrochemical detection method is more sensitive due to the relatively poor sensitivity of acetaminophen by electrospray mass spectrometry, due to its low mass and the presence of interfering endogenous ions. Acetaminophen is both small and highly polar, making it particularly susceptible to suppression effects from other components in the sample (264).

A similar picture was observed in human liver microsomes, with unchanged acetaminophen the only drug related component detected by LC/MS. Again, this is unsurprising since the primary in vitro metabolic route is expected to be oxidation to form NAPQI (213). This result is consistent with numerous reports in the literature (213, 268, 269).

The inability for LC/MS to detect the NAPQI metabolite is unfortunate as it makes an accurate assessment of the conversion of acetaminophen to NAPQI problematic. However, given the high covalent binding values observed for both the CYP2E1 Supersomes™ and human liver microsomes, oxidation to NAPQI must be taking place, even though the marker for this conversion was not detected in the incubation supernatant. An electrochemical detector was not available in the laboratory, therefore the results described by Chen et al., were not reproduced, however this would have been a useful validation of the viability of the incubation.

3.3 Glutathione trapping of the reactive metabolite of acetaminophen

Acetaminophen was incubated with CYP2E1 and human liver microsomes in the presence of reduced glutathione in an effort to trap and stabilise the reactive metabolite NAPQI with glutathione, as described in Experimental Section 2.6, therefore confirming the oxidation of acetaminophen to NAPQI by the metabolising enzyme systems. No glutathione adducts were
detected in the incubation of CYP2E1. This may be due to poor conversion of acetaminophen to NAPQI by the recombinant CYP2E1 enzyme, although this is difficult to evaluate as discussed above. In the incubation with human liver microsomes and reduced glutathione, a small peak was detected in the MS data corresponding to the glutathione adduct of acetaminophen. An extracted ion chromatogram, mass spectrum and high collision energy MS² spectrum for the glutathione conjugate of acetaminophen ([M+H]⁺, m/z 457.1) are shown in Figure 3.12. The exact mass for the ion, shown in Figure 3.12 (457.1403 Da) is in good agreement with the expected exact mass for a glutathione conjugate of acetaminophen (C₁₈H₂₅N₄O₈S, exact mass 457.1388) with an error of 3.28 ppm, well within the acceptable error (± 5 ppm) for the QTof Premier instrument.

![Glutathione conjugate of acetaminophen](image-url)

The exact mass for the ion, shown in Figure 3.12 (457.1403 Da) is in good agreement with the expected exact mass for a glutathione conjugate of acetaminophen (C₁₈H₂₅N₄O₈S, exact mass 457.1388) with an error of 3.28 ppm, well within the acceptable error (± 5 ppm) for the QTof Premier instrument.
Figure 3.12: LC/MS data from the analysis of the supernatant from an incubation of acetaminophen and human liver microsomes, in the presence of an excess of reduced glutathione, showing (A) the extracted ion chromatogram (m/z 457.1, 0.2 Th window) for the expected m/z of a glutathione conjugate of acetaminophen, (B) mass spectrum for the peak observed at retention time 6.5 minutes in (A) and, (C) the high collision energy MS\(^{E}\) mass spectrum at retention time 6.5 minutes. The exact mass for the ion shown in (A) is in good agreement with the expected exact mass for a glutathione conjugate of acetaminophen.

The fragment ions observed in the high collision energy MS\(^{E}\) data also confirm the assignment of the peak as a glutathione conjugate of acetaminophen. The ion at m/z 382 corresponds to the loss of glycine from the glutathione moiety of an acetaminophen glutathione conjugate, with the ions at m/z 208, m/z 182, m/z 166 and m/z 140 all appear to be related to acetaminophen, with part of the glutathione moiety remaining on the aromatic ring of acetaminophen (see Figure 3.12 (C)).

A bulk incubation was prepared for preparative chromatography and \(^1\)H NMR analysis (conducted by Rita Tailor (preparative chromatography) and Andrew Roberts (\(^1\)H NMR analysis), both GSK, Ware). This large volume incubation (20 mL) was prepared by combining 20 separate incubations prepared using the experimental procedure described in Experimental section 2.6, with the exception that a more concentrated solution of acetaminophen (10 mM in ethanol) was used. The preparative LC and \(^1\)H NMR analysis is described in Experimental Section 2.7. Briefly, the combined incubation was separated using semi-preparative chromatography and fractions containing the glutathione conjugate (based on online mass spectrometric detection) reduced to dryness under vacuum and reconstituted.
in 50:50 (v/v) D₂O: acetonitrile for ¹H NMR analysis. ¹H NMR spectra for an authentic standard of acetaminophen and for the putative glutathione conjugate of acetaminophen are shown in Figure 3.14 and 3.15, respectively.

Comparison of the ¹H NMR spectra for the glutathione conjugate of acetaminophen and a standard of unmodified acetaminophen shows that protons a and b are largely unaffected. The addition of the glutathione moiety means the benzene ring is no longer symmetrical, therefore an additional single proton d is observed. The chemical shift for proton d is consistent with predicted values (270) for a proton ortho to an amide moiety, rather than a hydroxy, therefore the glutathione must be situated in the remaining position, ortho to the hydroxy on the benzene ring. The glutathione structure is confirmed by the characteristic signals for the cysteine –a, and –b protons (at approximately 3.40 ppm for the cys-a signals, and 3.15 ppm and 3.32 ppm for the cys-b protons, respectively), the glutamic acid –a, -b and –c protons (at approximately 3.79 ppm for glu-a, and 2.40 ppm for the glu-c signals, respectively) and the signals for the glycine moiety at approximately 3.80 ppm. The signals for the glu-b protons and the protons for the methyl group (labelled c) are both obscured underneath the large background proton signals for acetonitrile at approximately 2.05 ppm.

The ¹H NMR data shown in Figures 3.14 and 3.15 unambiguously assign the structure of the glutathione adduct formed after incubation of acetaminophen with human liver microsomes, in the presence of an excess of reduced glutathione (Figure 3.13).

![Figure 3.13: The structure of the glutathione adduct of acetaminophen](image)

Elemental formula of [M+H]⁺: C₁₈H₂₅N₄O₉S

[M+H]⁺ m/z 457.1388
Figure 3.14: $^1$H NMR data on an authentic standard of acetaminophen (generated by Andrew Roberts, GSK Ware)
Figure 3.15: $^1$H NMR data on a glutathione conjugate of acetaminophen generated in human liver microsomes (generated by Andrew Roberts)
The addition of glutathione mimics a common Phase II conjugation reaction \textit{in vivo} which acts to protect the body from reactive metabolites. Glutathione S-transferase, which can be found widely distributed in organs and tissues, acts as a catalyst for this reaction \textit{in vivo}, with glutathione conjugates being excreted into the bile. However, in cases where a drug molecule is primarily metabolised to a reactive species such as a quinone imine (as is the case with acetaminophen and its oxidation to NAPQI) and the levels circulating in plasma or tissues are high e.g. where a large dose has been administered, levels of glutathione can be depleted, leaving reactive metabolites unconjugated and free to covalently bind to proteins. Although the quinone imine structure has two potential positions of attack by nucleophiles, the higher electronegativity value of oxygen rather than nitrogen means that the more likely site of attack is adjacent to the carbonyl. This is supported by the NMR data shown in Figure 3.15, with the structure of the glutathione adduct indicating that the addition of glutathione to NAPQI appears to proceed via a similar mechanism to that postulated in Figure 3.3 for the addition of cysteine. The proposed mechanism is shown in Figure 3.16.

![Mechanism for the formation of a glutathione conjugate of acetaminophen](image-url)
The failure to detect glutathione adducts in the incubation with CYP2E1 Supersomes™ with LC/MS, and the poor LC/MS data generated from the human liver microsome incubation sample is disappointing. One way to improve the sensitivity of MS detection would be to use a tandem in space mass spectrometer such as a triple quadrupole mass spectrometer, and to devise a method based on either multiple reaction monitoring (MRM) or constant neutral loss scanning (MS scanning modes described in the Introduction). These analytical modes are generally more sensitive due to their very high selectivity, resulting in a much reduced MS background and higher signal to noise ratio. MS fragmentation of glutathione conjugates of small molecules can often result in the loss of the pyroglutamic acid (C$_3$H$_7$NO$_3$), which means the constant neutral loss of 129 Da can be monitored to identify glutathione adducts in a complex sample (113, 271). Where pyroglutamic acid is not lost from the glutathione conjugate when fragmented in the MS collision cell, the loss of glutathione (307 Da) or glutamic acid (147 Da) may be viable alternatives (109).

### 3.4 Chemical production of NAPQI using silver oxide

Damsten et al. (272) showed the utility of using chemical methods for the production of stable NAPQI, the reactive metabolite of acetaminophen. The group showed that by reacting the chemically produced NAPQI metabolite, firstly to a tripeptide cysteine$_{34}$-proline-phenylalanine (CPF) and then to human serum albumin, they were able to produce reference standards of CPF-NAPQI adducts which aided the identification of these adducts in human plasma samples taken from patients exposed to a high dose of acetaminophen. The methodology described in this paper was applied here to produce stable NAPQI in order to demonstrate binding to glutathione. This was then used to evaluate the potential stability of NAPQI-protein adducts under sample preparation and analysis conditions. Secondly, NAPQI was also used to bind non-enzymatically to CYP2E1 Supersomes™. Following digestion with trypsin, the resultant NAPQI-peptide adduct was used as a reference standard to aid the identification of any adducts formed via the in vitro incubation of CYP2E1 Supersomes™ and acetaminophen. The experimental details for the formation of NAPQI via the chemical reaction of acetaminophen with silver oxide are given in Experiment Section 2.8.

A small amount of the reaction mixture was analysed using the LC/MS method described in Experimental Section 2.12 to assess the efficiency of the conversion of acetaminophen to NAPQI. A single peak was noted in the analysis at m/z 152.0704, corresponding to acetaminophen itself. This reinforces that while NAPQI is stable in an organic environment such as chloroform, it is easily reduced back to acetaminophen in the predominantly aqueous environment of the chromatographic separation (266). This makes a mass spectrometric
assessment of the conversion of acetaminophen to NAPQI by reaction with silver oxide non-viable. However the colour change of the solution (from colourless to pale yellow) was indicative of quinone imine formation. The colour changes associated with benzoquinone imines are frequently used within the dyeing industry and in colour photography (273, 274). A similar colour change was noted on the oxidation of amodiaquine to amodiaquine quinone imine AQQI (See Chapter 5). The mass spectrum is shown in Figure 3.17 (A), with the product ion spectrum shown in Figure 3.17 (B). These data are summarised in Table 3.3.

Figure 3.17: MS data of the direct infusion of the chemically generated NAPQI solution into the MS source, (A) mass spectrum of the reaction mixture in an aqueous environment, showing the reduction of NAPQI back to acetaminophen, and (B) product ion spectrum for acetaminophen (m/z 152)
Incubation of chemically-formed NAPQI with excess cysteine and glutathione (Experimental Section 2.9) demonstrated the chemical reactivity of the NAPQI species as a soft nucleophile, with two cysteine and two glutathione conjugates formed in each incubation, respectively. Extracted ion chromatograms for the unreacted acetaminophen (m/z 152.1, 0.1 Th window) and, cysteine (m/z 271.1, 0.1 Th window) and glutathione conjugates (m/z 457.1, 0.1 Th window) detected after incubation of chemically-generated NAPQI with excess cysteine and glutathione are shown in Figures 3.18 and 3.19, respectively.

Figure 3.18: Extracted ion chromatograms for (A) the unreacted acetaminophen (m/z 152, 0.1 Th window), and (B) acetaminophen cysteine conjugates (m/z 271, 0.1 Th window), following LC/MS analysis of the incubation of chemically generated NAPQI with an excess of cysteine.
Figure 3.19: Extracted ion chromatograms for (A) the unreacted acetaminophen (m/z 152, 0.1 Th window), and (B) acetaminophen glutathione conjugates (m/z 457, 0.1 Th window), following LC/MS analysis of the incubation of chemically generated NAPQI with an excess of glutathione.

Full scan mass spectra and product ion spectra for the cysteine and glutathione conjugates are shown in Appendix 2, A2.4 and A2.5, and A2.6 and A2.7, respectively. The MS data for the glutathione and cysteine conjugates are summarised in Table 3.3.
Table 3.3: Summary of MS data for acetaminophen and its cysteine and glutathione conjugates

<table>
<thead>
<tr>
<th>Observed [M+H]⁺ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
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<td>m/z 134: [M+H]⁺ - H₂O</td>
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<td>Empirical Formula: C₁₄H₁₅N₂O₄S⁺</td>
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<td></td>
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<td>Observed [M+H]^+ (m/z)</td>
<td>Proposed Structure</td>
<td>Diagnostic Fragment Ions (m/z)</td>
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</tbody>
</table>
| 457.1379                | ![Proposed Structure](image1) | m/z 382: ![Fragment](image2)  
  m/z 208: m/z 225 - NH₃  
  m/z 194: ![Fragment](image3)  
  m/z 336: m/z 382 - CO - H₂O  
  m/z 328: ![Fragment](image4)  
  m/z 182: ![Fragment](image5)  
  m/z 166: ![Fragment](image6)  
  m/z 140: ![Fragment](image7)  
  m/z 269: ![Fragment](image8)  
  m/z 225: ![Fragment](image9)  |
| 457.1371                | ![Proposed Structure](image10) | ![Fragment](image11)  
  ![Fragment](image12)  
  ![Fragment](image13)  
  ![Fragment](image14)  |

In both cases, incubation of chemically-produced NAPQI with cysteine and glutathione resulted in the formation of two positional isomers, most likely due to the sulphur lone pair attacking the carbons adjacent to both the carbonyl and to the imine moieties. For the glutathione conjugates, comparing the retention times of the peak detected in the incubation of acetaminophen with human liver microsomes and excess glutathione (for which ^1H NMR data
was generated to definitively assign the structure) and the peaks in the above analyses, the peak at the approximate retention time of 6.5 minutes is the same in both samples i.e. is consistent with a structure where glutathione is attached to the benzene ring adjacent to the hydroxyl. Therefore, it can be assumed that the glutathione peak at the approximate retention time of 5.9 minutes is the glutathione conjugate where glutathione is attached to the carbon adjacent to the amide nitrogen. Similarly, the structures given for the cysteine conjugates in Table 3.3 have been assigned, based on the relative retention times of the two glutathione conjugates and their structures, such that the cysteine conjugate where cysteine is adjacent to the amide nitrogen has eluted before the cysteine adjacent to the hydroxy; however, these structural assignments would need to be confirmed by $^1$H NMR analysis of the reaction mixture.

Interestingly, a large proportion of acetaminophen in the samples remained unreacted. This could be for two reason; firstly the original conversion of acetaminophen to NAPQI was incomplete, or secondly, the chemical reaction of NAPQI with a large excess (100-fold molar excess) of glutathione or cysteine in aqueous solution is inefficient. Either not all of the NAPQI molecules react with glutathione or the NAPQI is converted back to acetaminophen in the aqueous conditions. Glutathione itself can also act as a reducing agent (275) with a primary function of glutathione in the body being to keep free thiol groups on proteins in a reduced state and to prevent oxidative stress in cells. A similar result was observed for the chemical reaction between electrochemically-generated amodiaquine quinone imine and glutathione in solution, as detailed in Section 6.2.

The samples containing cysteine and glutathione conjugates were each incubated at 37°C and 50°C overnight in water baths to replicate the conditions experienced by the sample during trypsin or pronase digestion, and to evaluate the thermal stability of any acetaminophen-peptide adducts formed. The mass chromatograms generated post-incubation are shown in Figures 3.20 and 3.21. The data shows both the cysteine and glutathione conjugates to be stable under these thermal conditions. The slight peak splitting observed in Figure 3.21 is most likely due to column degradation rather than an artefact of the raised temperature as this effect did not occur after changing the column (Figure 3.20).
Figure 3.20: Extracted ion chromatograms for (A) the unreacted acetaminophen (m/z 152, 0.3 Th window), and (B) acetaminophen glutathione conjugates (m/z 457, 0.3 Th window), following incubation overnight at 37°C

Figure 3.21: Extracted ion chromatograms for (A) the unreacted acetaminophen (m/z 152, 0.1 Th window), and (B) acetaminophen glutathione conjugates (m/z 457, 0.1 Th window), following incubation overnight at 50°C
3.5 Detecting covalent protein-adducts of acetaminophen using *in vitro* systems

The formation of covalent adducts of acetaminophen with proteins was investigated by incubation of CYP2E1 Supersomes™ and human liver microsomes with acetaminophen as described in Experimental Section 2.2. The quantitative covalent binding data shown in Table 3.1, generated using radiolabelled acetaminophen shows a distinct retention of radiolabelled material associated with the protein. This indicated that further investigation of the proteins themselves may yield the structure of the reactive species while bound to protein, and its target location. This was reinforced by the metabolism and glutathione data generated showing the capacity for acetaminophen to be metabolised to the reactive metabolite NAPQI which can be trapped by a soft nucleophile such as cysteine or glutathione. For simplicity, the primary focus of investigation for these additional experiments involved using the simpler CYP2E1 Supersomes™ incubations, however data was also generated using the human liver microsomes incubations. This was attempted using three primary methods; firstly LC/MS analysis of the intact protein to monitor for any shift in molecular mass due to adduction by acetaminophen, secondly to use protease digestion and LC/MS to identify any mass changes to peptides in the mixture, and finally to use protease digestion and LC/MS to monitor mass shifts at the amino acid level upon incubation with acetaminophen.

3.5.1 Detection of modification of intact proteins by LC/MS

A reversed-phase LC/MS method was developed to separate the component proteins within the Supersome™ incubation. As discussed previously, the protein content in Supersomes™ is primarily cytochrome P450 CYP2E1, cytochrome P450 reductase and cytochrome b5 but by virtue of their production, they may also contain small amounts of endogenous proteins.

All incubations and LC/MS experimental procedures were conducted as described in Experimental Section 2.13. Initial LC/MS conditions were developed using standard proteins such as myoglobin and enolase dissolved in water at a concentration of 5 pmol/µL (therefore 25 pmol injection on column). Chromatographic peak shape and retention time properties for these standard proteins were generally good, as demonstrated by the myoglobin data shown in Figure 3.22. Analysis of the neat CYP2E1 Supersome™ mixture (12 mg/mL total protein content, therefore 60 µg protein on column) was less successful. The total ion current trace depicted in Figure 3.23 shows a very high level of background was observed in the analysis, completely masking any MS signals related to the intact proteins in the sample, making it impossible to extract a mass chromatogram for the protein components. This is most likely to be due to the buffer composition of the Supersomes™ themselves.
Figure 3.22: LC/MS method development showing (A) a mass chromatogram of the 21+ charge-state of myoglobin m/z 808.2 (0.2 Th window), and (B) the full scan mass spectrum for intact myoglobin.

Figure 3.23: Total ion current chromatogram for the analysis of the neat CYP2E1 Supersome™ mixture (12 mg/mL total protein content)

While the buffer composition for the Supersome™ product is proprietary information owned by Gentest, similar products (276) contain high concentrations of detergents such as 3-[3-cholamidopropyl]dimethylammonio]-propanesulfonic acid (CHAPS) and liposomes to
solubilise the proteins and buffering agents such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain the pH of the solution at 7.4. As discussed in the introduction to this thesis, the cytochrome P450 enzymes are lipophilic membrane-bound proteins therefore they require a mixture of detergents, buffers and liposomes to maintain the enzymes in solution without denaturing or deactivating them. However, these detergents are commonly thought to cause ion suppression (277, 278) and to adversely affect the MS signal for proteins (260, 279) and, as shown in Figure 3.23, require removal before MS analysis. Extraction of the protein and resolubilisation was also unsuccessful, with no protein signal detected in the sample. This is not surprising due to the lipophilic nature of the protein. Efforts to detect the intact cytochrome P450 protein by MS were halted at this point. An important experiment to conduct in any future work would be to spike a concentration of myoglobin into the Supersome™ product prior to analysis by LC/MS comparison of the Supersome™ sample with an equivalent concentration of myoglobin in water would give an assessment of the suppression effects observed due to the detergents present in the Supersome™ sample.

However, in common sense terms, the detection of a mass shift in the intact molecular weight of the protein of interest remains the simplest method for confirming the presence of a covalent modifier to a protein. In addition, the stoichiometry of the addition reveals important information about the number of binding sites on a protein, since the molecular weight of the protein should only differ from the control by multiples of the small molecule drug modifier, within experimental error. Therefore the development of methods to detect the intact proteins such as the cytochrome P450s by mass spectrometry has future utility. A number of groups have been successful (280, 281) using alternative chromatographic separation methods and protein products. One area of investigation could be the use of mass spectrometry compatible surfactants such as (1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) (282) for MALDI analysis or n-dodecyl-β-D-maltoside (DDM) (283) for electrospray.

3.5.2 Detection of modified peptides by LC/MS

While the analysis of intact cytochrome P450 CYP2E1 was unsuccessful, digestion of the protein using a protease such as trypsin offers a useful alternative. Peptide analysis is generally simpler and more amenable to LC/MS than analysis of intact proteins due to the highly polar and soluble nature of most peptides, and the specificity of the protease used means that any modification to the protein is unlikely to be removed during the proteolysis process. This has been proven in several academic papers where modifications to proteins due to
acetaminophen addition have been detected after protein digestion with trypsin (133, 272, 281). In addition, experiments conducted with chemically-generated acetaminophen glutathione adducts (Section 3.3) have shown that any peptide-drug adducts formed should be stable under the thermal conditions used as part of the digestion process.

The experimental procedures describing incubation, protease digestion and LC/MS analysis are described in Experimental Sections 2.2, 2.14 and 2.15, respectively. After incubation of acetaminophen with CYP2E1 Supersomes™ the incubation mixture was centrifuged to separate the protein content from the rest of the mixture. This protein pellet was retained and washed three times with phosphate buffer to ensure the complete removal of any un-bound drug-related material. This protein pellet was resolubilised in 0.1% (w/v) RapiGest SF in 50 mM ammonium bicarbonate and subjected to digestion overnight at 37°C with trypsin. As discussed previously, the use of RapiGest surfactant aids the solubilisation of the CYP2E1 enzymes and therefore improves the protein coverage detected by LC/MS. Suitable control samples were prepared alongside the samples; incubations in the absence of drug and NADPH cofactor, and as time 0 hour controls, which were all analysed to eliminate false positives during data processing.

Initial analyses were conducted using in-solution digestion with analysis by LC/MS using a chromatographic method on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled to a Waters QTof Premier mass spectrometer. While the Acquity UPLC system and QTof Premier mass spectrometer were primarily selected due to their availability in the laboratory, they are suitable instruments for these kinds of analyses. Nano-LC and nanospray are commonly used for LC/MS protein identification and confirmation experiments such as peptide mass fingerprinting and peptide mapping (284-287) due to the advantages of nanospray in terms of reduced suppression effects (288, 289) and reduced sample consumption with the maintenance of sensitivity (290). The low flow rates (approximately 20 nL/min) associated with nano-LC means that it can be technically challenging and difficult to maintain. Where sample volume or concentration is not a factor, UPLC can offer a viable alternative. The smaller column particle sizes and column widths (typically 1.7 μm and 2.1 mm respectively) used in UPLC result in smaller peak widths which improves both the analytical sensitivity and peak resolution compared to traditional HPLC methods (291). The higher flow rates (approximately 200 μL/min for protein analysis) mean the system is easier to set-up, maintain and troubleshoot than an equivalent nano-LC system, without a significant loss of sensitivity. As discussed in the introduction to this thesis, quadrupole- time-of-flight tandem mass spectrometers are suitable for protein and peptide analysis due to their large mass
range, high mass accuracy (typically < 5 ppm), high mass resolution (depending on the instrument used approximately 10-40,000) and their capacity for collision induced dissociation (CID) fragmentation experiments which can aid in the confirmation of protein identification, or in the identification of modifications.

An MS<sup>E</sup> method was devised for the identification of the digest peptides analysed. During MS<sup>E</sup> (263, 292) the mass spectrometer alternates between a scanning mode where the collision cell energy is low and a second mode where the collision cell energy is high, thereby inducing CID fragmentation of all ions as they pass through the collision cell to provide complementary product ion data. This has a major advantage over data dependent acquisition (DDA) methods (293-295) which are commonly cited in the literature. DDA requires an initial MS scan to identify peptides followed by a number of MS/MS fragmentation scans on the peptides identified, usually based on their signal abundance. The duty cycle required to complete a DDA cycle can mean that where the sample is complex or where peak resolution is poor, insufficient time is available to generate MS data on all of the peptides eluting from the column. For MS<sup>E</sup>, high and low collision energy MS data is collected over the whole of the analysis time, therefore exact mass and product ion data is generated on all peptides within the mixture (296, 297). LC/MS data generated on the tryptic digestion of the 0, 1, 2 and 4 hour incubations, with no drug and no cofactor control incubations used to provide a comparison, were processed and compared using BioPharmaLynx.

The BioPharmaLynx method used the protein sequences of human cytochrome P450 2E1 (193) NADPH cytochrome P450 reductase (298), and cytochrome b<sub>5</sub> (299) with trypsin as the digest reagent and 2 missed cleavages allowed.

The mass tolerance was 50 ppm for the MS match and 100 ppm for the MS<sup>E</sup> fragment ion match. Although these values appear to be very high, they are reasonable for these analyses since no lock mass correction was used. Carbamidomethylation on cysteine residues (+57 Da) and the addition of acetaminophen to cysteine residues (+149.1 Da) were selected as variable modifications, with no maximum number of modifications given. These relatively wide variables were selected to do an initial assessment to identify potential candidates of peptides modified with acetaminophen, with the intention that any peptides identified would be subjected to more vigorous investigation such as MS/MS. In addition to the automatic data interrogation by BioPharmaLynx, manual data interrogation was carried out. This involved looking for postulated modified peptide m/z values in the MS data by extracting mass chromatograms for m/z values of interest and comparing against the equivalent mass
chromatogram for the control samples. The m/z values of the postulated modified peptides were based on \textit{in silico} digestion of cytochrome P450 CYP2E1 and assuming modification of any cysteines in the protein sequence.

Initial attempts to identify acetaminophen-modified peptides in an in-solution tryptic digest of the CYP2E1 Supersome™ incubation were unsuccessful, with no modified peptides identified. This could have been for a number of reasons. As shown in Table 3.1, the percentage of the protein modified by acetaminophen is very small (approximately 2%). This means that the abundance of any modified peptides will be very small, compared to the unmodified peptides in the matrix, therefore, the sensitivity of the analysis may not be sufficient. In addition, while there are only three major proteins present, the resulting digest is still a highly complex sample, therefore, the method may not be sufficiently selective.

Addressing the selectivity issue first, two methods were identified to reduce the complexity of the sample. Firstly, 1D gel electrophoresis was used to separate the proteins in the incubation mixture and to remove other components such the potassium phosphate sodium bicarbonate buffers still present which could potentially have a detrimental effect on MS response (300). In-gel digestion with trypsin and analysis of the peptides by LC/MS was then conducted.

Secondly, the use of [\textsuperscript{14}C] radiolabelled acetaminophen and offline radiodetection using TopCount™ and Accelerator Mass Spectrometry (AMS) were employed to generate radiochromatograms of the digested peptide analyses, to help refine the search for modified peptides based on retention time.

In terms of improving the sensitivity of the analyses, firstly, the isolated protein from the incubation was digested with pronase to reduce the proteins to their constituent amino acids and analysed by LC/MS. The advantages of amino acid analysis are a reduction in the sample complexity, with a subsequent improvement in signal to noise, since all of the modified amino acids should co-elute i.e. all modified cysteines will co-elute irrespective of their protein/peptide of origin. Secondly, alternative LC/MS scanning methods such as precursor ion scanning and multiple reaction monitoring (MRM) were explored to improve MS sensitivity.

\textbf{3.5.3 In-gel digestion and LC/MS analysis}

SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) is commonly used to separate protein components in a complex mixture prior to digestion and LC/MS analysis (191, 301, 302). The protein is dissolved in a denaturing solution containing SDS and a reducing agent such as DTT, and heated. This destroys the secondary and tertiary structure of the
proteins and applies negative charges, proportional to their molecular weight, to the proteins. The protein is then loaded onto a polyacrylamide gel in a running buffer and a voltage applied, causing the negatively charged proteins to migrate through the gel. The distance travelled by the protein is dependent on its molecular weight, with smaller molecular weight proteins travelling further through the gel. A marker protein mixture is often used to provide a comparison of the molecular weights of the sample proteins with known standards, for molecular weight determination (303, 304). In-gel digestion of each protein band (191) can then be carried out to generate peptides for LC/MS analysis. The advantage of using in-gel digestion is that it can increase the dynamic range of the LC/MS experiment. The separation of the proteins in the mixture on the gel prior to digestion means that lower abundant peptides may be more easily detected since the analysis of each separate band is likely to be significantly less complex than digestion of the mixture as a whole.

The experimental procedures describing the incubation, SDS-PAGE and in-gel digestions are described in Experimental Sections 2.2, 2.16 and 2.17, respectively. The coomassie-blue stained gel prior to in-gel digestion is shown in Figure 3.24.

The gel (Figure 3.24) shows the presence of three primary gel bands; NADPH cytochrome P450 reductase (Mw 76690 Da, accession number P16435 (298)), cytochrome P450 2E1 (Mw 56849 Da, accession number P05181 (193)), and cytochrome b5 (Mw 15330 Da, accession number P00167 (299)) in the sample. All of the proteins appear in the gel in the correct order and the accuracy of the molecular weight for each protein is reasonably good, with the exception of cytochrome b5. However, there does appear to be some distortion of the molecular weight marker gel bands at the base of the gel so this may be due to experimental variability. Some minor gel bands are also noticeable which are postulated to be proteins from the insect-cells used to produce the Supersomes™. The intensity of the gel bands show that these background proteins are far less abundant in the Supersome™ mixture than the cytochrome P450-related proteins of interest.
Figure 3.24: Coomassie-blue stained SDS-PAGE gel prior to in-gel digestion. A Novex® sharp pre-stained molecular weight marker was used for molecular weight comparison on each of the outer gel lanes. The inner lanes contain digested protein pellets from CYP2E1 incubations: (from left to right) 0, 1, 2 and 4 hour, no cofactor and no drug controls.

All three primary gel bands from each sample were digested with trypsin and analysed using the LC/MS method described in Experimental Section 2.15. The data were processed using the BioPharmaLynx method described previously with the exception that carbamidomethylation was removed as a variable modification since reduction and alkylation was not conducted. The 0, 1, 2 and 4 hour samples were processed against the no drug and no cofactor controls to identify any modifications to the peptides detected. In addition, manual data processing was carried out as described previously.

As with the in-solution experiments described in Section 3.4.2, attempts to identify acetaminophen-modified peptides from the isolated gel bands from SDS-PAGE of a CYP2E1 Supersome™ incubation were unsuccessful, with no modified peptides identified for any of the proteins. This result, combined with the data from the in-solution digestion, points to the
sensitivity of the LC/MS method on the QTof Premier instrument as potentially being the issue preventing the detection of acetaminophen-modified peptides. However, even without additional sensitivity of analysis, retention time information on any modified peptide adducts would be extremely useful. Even with protein separation by SDS-PAGE, the digest sample is very complex, with peptides of varying abundance and MS signal intensity. If the retention time of the adducted peptide is known, this would allow for manual processing of the data in a much smaller window, even using MS data processing (background subtraction) to eliminate as much of the endogenous ions or non-adducted peptides as possible.

### 3.5.4 Radiodetection of adducted peptides

Since $^{14}$C-labelled acetaminophen was available from commercial suppliers, incubations of $^{14}$C acetaminophen with CYP2E1 Supersomes™ were conducted as described in Experimental Section 2.2. LC/MS analysis was conducted as described in Experimental Section 2.18 with the post-column solvent flow directed to a time-dependent fraction collector. Fractions were collected over the whole analysis time at a speed of 9.4 s per well into four 96-well plates containing a solid scintillant (yttrium silicate). These fractions were then analysed using a TopCount NXT (190) detector which uses photomultiplier tubes to measure the luminescence exhibited by the scintillant when excited by ionising radiation i.e. in any fractions containing $^{14}$C acetaminophen. The data from the scintillation counter was then combined with the fraction time to generate a radiochromatogram for each sample. A second injection of each sample was analysed by LC/MS immediately after fraction collection using the method described in Experiment Section 2.15 so that any peaks identified by radiodetection could be correlated to the MS data from the subsequent injection. An alternative method is to perform both fraction collection for radiodetection and LC/MS in the same analytical run by splitting the LC flow post-column, however, by performing the analyses by consecutive injection, this maximised the sensitivity of the radiodetection by TopCount.

0, 1, 2 and 4 hour incubations of $^{14}$C acetaminophen with CYP2E1 Supersomes™ together with no cofactor and no drug controls were analysed by LC/MS and fractions collected for TopCount radiodetection. In all cases, no peak was observed in the radiochromatograms (see Figure 3.25).
This is not surprising given the levels of radioactivity in the sample. TopCount has a limit of detection of approximately 5-20 disintegrations per minute (dpm)(305). Given that only a very small amount of the radioactivity is covalently bound, it is feasible that any acetaminophen adducted peptides would be below the limit of detection for the analysis. This may be further compounded if acetaminophen is bound in numerous places on the protein and numerous adducted peptides exist in the sample.

A more sensitive radio detection technique was therefore employed. As discussed in the introduction to this thesis, accelerator mass spectrometry (AMS) is an extremely sensitive technique first developed to measure the abundance of low-abundant isotopes in archaeological, geophysical and biomedical samples. It has a significantly lower limit of detection compared to more common radio detection techniques such as TopCount and liquid scintillation counting, and has a limit of detection of approximately 0.005-0.1 dpm per peak. Due to the size and cost of AMS instruments, they are not common-place within the pharmaceutical industry. In addition, the preparation of samples for analysis by AMS is a lengthy, complex and extremely costly process. Despite this, the use of AMS to investigate covalent binding of reactive metabolite to proteins offers significant advantages. The ability to generate a radio chromatogram for a tryptic digest of a protein where covalent binding is suspected, offers the ability to be able to assess the stoichiometry of binding by counting the
number of radio peaks, e.g. detecting one peak in a radio chromatogram suggests that binding occurs on only one amino acid of the protein. The second advantage is one of selectivity. As discussed previously, knowledge of the retention time of an adduct peptide in the analysis allows for the refinement of LC/MS data searching to a much smaller area, thus aiding the identification of unknown adducted peptides.

Due to the high cost and labour intensive nature of the sample preparation process for AMS, one sample, a tryptic digest of the 4 hour incubation of $[^{14}C]$ acetaminophen with CYP2E1 Supersomes™, was selected for AMS analysis. The sample was prepared using the protocol described in Experimental Section 2.19. Due to the highly specialised nature of sample preparation and analysis, the sample was prepared and analysed by AMS experts (Adrian Pereira and Claire MacIntyre for sample preparation, and Steven Corless for sample analysis.) The reconstructed AMS radiochromatogram is shown in Figure 3.26.

A well defined peak was observed in the chromatogram at a retention time of 15 minutes, suggesting the presence of at least one acetaminophen adducted peptide in the digest sample since any unbound acetaminophen or metabolites of acetaminophen should have been removed from the sample during sample preparation. To reinforce this, the polar nature of acetaminophen means that it is likely to elute from the column at an earlier retention time than the elevated region shown. While no other defined peaks were present in the chromatogram, a region of elevated signal was observed in the retention time region between approximately 4 and 10 minutes.
A comparable LC/MS method on an equivalent digest sample indicates that several peptides elute from the column in the 4 – 10 minute region so it is feasible that the broad radio peak represents several additional adducted peptides which are not well resolved by the chromatographic system. One of the disadvantages of the AMS sample preparation process is that it requires fractionation of the LC effluent. For very complex samples, this can mean the analysis can suffer from poor chromatographic resolution. The time interval set for the analysis is a balance between achieving sufficient resolution of the components of interest in the sample while minimising the number of total fractions collected, due to the labour intensive and costly sample preparation process. For this analysis, a total of 96 fractions (collected at 25 second intervals between 5 and 45 minutes of the LC analysis) were analysed by AMS. Looking at the extracted ion chromatograms for a selection of peptide peaks in an equivalent LC/MS analysis, the average peak width is approximately 30 seconds; therefore a 25 second interval for fractionation may not be sufficient to resolve co-eluting or closely eluting peaks. The restrictions of the analysis meant that it was not feasible to analyse equivalent control samples. Analysis of a no drug control sample might definitively show if the high background was compound related. Analysis of an equivalent sample using cold acetaminophen rather than $[^{14}\text{C}]$-labelled compound would also be helpful to indicate if this elevated background is
due to endogenous carbon in the components of incubation mixture, or is an artefact of the LC separation or fractionation. The highly sensitive nature of the technique means that sources of background \(^{14}\text{C}\) carbon need to be tightly controlled. For future investigations, in addition to the control experiments mentioned, it would be interesting to reanalyse the sample and collecting smaller time interval fractions e.g. 10 seconds, to investigate whether this region could be chromatographically resolved into a few separate peaks. The peak at 15 minutes may also be better defined because, as shown in Figure 3.26, this peak is approximately 30 seconds wide, therefore with a 25 second fraction interval the peak is only defined by one data point whereas fraction collection over a smaller time interval e.g. 10 seconds would generate more data points across the peak. Since the data is being used qualitatively in this instance to help direct data processing, the impact of any small error in peak height or shape is minimal, however, this would be particularly important if the data were used to quantify the amount of covalent binding observed.

Based on the retention time data from the AMS radiochromatogram, the LC/MS data for the 1,2 and 4 hour incubations of acetaminophen with Supersomes™ were re-interrogated in the regions around retention time 15 minutes, and between 4 and 10 minutes, using the manual data processing procedure described previously, and compared with the no drug and no cofactor controls. Again, no modified peptides were detected.

Despite improving the specificity of the analyses by using SDS-PAGE and in-gel digestion to simplify the sample prior to LC/MS analysis, and using a highly sensitive radiodetection method like AMS to generate a radio chromatogram of the chromatographically separated sample to highlight the retention time of any adducted peptides, no acetaminophen modified peptides were identified successfully. This implies that, whilst making the experiment more specific to simplify the data processing procedure making the detection of peptide adducts more likely any adducts present will still not be identified, if the analytical LC/MS method chosen is not sensitive enough.

In an effort to improve the sensitivity of the analysis, complete digestion of the proteins in the incubation to amino acids was conducted.
3.5.5 Digestion with pronase to amino acids and LC/MS analysis

Amino acid analysis (306) has been successfully used to identify covalent adducts (307, 308). A recent paper by Mitrea et al. (137) described the development of pronase digestion methods for the investigation of reactive metabolites binding to proteins. In the paper, modified cysteine residues were successfully identified for four drug compounds fipexide, β-estradiol, diclofenac, and acetaminophen, which had been incubated with rat liver microsomes.

The amino acids from the pronase digests were derivatised using 6-aminquinolyl N-hydroxysuccinimidyl carbamate (309) as shown in Figure 3.27, to increase the hydrophobicity of the amino acids, improve the chromatographic retention, and increase the MS sensitivity compared to non-derivatised amino acids. The addition of the labile 6-aminquinolyl N-hydroxysuccinimidyl carbamate group results in a distinctive m/z 171 fragment ion upon CID fragmentation of each amino acid (Figure 3.28).
Figure 3.27: Structures of the 6-aminoquinolyl N-hydroxysuccinimidy carbamate derivatised amino acids
Figure 3.28: Loss of the 6-aminoquinolyl N-hydroxysuccinimidyl carbamate fragment ion from derivatised amino acids

This distinctive fragment is extremely useful for the development of sensitive MS methods. For example, a highly selective precursor ion scanning MS method was developed to identify the precursors of the m/z 171 fragment ion. The method identifies any unmodified or modified amino acids in the mixture while eliminating any background endogenous ions, thereby reducing the analytical background and increasing the signal to noise of any ions of interest. An alternative method is to use multiple reaction monitoring (MRM), an MS method which monitors the transition from a specific parent ion to a specific fragment ion e.g. m/z 171 during CID fragmentation. Again, MRM is highly specific and sensitive as the analytical background is reduced, however, where numerous components need to be detected in a mixture, e.g. for amino acid analysis, the precursor ion scanning method represents a simpler method as the same scanning mode can be used to monitor for all ions of interest with the common fragment. Full mass range scanning and product ion scanning still have their utility in this experiment as they can provide important structural information about the ion in question.

Since the primary MS method used to identify amino acid adducts of acetaminophen was precursor ion scanning, it was necessary to move the LC/MS analysis to a tandem triple quadrupole mass spectrometer capable of performing scanning MS experiments before and after the collision cell, such as the API Sciex 5500. This was coupled to a Waters Acquity UPLC system. The LC method developed was based on the amino acid LC analysis methods developed by Waters as part of their AccQ.Tag Ultra system (310, 311). The LC/MS conditions are described in Experimental Section 2.22.

As discussed in the introduction to this chapter, based on the structure of NAPQI and its proposed mechanism of binding, the most likely amino acid binding site would be cysteine. An acetaminophen-cysteine adduct was prepared using the silver oxide oxidation method described in Experimental Section 2.9. This acetaminophen-cysteine was then derivatised with
6-aminoquinolyl N-hydroxysuccinimidy carbamate for use as an analytical reference standard, together with a derivatised amino acid mixture purchased from Waters.

The analysis of the amino acid reference standard is shown in Figure 3.29 (generated on the UPLC-QToF Premier instrument using a MS³ method described in Experimental Section 2.23) and Figure 3.30 (generated on the UPLC-API Sciex instrument, using a precursor ion scanning method, looking for the precursors of m/z 171, the 6-aminoquinolyl N-hydroxysuccinimidy carbamate fragment common to all derivatised amino acids, described in Experimental Section 2.22).

**Figure 3.29:** Extracted ion chromatogram showing the common fragment ion m/z 171 (0.2 Th window) in the high energy MS³ function (data generated on the Acquity QTof Premier system described in Experimental Section 2.23) of the derivatised amino acid reference mixture.
Figure 3.30: Precursor ion scanning total ion chromatogram for the derivatised amino acid mixture standard, showing precursors of m/z 171 (data generated on the Acquity AB Sciex Triple Quad 5500 system described in Experimental Section 2.22) of the derivatised amino acid reference mixture.

Tryptophan, glutamine and asparagine were absent from the amino acid standard supplied and so do not appear in the mass chromatogram. The chromatographic resolution of the amino acids was good; although for the API Sciex analysis, the peak shapes of each of the early running polar amino acids (histidine, arginine, serine, glycine, aspartic acid, glutamic acid, shown in Figure 3.30) were poor. This is most likely due to the polar needle wash solvent (50:50 (v/v) isopropranol: acetonitrile) affecting the solvent composition of the samples, resulting in peak broadening for those amino acids which elute from the column at low organic solvent composition. Where a less polar wash solvent was used (e.g. 50:50 (v/v) water: acetonitrile, on the QTof Premier Acquity system, shown in Figure 3.29) the early eluting amino acids were not affected. The amino acid peaks were slightly wider in the UPLC-API Sciex analysis due to a larger distance between the column and the MS source, resulting in a larger system dead-volume. All the amino acids were resolved with the exception of arginine and serine, and tyrosine and lysine for the data generated on the API Sciex system. Most importantly, a well resolved peak was observed for derivatised cysteine at a retention time of approximately 10 minutes. This peak corresponds to a precursor ion m/z 581, a dimer of derivatised cysteine. This formation of a dimer is expected since free thiol moieties are highly
reactive. In both chromatograms, a large peak representing dimerised derivatising agent was also detected.

The structure of the derivatised cysteine dimer and the dimerised derivatising agent are shown in Table 3.4. Exact mass and product ion spectra were acquired to confirm the postulated structure using the method described in Experimental Section 2.23. The supporting data are shown in Appendix 2, A2.8 – A2.10.

**Table 3.4: Summary of MS data for the derivatised cysteine dimer and the dimerised derivatising agent**

<table>
<thead>
<tr>
<th>[M+H]+ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315.1231</td>
<td><img src="image1" alt="Proposed Structure" /></td>
<td>m/z 171: <img src="image2" alt="Diagnostic Fragment" /> m/z 145: <img src="image3" alt="Diagnostic Fragment" /></td>
</tr>
</tbody>
</table>
|              | **Empirical Formula:** C_{36}H_{37}N_{14}O_{10}^{+}  
**Theoretical m/z:** 315.1240  
**Difference:** 2.9 ppm | |
| 581.1295     | ![Proposed Structure](image4) | m/z 411: ![Diagnostic Fragment](image5) m/z 290: ![Diagnostic Fragment](image6) m/z 241: ![Diagnostic Fragment](image7) m/z 171: ![Diagnostic Fragment](image8) m/z 145: ![Diagnostic Fragment](image9) |
|              | **Empirical Formula:** C_{36}H_{37}N_{14}O_{10}^{+}  
**Theoretical m/z:** 581.1272  
**Difference:** 3.96 ppm | |
The chemically-generated acetaminophen-cysteine adduct was analysed by LC/MS using a precursor ion scanning method, looking for the precursors of m/z 171, as described in Experimental Section 2.22.

Figure 3.31: Total ion chromatogram for the LC/MS analysis of a chemically generated acetaminophen-cysteine adduct, using a precursor ion scanning MS method (precursors of m/z 171)

The precursor ion scan total ion chromatogram (showing precursors of m/z 171) is shown in Figure 3.31. An acetaminophen-cysteine adduct was detected at a retention time of 8.7 minutes. The assignment of this peak was based on the extracted spectrum under the peak at 8.7 minutes, showing an ion at m/z 441 (data not shown). The same sample was then analysed using the LC/MS method described in Experimental Section 2.23 to generate some structure information to confirm the assignment. These data are summarised in Table 3.5, with the spectra shown in Figure 3.32.

CYP2E1 Supersome™ and human liver microsome incubations with acetaminophen were prepared using the method described in Experimental Section 2.2. These were digested to their constituent amino acids with pronase, using the method described in Experimental Section 2.20, derivatised using the method described in Experimental Section 2.21, and analysed using the LC/MS method described in Experimental Section 2.22, using a precursor ion scanning mode monitoring the precursors of the common fragment ion m/z 171. The
chemically generated acetaminophen-cysteine adduct was used as a reference standard to compare retention time of any cysteine adduct present.

Table 3.5: Summary of MS data generated on the chemically generated acetaminophen-cysteine adduct

<table>
<thead>
<tr>
<th>[M+H]^+ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>441.1226</td>
<td><img src="image" alt="Proposed Structure" /></td>
<td><img src="image" alt="Diagnostic Fragment Ions" /></td>
</tr>
</tbody>
</table>

Empirical Formula: C_{21}H_{28}N_{2}O_{3}S

Theoretical m/z: 441.1227

Difference: 0.2 ppm
Figure 3.32: MS$^E$ mass spectra for the chemically generated acetaminophen-cysteine adduct at (A) low collision energy, and (B) high collision energy.
Figure 3.33: The extracted ion chromatograms for ion m/z 441, from the precursor ion scanning LC/MS analysis of the pronase digestion of the protein pellets retained from the incubation of human liver microsomes; (A) shows the no drug control, and (B) the 4 hour incubation with acetaminophen.
While no adducts were noted in the CYP2E1 incubation, a small peak was noted in the human liver microsomes incubation at retention time 8.9 minutes (Figure 3.33). Within variation of the chromatography system (which can be caused by column use and replenishment of solvents) this is in good agreement with the reference standard of an acetaminophen-cysteine adduct.

The extracted ion chromatograms for ion m/z 441, from the precursor ion scanning MS data for the pronase digested 4 hour incubation of human liver microsomes with acetaminophen, and the equivalent no drug control sample are shown in Figure 3.33. While a small peak is visible at the correct retention time in the 4 hour sample, a similar area was noted in the no drug control. The small size of the peak makes it difficult to reliably judge the presence of the acetaminophen-cysteine adduct in the sample. Therefore, a more sensitive and selective MRM method was developed to monitor the transition between the acetaminophen-cysteine adduct (m/z 441) and its fragment ion (m/z 271). The details of the MRM mode LC/MS analysis are shown in Experimental Section 2.24.

As with the precursor ion scanning method, no acetaminophen-cysteine adducts were present in the CYP2E1 Supersome™ incubation. However, a peak was detected at retention time 8.9 minutes in the human liver microsome incubation, which corresponds to the peak identified in the precursor ion scanning data and the reference standard. These data are shown in Figure 3.34.
Figure 3.34: Extracted MS data showing the MRM transition m/z 441 → 271 from the LC/MS analysis of the pronase digestion of the protein pellet following incubation of human liver microsomes; (A) no drug control, and, (B) the 4 hour incubation with acetaminophen

The lack of a detectable peak in the CYP2E1 Supersome™ incubation, despite the presence of a peak in the human liver microsome incubation is not surprising considering the difference in
the quantitative covalent binding between the two incubations (2-3 fold lower, see Tables 3.1 and 3.2). The amino acid method detects all of the acetaminophen-cysteine adducts in the sample as a single peak, irrespective of the protein from which the adduct is derived, therefore it is reasonable to suppose that where the total amount of binding is higher, the detection of a single amino acid adducts should be easier.

This data reinforces the hypothesis that the key to the successful detection of covalent adducts is the sensitivity of the method utilised. For example, the sensitivity of full scan MS analysis on the QTof Premier instrument was not sufficient to detect acetaminophen–adducts in the sample against a biologically-derived background, with the exception of the significantly more abundant chemically derived adduct. The more sensitive and selective precursor ion scanning method (precursors of the common fragment ion m/z 171), was successful in identifying an acetaminophen-cysteine adduct in the human liver microsome incubations, however, this peak was very small and, on its own, the precursor ion scanning data was not sufficient to confirm the presence of the adduct in the sample. In contrast, the MRM method, developed to detect ions the m/z 171 fragment ion of precursor m/z 441 identified a peak of significant size thus increasing the confidence in the analysis.

The second point to note from the amino acid analysis is the importance of reference standards, if available. In this case, the ability to generate a sample of the acetaminophen-cysteine adduct of high concentration was invaluable, not only to confirm the assignment of the peaks detected in the precursor ion scanning and MRM analyses by retention time comparison, but also to provide some confirmatory structural information by analysis in full scan mode. The major disadvantage of the precursor and MRM scanning modes is that, whilst the sensitivity of the analysis is significantly increased, the ability to derive structural information is diminished. For an MRM method to be developed, the identity of the precursor and fragment ions related to the component of interest must be known or assumed beforehand. For precursor ion scanning, the information derived from the analysis is not sufficient to derive a significant amount of structural information, beyond molecular mass. The mass resolution of the triple quadrupole instrument used here is not sufficient to generate accurate fractional mass MS data; therefore, it cannot be used to derive the empirical formula of the precursor of interest.

One improvement to the experiment could be to use a quadrupole-time-of-flight instrument in precursor ion scanning mode. While true precursor ion scanning cannot be achieved, all fragment ions may be detected in the time-of-flight (ToF) spectrometer whilst the quadrupole
is scanning, therefore, data processing techniques can be used to correlate fragments with the precursor ions appropriately (312). The increased mass resolution of the Tof spectrometer can be used to more accurately discriminate between the fragment ion of interest and background ions, thereby increasing the selectivity of the analyses and reducing false positives. Traditionally, the increased duty cycle of the Tof spectrometer has meant that the sensitivity of the analysis was an order of magnitude lower than the equivalent analysis in a triple quadrupole MS (313), however, this has been addressed in modern QTof mass spectrometers (312).

In terms of future applications of amino acid analysis for detecting acetaminophen-amino acid adducts, the methods described in this section could be tailored to investigate the binding of NAPQI to other amino acids such as lysine and histidine (314).

Due to the success of the MRM MS analysis with amino acids, this was applied to the reanalysis of samples containing tryptic peptides from CYP2E1 Supersome™ and human liver microsome incubations, originally analysed in full scan mode using the QTof Premier MS instrument (see Section 3.4.2) were reanalysed using similar methodology in an effort to identify any peptide adducts.

3.5.6 Digestion to peptides and LC/MS analysis using MRM

To aid the location and identification of any acetaminophen-peptide adducts, chemical formation of NAPQI using silver oxide oxidation and incubation with CYP2E1 Supersomes™ was utilised to produce a sample with a high concentration of a CYP2E1 acetaminophen-peptide adduct which could be used as reference standard. The experimental procedure is detailed in Experimental Section 2.11.

MRM methods were developed based on the in silico digestion of cytochrome P450 CYP2E1, with trypsin, with two missed cleavage sites allowed, and taking into account the mass change associated with carbamidomethylation (from the reduction and alkylation procedure during digestion). This in silico analysis was performed using BioLynx software from Waters (Manchester, UK) and was based on the sequence described in the Uniprot database, as described previously. Based on the modification of cysteines by acetaminophen, as shown with the amino acid analysis data described above, all cysteine containing peptides were selected and the mass modified by the addition of acetaminophen, to predict the resultant m/z value of a modified peptide. The +2, +3, +4 and +5 charge states of each peptide was considered up to m/z 2000. It was impossible to perform CID fragmentation of each postulated peptide in order
to optimise each MRM fragment ion transition, therefore, the expected \( y_1 \) fragment ion of each peptide was selected as this ion was considered likely to be present and of reasonable intensity. The m/z of the \( y_1 \) ion was predicted for each of the postulated peptides based on their structure and since trypsin was used as the protease of digestion, the fragment ion was either m/z 175 for arginine terminal peptides, or m/z 147 for lysine terminal peptides, with the exception of the terminal peptide of the protein.

![Figure 3.35: \( y_1 \) ions of lysine and arginine terminal peptides](image)

One of the advantages of using the \( y_1 \) ion, rather than other in silico predicted fragment ions for each postulated peptide was the simplicity in selecting only a limited number of fragment ions for all of the MRM transitions (\( y_1 \) was either m/z 175 for arginine terminal peptides, m/z 147 for lysine terminal peptides (as shown in Figure 3.35) or m/z 106 for the serine terminal peptide from the protein terminus), thereby greatly improving the simplicity of method development. Due to the duty cycle of the experiment, the MRM transitions were divided into batches of approximately 15 experiments per analysis, therefore, each sample was analysed several times in order to accommodate all of the MRM transitions. The MRM transitions used in these MS methods are detailed in Experiment Section 2.25.

The analysis of the reference incubation containing an excess of chemically generated NAPQI incubated with CYP2E1 Supersomes™ in the absence of NADPH cofactor, compared against a no drug control, revealed the presence of two acetaminophen-adducted peptides in the sample. These peptides corresponded to MRM transitions m/z 425 \( \rightarrow \) m/z 175, at a retention time of approximately 15 minutes, and m/z 667 \( \rightarrow \) m/z 175, at a retention time of approximately 24 minutes. The transition for the first modified peptide corresponds to the acetaminophen modified doubly charged T56 peptide (LCVIPR), and the second, the acetaminophen modified triply charged T54 peptide (DIDLSPIHIGFGCIPPR). These MS data are shown in Figures 3.36 and 3.37.
The sample was also analysed by LC/MS using full scan and product ion scanning methods on the QTof Premier MS, to gain some structural information on the modified peptides observed in the MRM analysis. These data are shown in Figure 3.38.
Figure 3.36: Extracted MS data showing the MRM transition m/z 425 → 175 for (A) the CPY2E1 Supersome™ no drug control incubation, and (B) CYP2E1 Supersome™ incubated with an excess of chemically generated NAQPI (no co factor)
Figure 3.37: Extracted MS data showing the MRM transition m/z 667 → 175 for (A) the CYP2E1 Supersome™ no drug control incubation, and (B) CYP2E1 Supersome™ incubated with an excess of chemically generated NAQPI (no cofactor)
Figure 3.38: Extracted ion chromatograms showing (A) the unmodified T56 peptide ([M+2H]2+ m/z 350.8, 0.3 Th window) and, (B) the acetaminophen-modified T56* peptide ([M+2H]2+ m/z 425.3, 0.3 Th window)

No peak was detected for the modified T54 peptide (m/z 667, [M+3H]3+), however, the modified T56 peptide (m/z 425, [M+2H]2+) was detected, as shown in Figure 3.38. Interestingly,
a significant proportion of the T56 peptide remains unmodified despite an excess of the chemically generated NAPQI. This is consistent with the result observed in the glutathione trapping experiment with NAPQI, and suggests that either the binding between NAPQI and peptides is slow and inefficient or that a significant proportion of NAPQI is converted back to non-reactive acetaminophen under the aqueous conditions used in the incubation.

The product ion spectrum for the CID fragmentation of the acetaminophen modified T56 peptide is shown in Figure 3.39. The presence of unmodified y1 to y4 ions and an acetaminophen modified y5 ion confirms that acetaminophen is bound to the cysteine moiety in the peptide. This is reinforced by the presence of a modified b2 ion (m/z 324), which represents the addition of acetaminophen with the subsequent loss of an acetyl group from the acetaminophen moiety itself. The proposed structure of the modified T56 peptide is shown in Figure 3.40.

![Proposed structure of the acetaminophen-modified T56 peptide (LC*VIPR)](image)

**Figure 3.40: Proposed structure of the acetaminophen-modified T56 peptide (LC*VIPR)**

CYP2E1 Supersome™ and human liver microsome incubations with acetaminophen in the presence of an NADPH cofactor were prepared as detailed in Experimental Section 2.2. The protein pellets from these incubations were digested with trypsin, as described in Experimental Section 2.14, and then analysed using LC/MS (MRM mode) as described in Experimental Section 2.25. No drug and no cofactor controls were also analysed for comparative purposes.
The MRM MS data generated for the CYP2E1 and human liver microsome incubation samples were compared manually with the data for the equivalent no cofactor and no drug controls to identify any peaks present in the samples which were not also in the controls. Any peaks identified uniquely in the samples were identified as potential acetaminophen-peptide adducts. In addition, these data were compared to the MRM data generated for the CYP2E1 incubation with chemically-generated NAPQI.

The modified T56 peptide was noted in the CYP2E1 Supersome™ and the human liver microsome incubations at all time-points, using the sensitive MRM method. These data are shown in Figures 3.41 and 3.42, respectively. While the quantitative covalent binding value for the human liver microsome incubation was 2-3 fold higher than observed for the CYP2E1 Supersome™ incubation (see Tables 3.1 and 3.2), the peak observed for the modified T56 peptide in the human liver microsome incubation (Figure 3.42) was significantly smaller than the modified T56 peptide peak observed in the CYP2E1 Supersome™ incubation (Figure 3.41). This is most likely to be due to the fact, while the quantitative covalent binding values have been corrected for total protein content, there are significantly more protein types present in human liver microsomal incubations, than in the Supersome™ incubations (which contain three main proteins). It is likely that several proteins are modified in addition to cytochrome CYP2E1 in the human microsome incubation, resulting in a comparatively smaller peak observed for CYP2E1.
Figure 3.41: Extracted MS data showing the MRM transition m/z 425 → 175 for (A) the CYP2E1 Supersome™ no drug control incubation, and (B) CYP2E1 Supersome™ incubated with acetaminophen
Figure 3.42: Extracted MS data showing the MRM transition m/z 425 → 175 for (A) the human liver microsomes no drug control incubation, and (B) human liver microsomes incubated with acetaminophen

Peaks were detected in the MRM transition m/z 425 → 175 at a retention time of 15 minutes, which strongly suggests that they correspond to the acetaminophen-modified T56 (equivalent retention time to the chemically generated peptide). In both cases, the peaks are absent from
the respective no drug control incubations shown, suggesting that they are related to the addition of acetaminophen to the incubation. The low intensity of these peaks may explain why these peaks were not detected using the less sensitive full scan LC/MS method on the QTof Premier. Unfortunately, this also meant that it was not possible to generate any further data to confirm the structure, however, the retention time was consistent with the peak observed in the AMS radiochromatogram for CYP2E1, further reinforcing that it represents a real acetaminophen-modified peptide.

No additional peaks were detected in the MRM analyses of both the CYP2E1 and human liver microsome incubations with acetaminophen; however, to the author’s knowledge these data represent the first positive identification of any modification of the CYP2E1 enzyme after the in vitro incubation with acetaminophen. These data reinforce the importance of using highly sensitive methods for the detection of low level covalent adducts, as the adduct present in the sample could only be identified using a specific MRM method on a sensitive instrument and were not observed using full scan methodology. As discussed previously for the amino acid analysis, the real disadvantage of this is that these methods do not provide any structural data on the adducts detected, and the methods used to detect them can only be developed based on certain assumptions about the adduct and its formation. For example, the MRM methods developed as part of this investigation were based on the assumption that acetaminophen only modifies proteins through binding to cysteine residues exclusively; however, the structure of the reactive metabolite NAPQI means that protein modification by acetaminophen could potentially occur on both lysine and histidine residues, in addition to cysteine. Holtzmann (314) postulated the modification of lysine residues in microsomal proteins, therefore, in future work, it would be prudent to extend the development of MRM methods to include lysine and histidine modification, however, this poses problems in terms of the time needed for sample analysis. Five separate analyses were necessary to accommodate all of the MRM transitions to include all of the potential cysteine modifications of CYP2E1. However, the number of analyses would significantly increase in order to include lysine and histidine modifications in this list. In addition, since Supersomes™ also contain cytochrome P450 reductase and cytochrome b₅ proteins, these should also be included for completeness as it is conceivable that modification could also occur on these proteins. If all of the known proteins in human liver microsomes were included as potential target proteins, the number of MRM analyses required would increase exponentially. With this in mind, the development of a full scan LC/MS method with a similar degree of sensitivity would enable the untargeted detection
of any protein modification, regardless of target protein or amino acid modification, in one analysis.

Recent improvements in the sensitivity of MS instrumentation have potentially made this possible. For example the Synapt G2S from Waters (Manchester, UK) (315), employs StepWave™ ion optics to improve the efficiency of the transfer of ions into the mass spectrometer, resulting in up to a 30-fold increase in signal intensity, with greatly improved mass resolution (over 40,000 FMHM) and mass accuracy (<1 ppm RMS) for specificity, compared to older instruments such as the QTof Premier.

3.5.7 Digestion to peptides and LC/MS analysis using MS⁵ on the Synapt G2S

An LC/MS method using an Acquity UPLC coupled to a Waters Synapt G2S was devised incorporating low and high collision energy full scan MS⁵ functions, the details of which are recorded in Experimental Section 2.26. The experimental procedure for the incubation of CYP2E1 Supersomes™ and human liver microsomes with acetaminophen, and the subsequent digestion of proteins with trypsin, is detailed in Experimental Sections 2.2 and 2.5, respectively. 0, 1, 2 and 4 hour incubations were conducted, together with no drug and no cofactor controls for comparison. Data were processed using BioPharmaLynx using the protein sequences of human cytochrome P450 2E1 (193), NADPH cytochrome P450 reductase (298) and cytochrome b5 (299), and trypsin as the digest reagent with 2 missed cleavages allowed and leucine enkephalin (m/z 556.2771) was used for lock mass correction. The mass tolerance was 5 ppm for the MS match and 10 ppm for the MS⁵ fragment ion match due to the improved mass resolution and accuracy of the instrument, with carbamidomethylation on cysteine residues (+57 Da) and the addition of acetaminophen to cysteine and lysine residues (+149.05 Da) selected as variable modifications, with no maximum number of modifications given. In addition, a manual comparison of the sample data, with the no drug and no cofactor control data was carried out.

For all of the samples analysed, the protein coverage for all three proteins was in excess of 80%. An example is given in Figure 3.43 for the 2 hour incubation of acetaminophen with CYP2E1 Supersomes™. Two regions of the protein were not detected in the LC/MS analysis; amino acids 1-51 and 199-233. For the region 1-51, the trypsin cleavage at the arginine residue (residue 24) may have been missed in the analysis; therefore the resulting peptide would be 51 residues long. The same is true for the region 199-233. Here, no arginine or lysine residues are present, again resulting in a large peptide (35 amino acids long). While electrospray is capable of ionising these large peptides, it is feasible that ionisation would occur on a large number of
sites on the peptide, resulting in the signal being distributed over several charge states, thereby reducing the MS sensitivity. Neither region contains cysteine residues so the absence of these peptides from the analysis is unlikely to prevent the identification of any cysteine-modified peptides. In future work, it would be prudent to alter the digestion procedure to include an alternative protease such as chymotrypsin, to allow for the possibility of binding to lysine and histidine residues.

**Figure 3.43:** Representative protein coverage for CYP2E1 following analysis of the 2 hour incubation of acetaminophen with CYP2E1 Supersomes™. Peptides observed in both the control and analyte samples are shown in green, with peptides observed in the control samples shown in blue and peptides unique to the analyte sample shown in orange.

In all samples analysed, several acetaminophen-modified peptides were identified by BioPharmaLynx. A summary of the data is shown in Table 3.6.

In all cases, the differences in the theoretical m/z values of the postulated acetaminophen-modified peptides compared to those observed were very small <3.5 ppm, implying a high likelihood that the assignment of the acetaminophen-modified peptides are correct. Despite the improved sensitivity of the Waters Synapt G2S instrument compared to the QTof Premier the peaks were still too small for confirmatory MS/MS experiments to be carried out successfully, therefore the assignments in Table 3.6 are based solely on molecular weight and empirical formulas.
Table 3.6: Summary of modified peptides observed in the CYP2E1 Supersome™ incubations with acetaminophen

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Sequence</th>
<th>Retention time</th>
<th>m/z</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 reductase</td>
<td>T58</td>
<td>EVGETLLYYGC*R</td>
<td>11.7 min</td>
<td>517.9269 [M+3H]⁺</td>
<td>2.0 ppm</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>T22</td>
<td>VC*AGEGLAR</td>
<td>14.8 min</td>
<td>512.7728 [M+2H]²⁺</td>
<td>1.8 ppm</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>T33</td>
<td>DLTDC*LLVEMEK</td>
<td>21.5 min</td>
<td>519.9296 [M+3H]³⁺</td>
<td>1.5 ppm</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>T56</td>
<td>LC*VIPR</td>
<td>15.3 min</td>
<td>425.2632 [M+2H]³⁺</td>
<td>0.6 ppm</td>
</tr>
</tbody>
</table>

A significant improvement in signal-to-noise would be required to interrogate the data sufficiently to have confidence in the definitive assignment of peptide modification. Future work that could be done to achieve this could be focused in two areas; sample enrichment and background reduction.

One way to achieve a reduction in the endogenous background might be to use ion mobility as an additional post-chromatographic dimension of separation, prior to MS detection. As discussed in the introduction to this thesis, ion mobility separation acts to separate ions based on their collision cross-sectional area. The collision cross-sectional area is dependent on the size, shape and charge of an ion, therefore ion mobility can be particularly useful for the separation of proteins and peptides as there can be a great deal of variation in conformation due to size and charge state (316, 317).

For protein digests, ion mobility separation can be used to distinguish and separate ions of different charge states. Typically, when the drift time of ions is plotted against their m/z the ions separate into bands across the ion mobility chromatogram (or mobilogram). Since the mobility of an ion is dependent on its charge state (as well as other factors such as size, shape and mass) peptides have been observed to formed diagonal bands within the mobilogram according to charge state. It should therefore be possible to separate the multiply-charge peptides formed during the digestion of a protein from the singly-charged background ions. By applying this ion mobility separation to the MS data generated, it should be possible to remove
any singly-charged ions from the data completely, thereby significantly reducing spectral complexity and theoretically improving the limits of detection (318, 319).

Ion mobility separation was utilised during the LC/MS analyses described above using the Synapt G2S instrument, with the mobilogram used to select only the MS data in the mobilogram bands corresponding to the multiply-charge ions for further interrogation; however, no additional peptides were identified (Figure 3.44).

Figure 3.44: Mobilogram plotting the m/z against their drift time of ions from the Synapt G2S analysis of the 1 hour incubation of CYP2E1 Supersome™ with acetaminophen. Singly-charged ions are shown in the region highlighted in white, double-charged ions in yellow and the triply-charged and higher ions highlighted in green.

Affinity chromatography may achieve enrichment of a sample and a reduction in endogenous background ions. It is based on the highly specific interaction between two moieties, for example, between an antigen and an antibody, or an enzyme and a substrate (320-322). It is often used as a method for the purification of recombinant proteins (323) using affinity tags such as glutathione-S-transferase (GST) (324) and histidine (325). Dennehy et al. (326) used biotin-tagged thiol-reactive electrophiles, coupled with biotin-avidin chromatography to identify the peptide adducts formed drug substrates and cytosolic and nuclear proteins. The advantage of using affinity chromatography is that this allows the enrichment of several injections of sample on a column, with complete removal of all un-tagged background ions, thus resulting in an increase in sensitivity. GST- and His-tagged cytochrome P450 enzymes are
available for some isoforms (327), however, tagging the P450 enzyme can only allow removal of non-protein related background ions, since both unmodified and modified proteins or peptides would be retained. Tagging of the drug substrate might be more successful, as affinity chromatography may separate and remove non-adducted proteins or peptides from the sample, whilst retaining any drug-related material including modified peptides or proteins. An example of this was reported by Lui et al. (328) for the identification of microsomal protein adducts of oxidised metabolites of biotinylated-raloxifene. The impact of the tag on the metabolism on the drug of interest should be evaluated. Lui et al. (329) do not report any disruption of the metabolic activity of raloxifene, due to the presence of biotin, however, in the case of raloxifene, its site of metabolic modification is remote from the biotin tag. In summary, affinity chromatography certainly warrants investigation as a method to improve sample detection.

3.6 Conclusions and future work

To the best of the author’s knowledge, the data reported here represent the first positive identification of any modification of the CYP2E1 enzyme after in vitro incubation with acetaminophen. Acetaminophen adducts of other microsomal proteins have been documented in the literature, as discussed in the Introduction (210). Whilst high levels of covalent binding have been reported for acetaminophen, there is no evidence in the literature of any metabolism-dependent inhibition or induction of the CYP2E1 enzyme by acetaminophen, suggesting that any binding that occurs to the metabolising enzyme does not inhibit the enzyme itself. A recent paper by Harrelson et al. (281) demonstrated the metabolism of a structural analogue of acetaminophen, N-acetyl-m-aminophenol (AMAP) to a reactive species which binds to the CYP2E1 protein, resulting in inhibition of the enzyme itself. Whilst they had been unable to identify the specific site of binding, the authors postulated the binding of metabolites of AMAP to cysteine residues and importantly to a cysteine residue adjacent to a substrate recognition site of the CYP2E1 protein, as defined by Gotoh (330), thus leading to the enzyme inhibition observed.

One location of binding for NAPQI as defined in this chapter is the cysteine residue of the T56 peptide (residue 488 of 493). The location of this cysteine is far from the substrate active site (residues 298-303, see Figure 3.45, shown in green) which would imply that binding at this residue should have a minimal impact on enzyme activity; however it is located within a substrate recognition site. Gotoh reports that these regions are responsible for the specificity of the individual cytochrome P450 enzymes, which generally have largely similar amino acid
sequences, therefore, it would be reasonable to assume that binding to amino acids within these regions would have some effect on the enzyme specificity. An important experiment to perform in the future would be to conduct enzyme inhibition studies (281) under identical incubation conditions used in this chapter to determine the effect of the covalently adducted acetaminophen on the in vitro enzyme activity.

MSALGVTLV LWAAFLLLV SMWRQVHSSW NLPPGPFFPLPII GN LFQEL KNIPKFTRL AQ RF GPVF TLPAPAFHAH RDGIIFNNGP TW KD IRR FS L TT LR NY GMKQGNESRIQRE AH F LLE ALRK T QGQPF DPTF LIGC A PC VI ADILFRKHFD YN DEK FRLLM
YLFNENF HLL SPW LQLYNN FPS FLHYLP G S RH K VKNVA EV KEY V SERV KEH HO SLP DN CP RL D LDC LLL VE ME KE K HS A E RL YTMD GIT VTV ADLFF AG TET TSSLR Y GL LIM KY P E IEE KL H EED RVIGPSRI P A IKD RQEM P YM DAVVHEIQRF ITLV PSNL PH EATR D TFGR Y LI PK GTVVV PTLD SV LDYN QEF PDPEKF PEHFL NE NGK FKYS DY FKPF STGK RV C AGE GLAR M E LF LL LC AILQHF NLKPLVD PKD ID LSPI HI FG C
IPPRY KLC V I PR S

**FAG T E T:** substrate binding site

**C:** Heme binding site

**C:** Cysteine

**...PRS:** substrate recognition site

**Figure 3.45: The amino acid sequence of CYP2E1, with cysteine residues highlighted. The substrate binding site is shown in green, with the heme binding site in purple (193, 330).**

The data from throughout this chapter highlights the limitations of current instrumentation in identifying the covalent adducts of reactive metabolites to protein targets and the difficulty in identifying and characterising any modifications observed. Whilst an acetaminophen protein adduct has been identified at the cysteine residue of the T56 peptide, this was only achieved through the utilisation of chemically-produced reference standards and using targeted mass spectrometric methods such as precursor ion scanning and multiple reaction monitoring, which rely on assumed knowledge of the mechanisms of binding derived from the glutathione trapping data i.e. that binding will occur on cysteine residues.. There are several disadvantages to this; firstly, as mentioned previously, these targeted mass spectrometric methods, whilst extremely sensitive, do not provide any useful structural information on the adduct with the exception of molecular weight. Secondly, since the development of these methods are based on assumptions being made on the mechanisms of binding, the analysis is biased to prove the expected mechanism, and is unlikely to identify any unexpected or novel adducts.
However, several of the techniques described in this chapter have future utility in identifying covalent adducts of reactive metabolites for other drug moieties. The use of metabolism studies and trapping techniques such as glutathione trapping can be very useful in postulating the potential mechanism of binding to proteins, and to develop selective and sensitive mass spectrometric methods to maximise the chances of identifying protein adducts. The use of accelerator mass spectrometry to produce a radiochromatogram showing the chromatographic separation of modified peptides can be very useful to provide retention time information thereby simplifying and refining MS data searching. Chemical production of reactive metabolites to react with target proteins of interest can be effectively used as reference standards, again to help refine data searching and to provide additional structural information such as peptide sequencing by CID fragmentation or identify the target amino acid. Finally, the use of new instrumentation such as the Synapt G2S instrument, which combines high mass resolution and sensitivity, and the option to use ion mobility as an additional dimension of separation, provides the best chance of generating structural information on any adducts identified.

Some of these techniques and methods will be further investigated in the next chapter to evaluate the binding of metabolites of an orexin antagonist, previously under development within GSK.
Detection of covalent protein-adducts of SB-649868, an Orexin 1 and 2 receptor antagonist

SB-649868 (N-[[2S]-1-[[5-(4-fluorophenyl)-2-methyl-4-thiazolyl]carbonyl]-2-piperidinyl][methyl]-4-benzofurancarboxamide), depicted in Figure 4.1, is a novel Orexin 1 and Orexin 2 (OX1/OX2) receptor antagonist which was previously in development within GlaxoSmithKline (GSK) for the treatment of insomnia.

![Structure of SB-649868](image)

**Figure 4.1: The structure of SB-649868**

Evidence within the literature suggests that orexin has a key role in the regulation of arousal and sleep/wake behaviour. Studies in rats have shown increased time spent awake and decreased REM and deep sleep (331), increased locomotor activity, and rearing and grooming behaviour (332) after intracerebroventricular injection of the orexin-A peptide. Studies using knock-out (KO) mice (333) have shown that, when devoid of the orexin precursor prepro-orexin, the KO mice exhibit behaviour similar to that of narcolepsy. In humans, Mignot *et al.* (334) observed that human narcolepsy is associated with a reduction in the levels of orexin in the cerebrospinal fluid, suggesting that antagonism of the orexin receptors in humans could reduce arousal and increase sleep. SB-649868 increased total sleep time and reduced sleep latency in a rat *in vivo* 18 hour sleep model and displayed similar activity in an *in vivo* sleep model in marmosets (335, 336).

During the development of this compound within GSK, pharmacokinetic, distribution, metabolism and elimination studies were performed *in vivo* in rats and dogs, the two species selected for the toxicological evaluation of the safety of SB-649868, after single and repeat oral dosing. In addition, *in vitro* studies in hepatocytes and isolated cytochrome P450 enzymes were performed to assess the enzymes involved in the metabolism of SB-649868 and to investigate the potential for SB-649868 to inhibit these enzymes. Metabolism and distribution data were also generated in humans using a [14C]-labelled analogue of SB-649868. The data
summarised here were collected by numerous scientists at GSK during the course of the compound’s development.

The mean oral bioavailability of SB-649868 was high in the rat (approximately 85%) and moderate in the dog (approximately 35%). Elimination of the dosed compound in rats was largely via metabolism, with metabolites being excreted primarily into the bile and/or faeces. In dogs, SB-649868 was eliminated largely unchanged via the faeces. The elimination half-life is short ($t_{1/2} < 1$ hour) in both rat and dog. Systemic exposure of SB-649868 generally increases with increasing dose in an approximately dose-proportional fashion.

**Figure 4.2**: QWBA data from rats dosed with [$^{14}$C] SB-649868 via IV (1 mg/kg) and oral (10 mg/kg) administration. The time-points displayed show the elimination of the radio labelled drug from the tissues over 35 days

The distribution of SB-649868 in rats was studied using quantitative whole body autoradiography (QWBA) at various time-points after oral and IV administration of [$^{14}$C]SB-649868 (337). Following either route of administration, radioactivity was widely distributed throughout most tissues at the first time point (15 minutes post dose for intravenous administration and 2 hours post dose for oral administration). Concentrations of radioactivity
steadily declined but were still quantifiable in the majority of tissues at 10 days post dose, and by 35 days post dose, radioactivity was still quantifiable in almost half of the tissues analysed (the highest concentrations being present in the pigmented fur and uveal tract (not shown in sections displayed in Figure 4.2)). As discussed in the Section 2.4.6, prolonged retention of drug-related material in tissues can be an indicator of covalent binding and thus was a flag for further investigation.

The metabolism and elimination of SB-649868 was investigated in vivo in rats (338) and dogs (339) (Figure 4.3). Metabolism occurs predominantly on the benzofuran moiety, with oxidation, ring-opening hydrolysis and oxidation to a carboxylic acid, and oxidation to a dihydrodiol together with oxidation of the methyl thiazole. Combinations of oxidation and glucuronide or glutathione conjugation on the benzofuran moiety were also noted. The presence of the dihydrodiol metabolite on the benzofuran ring is an indicator of the formation of a reactive epoxide metabolite (340). The formation of epoxides is most likely to be cytochrome P450 mediated (18, 340-344) with epoxidation a common metabolic route observed for unsaturated furan rings (341, 345). The formation of epoxide metabolites of furans has been linked to liver cell necrosis (63, 345-349), most likely caused by covalent binding of these reactive metabolites to endogenous proteins or DNA within the cell.
Figure 4.3: Summary of the metabolism of SB-649868 observed in vivo in rats and dog (338, 339)
Figure 4.4: The potential metabolic fate of epoxides within the body (adapted from (63, 350))

As shown in Figure 4.4, the reactive epoxide metabolite can either be detoxified via further reaction to hydroxy or dihydrodiol metabolites, or conjugation with glutathione. If not detoxified, the epoxide may react directly with proteins within the body. This reaction can either proceed via direct conjugation to amino acid residues such as cysteine or via the formation of an electrophilic reactive ring-opened aldehyde intermediate (Figure 4.5).

Aldehydes are extremely unstable in vivo, so it is likely that if the reaction proceeds via an aldehyde intermediate, electron rearrangement occurs to close the ring, resulting in the same protein adduct as seen for direct conjugation.
The extractability of drug-related material from plasma and liver tissue was also poor (at best, less than 55% of sample radioactivity was extracted in the rat) which, as discussed in the Introduction, can be indicator of covalent binding of reactive metabolites to proteins. The presence of a dihydrodiol metabolite and a glutathione conjugate (Figure 4.3) in both the rat and dog reinforce that covalent binding may be a factor in the poor extractability of drug related material from tissue. However, SB-649848 was well tolerated by rats and dogs after single and repeated dosing with low and moderate doses of SB-649868, with side-effects such as weight gain and emesis only observed at very high doses. Histopathological examination of the livers from rats and dogs revealed minimal to moderate centrilobular hypertrophy in the rat, which corresponded to an increase in liver weight, and can be a sign of enzyme induction. In dogs, minimal to mild hepatocyte vaculoation was observed at all doses; however this was attributed to fat accumulation and was not considered to be associated with cell injury. Both the rat and dog findings were not considered to be a risk to human subjects.

Based on these data, an investigation into the metabolic activation of SB-649868 was conducted within GSK using in vitro microsomal systems from human, rat, dog, mouse and cynomolgus monkey, and \[^{14}C\] radiolabelled drug substance. A high degree of unextractable radioactivity was noted for incubations with liver microsomes from all species. SDS-PAGE was conducted on the protein pellets from the 1 hour incubation of human, rat and dog liver microsomes with \[^{14}C\] SB-649868, with bands of radioactivity co-located with the microsomal protein present, demonstrating that the binding is covalent in nature, rather than
due to the presence of an insoluble or unextracted metabolite. When compared to the molecular weight markers used in these analyses, the radioactivity was observed to be bound in the 46 – 66 KDa molecular weight region, which is consistent with the molecular weight of the cytochrome P450 enzymes. The phosphoimage generated for the SDS-PAGE analysis of the 1 hour incubation with human liver microsomes is shown in Figure 4.6.

![Phospho-image for the SDS-PAGE analysis of the incubation of human liver microsomes with [14C] SB-649868](image.png)

**Figure 4.6: Phospho-image for the SDS-PAGE analysis of the incubation of human liver microsomes with [14C] SB-649868, where lane 1 shows the 0 hour time point and lane 2 the 1 hour time point. Lanes 3 and 4 show the incubation of [14C] acetaminophen with human liver microsomes at 0 and 1 hour, respectively, used here as a positive control. [14C] labelled molecular weight marker proteins are shown in the outer lanes for comparison (Image taken from (354), with permission)**

The images for the SDS-PAGE analysis of the incubations of [14C] SB-649868 with rat and dog liver microsomes were very similar to the image shown in Figure 4.6.

As shown in Figure 4.4, epoxide hydrolases are capable of detoxifying reactive epoxides by catalysing the reaction of epoxides to the stable dihydrodiol. A series of experiments were conducted to evaluate the effect of the addition of two epoxide hydrolase inhibitors valpromide (355) and elaidamide (356) on the levels of microsomal binding observed. Dave
and Nash (354) found that while the addition of these enzyme inhibitors to the incubation of \[^{14}\text{C}]\text{SB-649868}\) with liver microsomes was expected to increase the level of binding observed due to the elimination of the detoxification pathway to the dihydrodiol, no change in binding levels was observed. This suggested that the epoxide hydrolysis was occurring non-enzymatically and that the epoxides formed in the microsomal incubation were reactive enough to undergo spontaneous hydrolysis to the dihydrodiol.

Incubations of \[^{14}\text{C}]\text{SB-649868}\) with human liver microsomes were also conducted in the presence and absence of aminobenzotriazole, a potent, non-specific cytochrome P450 inhibitor (357) to evaluate the extent to which the levels of covalent binding were dependent on cytochrome P450-catalysed metabolism (354). An approximate 10-fold reduction in covalent binding was noted in the presence of aminobenzotriazole, indicating that the binding is both metabolism dependent, and that the formation of the reactive metabolite(s) is primarily via cytochrome P450 oxidative metabolism.

Based on these data, \textit{in vitro} investigations using individual cytochrome P450 enzymes were conducted, firstly to identify the cytochrome P450 enzymes responsible for the metabolism of \text{SB-649868}\) to the reactive epoxide, and secondly to determine the inhibitory effect of this reactive species on the metabolising enzymes themselves (358). \[^{14}\text{C}]\text{SB-649868}\) was incubated with human liver microsomes, and with Supersomes\(^\text{TM}\) of the individual cytochrome P450 enzymes CYP1A2, CYP2C8, CYP1C9, CYP2C19, CYP3A4 and CYP2D6, in the presence and absence of selective cytochrome P450 inhibitors azamulin, sulphaphenazole, quinidine, montelukast, furafylline and benzynirvanol (inhibitors of CYP3A4 (359), 2C9 (360), 2D6 (361), 2C8 (362), 1A2 (363) and 2C19 (364), respectively). In total, four major metabolites were identified in the incubation with human liver microsomes; an amine metabolite formed via amide hydrolysis of the benzofuran moiety, two benzofuran dihydrodiol stereoisomers and a benzofuran ring-opened carboxylic acid as shown in Figure 4.7.
Figure 4.7: The major metabolites of SB-649868 detected after LC/MS analysis of the incubation of $[^{14}C]$ SB-649868 with human liver microsomes (adapted from (358))

The presence of the dihydrodiol isomers was a strong indicator of epoxide formation by metabolising enzymes in human liver microsomes. In addition, the formation of a ring-opened carboxylic acid metabolite would most likely proceed via a reactive aldehyde which is then oxidised to the acid, a similar mechanism to the one detailed in Figure 4.5. The dihydrodiol isomers were detected in all the Supersome™ cytochrome P450 isoenzyme incubations analysed, with CYP3A4 generating the largest amount of these metabolites, suggesting CYP3A4 is the primary CYP isoform responsible for the formation of a reactive epoxide intermediate. This was reinforced by complete inhibition of dihydrodiol formation by azamulin, a potent inhibitor of CYP3A4 activity.

The likely cytochrome P450 mediated formation of a reactive epoxide metabolite of SB-649868 in *in vitro* incubations of human liver microsomes prompted an investigation into the inhibition of the cytochrome P450 enzymes by SB-649868 and its metabolites (365). The enzyme activities of phenacetin O-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), bupropion hydroxylase (CYP2B6), paclitaxel 6α-hydroxylase (CYP2C8), diclofenac 4'-hydroxylase (CYP2C9), S-mephenytoin 4'-hydroxylase (CYP2C19), bufuralol 1'-hydroxylase (CYP2D6), atorvastatin O-hydroxylase (CYP3A4), midazolam 1'-hydroxylase (CYP3A4) and nifedipine oxidase (CYP3A4) were investigated in human liver microsomes in the presence and absence of SB-649868. In addition, a range of concentrations of SB-649868 (0.1-100 μM) were incubated with each probe substrate and pooled human liver microsomes to evaluate mechanism-dependent (also known as metabolism-dependent) inhibition. All samples were analysed by LC/MS. Based on the results of these analyses, SB-649868 showed direct inhibition of all cytochrome P450
enzymes, most notably the metabolism of atorvastatin O-hydroxylase by CYP3A4. SB-649868 was a mechanism-dependent inhibitor of CYP1A2, 2C19, 2D6, and 3A4, with CYP3A4 exhibiting up to a 20-fold reduction in enzyme activity after incubation with SB-649868. The mechanism-dependent inhibition of CYP3A4 by SB-649868, coupled with the metabolism data generated in human liver microsomes and CYP3A4 Supersomes™, strongly suggested the formation of an epoxide metabolite, catalysed by CYP3A4, which then covalently binds to the CYP3A4 enzyme itself, irreversibly inhibiting the enzyme activity.

Trapping experiments were conducted in human liver microsome incubations with SB-649868 to further investigate the mechanism of binding of the reactive epoxide metabolite of SB-649868 to proteins (354). A reduction in the extent of binding was observed in the presence of glutathione, suggesting that the reactive epoxide is an electrophile which can bind to glutathione, thereby removing the reactive epoxide from the incubation mixture and preventing adduction to the microsomal proteins. LC/MS and \(^1\)H NMR analyses of these trapping experiments with glutathione revealed the presence of two hydrated glutathione adducts, together with the two dihydrodiol metabolites observed in previous analyses and a hydroxylated benzofuran metabolite (Figure 4.8).
Figure 4.8: Metabolites of SB-649868 detected by LC/MS and $^1$H NMR analysis of the human liver microsome incubation of SB-649868 in the presence of an excess of glutathione

The data generated for the incubation of SB-649868 with human liver microsomes in the presence of an excess of glutathione is a direct correlation with the reactions shown in Figure 4.4, showing the metabolic fate of expected epoxides within the body. These data show that where epoxides form, several protective mechanisms exist within the body to detoxify the epoxide, either via further oxidation to a hydroxy or dihydrodiol metabolite, or via conjugation with glutathione. However, the data from the in vitro covalent binding assessment and the enzyme inhibition studies imply that where the conversion of a drug to a reactive species such as an epoxide is prolific, or where the reactive species binds immediately upon formation, covalent binding to proteins can still occur despite these mechanisms being in place.

Single oral doses (10-80 mg) and repeated oral dosing of up to a maximum of 30 mg/day of SB-649868 was administered to a small number of healthy human subjects to evaluate the pharmacokinetics of the drug in humans (366). In addition a single oral dose of 30 mg of $[^{14}$C] SB-649868 was administered to a small number of human subjects to evaluate the disposition and metabolism of SB-649868 (367). SB-649868 was well tolerated in humans with the concentration of SB-649868 in plasma increasing in a dose-proportional manner and a half life
of approximately 3-6 hours. After repeated dosing, the plasma exposure was slightly higher than observed for single dose administration and was time and dose dependent (366). Upon co-administration with a fixed dosage of simvastatin, a CYP3A4 substrate, the plasma exposure of simvastatin increased with increasing dose of SB-649868, consistent with the in vitro data generated to suggest that SB-649868 is a potent inhibitor of CYP3A4 (366). Encouragingly, these early studies showed a dose-dependent relationship between the administration of SB-649868 and an improvement in total sleep time and a reduction in latency to persistent sleep (368).

From the [14C] SB-649868 study, drug related material was eliminated primarily in the faeces, with urinary excretion accounting for only 12% of the administered dose. While clearance of SB-649868 from the plasma was complete within 24 hours, the elimination of the radioactive dose was much slower, with radioactivity still detectable in faeces up to 9 days after dosing. This suggests both the delayed formation of metabolites and significantly slower clearance of metabolites from the body compared to unchanged SB-649868. The major metabolite detected in plasma was a hemiaminal metabolite, most likely formed via metabolism to the epoxide with subsequent benzofuran ring-opening (as postulated in Figure 4.5) and reaction of the reactive aldehyde with the amide nitrogen to form a six-membered ring (Figure 4.9). The benzofuran ring-opened carboxylic acid was also observed in the plasma at later time points, suggesting slower formation of this metabolite compared to the hemiaminal.
Figure 4.9: A summary of the metabolism of SB-649868 observed in humans (adapted from (367))
Excretion of SB-649868 was primarily via metabolism, with very little SB-649868 detected in urine or faeces. The benzofuran ring-opened carboxylic acid was the principle drug related component, accounting for approximately 12% of the administrated dose. The benzofuran hydroxy metabolite was also observed in the excreta, together with the benzofuran dihydrodiol isomers. The data generated in human were highly consistent with the in vitro data generated in human liver microsomes and individual cytochrome P450 isoenzymes, suggesting that, in the case of SB-649868, in vitro systems are a good approximation of the metabolism occurring in vivo, mainly due to the predominance of oxidation of the benzofuran moiety as the major metabolic route in man. Interestingly, no glutathione or glycyl cysteine adducts were detected in human, despite the majority of the metabolic routes observed proceeding via the reactive epoxide intermediate. This could be due to the efficiency of alternative oxidative routes in detoxifying any epoxides formed, as demonstrated by the detection of the ring-opened carboxylic acid, the hemiaminal and the dihydrodiol metabolites. Alternatively, a portion of epoxide could be binding to proteins or other endogenous material within the body; some indication of this was observed. The low recovery of both the faecal and plasma extractions, particularly at the later time points, was consistent with this hypothesis.

The development of SB-649868 as a potential drug for sleep disorders was discontinued recently due to toxicity issues in the rat, unrelated to the formation of reactive epoxide species. However, the wealth of information related to this compound and its metabolism to a reactive species meant that it was an excellent tool substrate for the methods developed and discussed in Chapter 3, to directly explore and evaluate the binding of reactive species to proteins. The work detailed in this chapter describes the application of the methods developed with the tool substrate acetaminophen to directly identify covalent adducts of SB-649868 to proteins. As with acetaminophen, in vitro systems were used to reduce the complexity of the sample by minimising the number of proteins present. Both human liver microsome and CYP3A4 Supersome™ incubations with SB-649868 were selected for these experiments because in vitro experiments conducted during the development of SB-649868 and discussed previously, indicate that both systems are likely capable of producing reactive epoxide species.

We know from the previous in vitro assessments detailed that covalent binding was observed in incubations with human liver microsomes, and with CYP3A4 Supersomes™ (shown by the inhibition of enzyme activity). We also know, from the comparison of the metabolites observed in in vitro incubations to those observed in humans, that SB-649868 in vitro assessments are a good approximation for the metabolic processes occurring in vivo. It is
reasonable to assume that any data on protein binding generated would have real relevance to
the clinical setting.

4.1. Quantitative assessment of the covalent binding of [\(^{14}\)C] SB-649868 in CYP3A4
Supersomes™ and human liver microsomes

A quantitative assessment of the degree of covalent binding observed in an in vitro incubation
of [\(^{14}\)C] SB-649868 and CYP3A4 or human liver microsomes was conducted using the
experimental procedures described in Experimental Section 2.3. These data are shown in
Tables 4.1 and 4.2. A significant degree of binding was observed at all time points. The data
generated for the 1 hour time point incubation with human liver microsomes is in good
agreement with data generated within GSK for the equivalent incubation, which was
approximately 400 pmol/mg protein (354). In contrast to the quantitative covalent binding
data generated for [\(^{14}\)C] acetaminophen, the cofactor independent binding was low, suggesting
that any covalent binding that had occurred was predominantly a result of cofactor driven
metabolism to a reactive metabolite or metabolites. This was anticipated based on previous
knowledge of the compound, because the formation of an epoxide moiety would require
cofactor-driven oxidation of the benzofuran ring.

As with the acetaminophen incubations in Chapter 3, the levels of covalent binding are almost
2-fold higher for the human liver microsome incubations than for the isolated enzyme
incubations. This implies the involvement of other cytochrome P450 enzymes, as well as
CYP3A4, in the conversion of SB-649868 to a reactive species. This correlates well with the
data generated by Taylor et al. (358) who noted the presence of dihydrodiol isomers in all of
the individual cytochrome P450 isoenzyme incubations analysed.

Interestingly, the data for both the human liver microsome and CYP3A4 Supersome™
incubations show an increase in covalent binding with an increase in incubation time. Whilst
this increase was relatively small (a less than 2-fold increase in binding was observed in the
binding seen in the 1 hour compared to the 4 hour incubations), this finding does correlate
with the human metabolism data which reported an apparent delayed formation of
metabolites in vivo and a larger degree of unextracted radioactivity for the later faecal and
plasma sample extractions (367). However, due to the relatively small increase according to
time, it is impossible to completely eliminate analytical variability as a cause of this change.
Table 4.1: Quantitative assessment of covalent binding of $^{14}$C SB-649868 and CYP3A4 Supersomes™

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time point (hours)</th>
<th>dpm/sample $^1$</th>
<th>pmol/sample $^2$</th>
<th>pmol/mg of protein $^3$</th>
<th>Cofactor dependent/ independent binding (pmol/mg)$^4$</th>
<th>Total covalent binding to protein (pmol/mg)$^5$</th>
<th>Approximate % protein modification$^6$</th>
<th>Approximate % radioactivity bound$^7$</th>
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Notes:
1: dpm/sample value generated by LSC of the filter papers post-analysis.
2: dpm/sample converted by dividing by radioactivity present in incubation e.g. 26 dpm/pmol
3: pmol/mg protein converted by dividing by amount of protein present in incubation e.g. 0.6 mg
4: Cofactor dependent binding = (cofactor binding at x hours – cofactor binding at 0 hours) – (cofactor independent binding at x hours – cofactor independent binding at 0 hours). Cofactor independent binding = (cofactor independent binding at x hours – cofactor independent binding at 0 hours).
5: The total covalent binding value represents the combination of the cofactor dependent and cofactor independent values.
6: Approximate % protein modification is based on the Mw of CYP3A4 as 57343Da (Uniprot, accessed 15 August 2012)
7: Approximate % radioactivity bound is based on the comparison of radioactivity bound with the equivalent volume of stock solution added to the incubation i.e. 5 µL.
Table 4.2: Quantitative assessment of covalent binding of $[^{14}C]$ SB-649868 and human liver microsomes

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<th>pmol/sample$^2$</th>
<th>pmol/mg of protein$^3$</th>
<th>Cofactor dependent/independent binding (pmol/mg)$^4$</th>
<th>Total covalent binding to protein$^5$ (pmol/mg)</th>
<th>Approximate % protein modification$^6$</th>
<th>Approximate % radioactivity bound$^7$</th>
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<tr>
<td>$[^{14}C]$ SB-649868 stock solution</td>
<td>NA</td>
<td>543050</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1: dpm/sample value generated by LSC of the filter papers post-analysis.
2: dpm/sample converted by dividing by radioactivity present in incubation e.g. 26 dpm/pmol
3: pmol/mg protein converted by dividing by amount of protein present in incubation e.g. 0.5 mg
4: Cofactor dependent binding = (cofactor binding at x hours – cofactor binding at 0 hours) – (cofactor independent binding at x hours – cofactor independent binding at 0 hours). Cofactor independent binding = (cofactor independent binding at x hours – cofactor independent binding at 0 hours).
5: The total covalent binding value represents the combination of the cofactor dependent and cofactor independent values.
6: Approximate % protein modification is based on the assumption that the numerous proteins in human liver microsomes have an average Mw of 50 kDa
7: Approximate % radioactivity bound is based on the comparison of radioactivity bound with the equivalent volume of stock solution added to the incubation i.e. 5 µL.
In their original experiment, Patel et al. (365) found that the activity of CYP3A4 was significantly inhibited after only 20 minutes of incubation with SB-649868, thereby suggesting mechanism-dependent inhibition due to formation of reactive metabolites which bind to and inactivate the enzyme. The covalent binding data shown in Tables 4.1 and 4.2 appear to support this as in both cases we see the largest increase in covalent binding over the first hour of the experiment, with the rate of increase in covalent binding slowing as the experiment proceeds. This may be indicative of mechanism-dependent inhibition of the enzyme by reactive species, i.e. as reactive epoxide species are formed, these bind and inhibit the enzyme, thereby reducing the formation of reactive species as the experiment proceeds. For future investigations it might be prudent to evaluate the variability of the quantitative covalent binding data by repeating these experiments several times, to see if the time-dependence is a real result, and increase the incubation time to 24 hours, although the viability of in vitro systems is generally expected to decrease with time.

As for acetaminophen, the level of covalent modification of the proteins is small, with, at best, only 2% of the protein modified by reactive species. For SB-649868, whilst metabolic turnover should be relatively good in both CYP3A4 Supersomes™ and human liver microsomes, oxidation to form an epoxide reactive species is not the only metabolic route occurring in the incubation. As discussed by Dave et al. (354), and shown in Figure 4.7, a metabolite formed via amide hydrolysis was significant in human liver microsomes, a metabolic route which was not initiated by the formation of an epoxide. In addition, the dihydrodiol isomers were also detected, suggesting that not all of the reactive epoxide species formed binds to proteins, with a proportion is metabolised further by epoxide hydrolases to form the stable dihydrodiol metabolites. It is likely that despite good conversion of parent compound by the metabolising enzymes, a relatively small amount of epoxide is formed, of which only a small proportion is available to bind to the protein. As with acetaminophen, the relatively low modification of protein makes the detection of any covalent adducts at the protein/peptide level technically challenging which again is compounded if the modification occurs on multiple residues of each protein.
4.2 Metabolites of SB-649868 detected by LC/MS following incubation of [\(^{14}\text{C}\)] SB-649868 with CYP3A4 and human liver microsomes

LC/MS analysis of the supernatant from the incubation of [\(^{14}\text{C}\)] SB-649868 with human liver microsomes and CYP3A4 Supersomes™ were examined to assess the metabolic turnover of SB-649868 and the metabolic routes observed. The supernatants from the incubations of [\(^{14}\text{C}\)] SB-649868 with CYP3A4 Supersomes™ and human liver microsomes were collected and analysed by LC/MS and off-line fraction collection with TopCount radiodetection to assess metabolic routes of the compound using in vitro systems. The experimental procedures are detailed in Experimental Section 2.2 and 2.27. Radiochromatograms showing the no cofactor control and the 0, 1, 2, and 4 hour Supersome™ incubations are depicted in Figure 4.10.
Figure 4.10: Reconstructed radiochromatograms for the analysis of the supernatants retained from the CYP3A4 Supersome™ incubations with [14C] SB-649868 showing the (A) no cofactor control, (B) 0 hour, (C) 1 hour, (D) 2 hour and (E) 4 hour time points, by LC and offline fraction collection and radiodetection by TopCount. The peak at retention time 8.5 minutes is [14C] SB-649868 is assigned based on comparison with the retention time of a standard of SB-649868, analysed using the same analytical method.

LC/MS and radiochromatograms data were generated for the incubations with human liver microsomes; however these were identical, in terms of the metabolites identified, to those shown for CYP3A4 and as such will not be discussed further. These data can be found in Appendix 3, A3.1. In addition, the same metabolites were detected in the samples from all time points, therefore only representative data are discussed here.
For the no cofactor control seen in Figure 4.10 (A), whilst the major peak present corresponded to unchanged [\(^{14}\)C] SB-649868, some very small radio-peaks were observed in the baseline of the radiochromatogram; these are most likely due cofactor independent metabolism. As shown in Figure 4.10 (B), metabolism by CYP3A4 occurs rapidly, with metabolite peaks observed the 0 hour time point sample. While every effort was taken during sample preparation to keep the incubation time of the 0 hour sample as short as possible, the logistics of the incubation procedure meant that it was impossible to keep the time between adding the [\(^{14}\)C] SB-649868 substrate and terminating the reaction by centrifugation and removal of the protein to 0 minutes. Given the data from both the no cofactor and 0 hour controls, these samples were deemed unsuitable for future data processing and comparison with the 1, 2 and 4 hour controls, for which the no drug control was utilised instead.

A notable decrease in [\(^{14}\)C] SB-649868 was observed in the radiochromatograms for the 2 and 4 hour time points, compared to the 1 hour time point, reinforcing the hypothesis that the enzyme viability was maintained throughout the duration of the experiment. Whilst the chromatographic resolution of the metabolites is poor, it is clear that [\(^{14}\)C] SB-649868 is extensively metabolised by both human liver microsomes and CYP3A4 Supersomes™. The data in Figure 4.11 (and summarised in Table 4.3) shows the MS spectrum under the peak at 8.5 minutes, which was assigned as unchanged [\(^{14}\)C] SB-649868 (m/z 480.1629) based on exact mass (which was in good agreement with the expected exact m/z 480.1628, difference 0.21 ppm) and the comparison of the retention time with analysis of an authentic standard of [\(^{14}\)C] SB-649868 using the same analytical method. The product ion spectrum for the ion m/z 480.2 (shown in Figure 4.11, with assignment of the fragment ions in Table 4.3) confirms the assignment.
Figure 4.11: LC/MS analysis of a 4 hour CYP3A4 Supersome™ incubation with [14C] SB-649868 showing (A) the mass spectrum for the peak at retention time 8.5 minutes, showing ion m/z 480.1629 assigned as unchanged [14C] SB-649868, with sodium and potassium adducts corresponding to the ions at m/z 502.1 and m/z 518.1, respectively, with the in-source loss of water from [14C] SB-649868 shown by the ion at m/z 462.2 and, (B) the product ion spectrum for ion m/z 480.2

The same sample was analysed using the LC/MS method developed for peptide mapping experiments (see Experimental Section 2.15) to achieve better chromatographic resolution. A representative radiochromatogram is shown in Figure 4.12 for the CYP3A4 Supersome™ 4 hour incubation, and in Appendix 3, A3.2 for the corresponding human liver microsome sample.
Figure 4.12: Representative radiochromatogram for the metabolites observed after a 4 hour CYP3A4 Supersome™ incubation with [14C] SB-649868, using the method described in Experimental Section 2.18, with off-line fraction collection and radiodetection by TopCount.

A summary of the major metabolites identified is shown in Table 4.3, with individual extracted ion chromatograms and full scan and product mass spectra collated in Appendix 3, A3.3 – A3.16. The metabolites observed in both the CYP3A4 Supersome™ and human liver microsome incubations with [14C] SB-649868 correlate well with the expected in vitro metabolic routes observed in previous work conducted within GSK (354, 358), with several metabolites formed via either oxidation to the piperidine ring, or most notably, to the benzofuran moiety.

Of particular importance is the presence of dihydrodiol metabolites and a benzofuran ring-opened carboxylic acid metabolite (both m/z 514, retention times 2 and 4 minutes, respectively, based on the 15 minute method described in Experimental Section 2.27), which are assumed to be formed via the reactive epoxide intermediate.
Table 4.3 Summary of the MS data for $[^{14}C]$ SB-649868 and its metabolites

<table>
<thead>
<tr>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
</table>
| 480.1629         | ![Proposed Structure](image) | m/z 462: [M+H]$^+$ - H$_2$O  
m/z 319: F1  
m/z 318: F2  
m/z 242: F3 | 496.1586         | ![Proposed Structure](image) | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
|                  |                    | m/z 241:                       |                  |                    | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
|                  |                    | m/z 222: F4                    |                  |                    | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
|                  |                    | m/z 151: F5                    |                  |                    | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
|                  |                    | m/z 145: F7                    |                  |                    | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
|                  |                    | m/z 98: F8                     |                  |                    | m/z 318: F2  
m/z 222: F5  
m/z 151: F6 |
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<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
</table>
| 496.1556       | ![Proposed Structure](image1) | m/z 319: F1  
m/z 258: F3 + [O]  
m/z 151: F6 | 496.1544       | ![Proposed Structure](image2) | m/z 319: F1  
m/z 240: F3 + [O] – H₂O  
m/z 151: F6 |
| 496.1600       | ![Proposed Structure](image3) | m/z 319: F1  
m/z 240: F3 + [O] – H₂O  
m/z 151: F6 | 496.1574       | ![Proposed Structure](image4) | m/z 335: F1 + [O]  
m/z 334: F2 + [O]  
m/z 258: F3 + [O]  
m/z 222: F5  
m/z 145: F7  
m/z 114: F8 + [O] |
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<td>496.1599</td>
<td><img src="image2.png" alt="Image" /></td>
<td>m/z 319: F1&lt;br&gt;m/z 240: F3 + [O] − H$_2$O&lt;br&gt;m/z 222: F5</td>
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<td>498.1755</td>
<td><img src="image3.png" alt="Image" /></td>
<td>m/z 337:&lt;br&gt;m/z 336:&lt;br&gt;m/z 222: F5&lt;br&gt;m/z 145: F7</td>
<td>512.1518</td>
<td><img src="image4.png" alt="Image" /></td>
<td>m/z 351: F1 + 2[O]&lt;br&gt;m/z 222: F5</td>
</tr>
</tbody>
</table>

Empirical formula: $\text{C}_2\text{H}_9\text{N}_2\text{O}_2\text{S}_2$<br>Empirical formula: $\text{C}_2\text{H}_9\text{N}_2\text{O}_2\text{S}_2$<br>Empirical formula: $\text{C}_2\text{H}_9\text{N}_2\text{O}_2\text{S}_2$<br>Empirical formula: $\text{C}_2\text{H}_9\text{N}_2\text{O}_2\text{S}_2$
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<th>[M+H]+ (m/z)</th>
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</tr>
</thead>
<tbody>
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<td><img src="image1" alt="Proposed Structure 1" /></td>
<td>m/z 335: F1 + [O] m/z 238: F5 + [O] m/z 222: F5 m/z 151: F6</td>
<td>512.1573</td>
<td><img src="image2" alt="Proposed Structure 2" /></td>
<td>m/z 319: F1 m/z 222: F5</td>
</tr>
<tr>
<td>514.1650</td>
<td><img src="image3" alt="Proposed Structure 3" /></td>
<td>m/z 276: F3 + 2[O] + 2[H] m/z 240: m/z 276 – 2H2O m/z 222: F5 m/z 178:</td>
<td>514.1697</td>
<td><img src="image4" alt="Proposed Structure 4" /></td>
<td>m/z 319: F1 m/z 240: F3 + 2[O] + 2[H] -2H2O m/z 222: F5 m/z 151: F6 or m/z 98: F8</td>
</tr>
</tbody>
</table>
Given the substantial metabolic turnover of $[^{14}\text{C}]$ SB-649868 by both human liver microsomes and CYP3A4 Supersomes™, the presence of metabolites formed via reactive epoxide intermediates and the high levels of covalent binding observed, these incubation experiments were deemed viable for further examination of the protein pellet to determine the nature of the binding of the reactive species with proteins.

Trapping experiments with glutathione were initially conducted to gain more insight into the mechanism of binding as demonstrated previously for acetaminophen. The glutathione trapping data was utilised to predict which amino acids NAPQI would bind to. This information was then successfully used to devise highly sensitive multiple reaction monitoring MS experiments to detect modified peptides after the protein was digested with trypsin.

### 4.3 Glutathione trapping of reactive metabolites of $[^{14}\text{C}]$ SB-649868

$[^{14}\text{C}]$ SB-649868 was incubated with CYP3A4 Supersomes™ and human liver microsomes in the presence of reduced glutathione in an effort to trap and stabilise the reactive epoxide intermediate. The resultant supernatants were retained and analysed by LC/MS and off-line radiodetection. The experimental methods are detailed in Experimental Sections 2.6 and 2.27.

The reconstructed radiochromatogram for the 4 hour incubation of $[^{14}\text{C}]$ SB-649868 with CYP3A4 Supersomes™ in the presence of glutathione is shown in Figure 4.13. As expected, based on the in vitro metabolite identification data described in Section 4.1, the corresponding radiochromatogram for the human liver microsome incubation was identical, (see Appendix 3, A3.17, with corresponding MS data in A3.18 and A3.19) therefore, only the data from the CYP3A4 Supersome™ incubation is discussed further.
Figure 4.13: Representative Radiochromatogram for LC/MS analysis with off-line fraction collection and radiodetection by TopCount of the 4 hour incubation of CYP3A4 Supersomes™ with $^{[14]}$C SB-649868 in the presence of an excess of glutathione
Figure 4.14: LC/MS analysis of the 4 hour incubation of $[^{14}\text{C}]$ SB-649868 with CYP3A4 Supersomes™, in the presence of an excess of glutathione, showing (A) the extracted ion chromatogram for m/z 803.3 (0.3 Th window, retention time 3.9 minutes) and (B) mass spectrum for the peak at retention time 3.9 minutes, the postulated hydrated glutathione conjugate of $[^{14}\text{C}]$ SB-649868
Figure 4.15: LC/MS analysis of the 4 hour incubation of [14C] SB-649868 with CYP3A4 Supersomes™, in the presence of an excess of glutathione, showing the high collision energy MS² spectrum showing the fragment ions for the peak at 3.9 minutes, the postulated hydrated glutathione conjugate of [14C] SB-649868

Glutathione was in large excess in the incubations (100-fold) so it was anticipated that any epoxide formed would be trapped by the spontaneous reaction between the epoxide electrophile and the free thiol of the glutathione moiety. One major radio peak at 3.9 minutes was present in the radiochromatogram generated from analysis of the 4 hour incubation of [14C] SB-649868 with CYP3A4 Supersomes™, shown in Figure 4.13. No [14C] SB-649868 was detected (expected retention time of 8.5 minutes) indicating that the entire drug has been metabolised in this instance. Small peaks corresponding to other oxidised metabolites noted in the previous in vitro incubations were observed, however, the presence of the hydrated glutathione conjugate as the largest radio peak indicates that the majority of the drug has been metabolised via a reactive epoxide which has been trapped by glutathione preventing further metabolism.

An extracted ion chromatogram for the postulated hydrated glutathione (m/z 803.2, 0.3 Th isolation window) is shown in Figure 4.14 (A) at retention time 3.9 minutes, directly correlating with the retention time of the peak observed in the radiochromatogram (Figure 4.13), with the mass spectrum shown in Figure 4.14 (B). Interestingly, while the pseudo molecular ion is present, a larger peak at m/z 785.2 was detected, suggesting the in-source loss of water from the [M+H]⁺ ion. The size of this peak, relative to the size of the intact pseudo molecular ion
suggests that water can be lost very easily from the pseudo molecular ion structure. Additional ions, most likely due to the in-source fragmentation of the pseudo molecular ion m/z 803.2, were also observed, most notably m/z 656.2, 480.2 and 222.0, which all correspond to potential fragment ions for the hydrated glutathione adduct (summarised in Table 4.4). The large peak at m/z 491.3 was not compound related and was noted at the same retention time in the no drug control incubation. The presence of ions due to in-source fragmentation can be an indicator of unfavourable ion source parameters, however these ions were not eliminated by changing the ion source conditions e.g. by reducing the cone voltage, source temperature and source gas pressures, suggesting that the hydrated glutathione conjugate is particularly susceptible to fragmentation, even in relatively mild conditions. Sodium and potassium adducts of the parent ion were also noted, at m/z 825.2 and 841.2 respectively.

Unfortunately, data from the product ion scanning experiment on ion m/z 803.2 became corrupted and were unusable; however, the data from the high collision energy function of the MS$^5$ experiment have been included. The spectrum was generated by the sum of data under the peak at retention time 3.8 minutes, with background subtraction used to remove any ions not related to the hydrated glutathione of interest. This spectrum is shown in Figure 4.15, with all of the MS data summarised in Table 4.4.

The data from the high collision energy MS$^5$ experiment (Figure 4.15) show several fragments that are characteristic of the addition of glutathione to the drug moiety, particularly the loss of glycine (m/z 710) and pyroglutamic acid (m/z 656), components of the glutathione moiety from the m/z 785 ion ([M+H]$^+$ - H$_2$O). The ion at m/z 512 corresponds to [$^{14}$C] SB-649868 with the addition of a sulphur atom, reinforcing that conjugation occurs via nucleophilic attack by the lone pair of the sulphur atom of the glutathione moiety. The presence of m/z 319 and m/z 151 ions, which are also observed in the high collision energy MS$^5$ spectrum of unchanged [$^{14}$C] SB-649868 confirm that glutathione conjugation has taken place on the benzofuran portion of the molecule, with the most likely site of attack being the double bond in the furan ring. The addition of 18 Da to the mass of [$^{14}$C] SB-649868 in addition to 305 Da for the glutathione, and the facile loss of water, suggest that the trapped species is a hydrated glutathione conjugate, with the oxygen atom of the epoxide situated on the adjacent carbon to that with the glutathione moiety attached.

Preparative HPLC of a bulk incubation and $^1$H NMR analysis of the isolated hydrated glutathione conjugate conducted as part of metabolism investigations within GSK (354), confirmed the definitive structure shown in Figure 4.16, and concurred with the data presented here.
### Table 4.4 Summary of the LC/MS data generated for the hydrated glutathione conjugate of [\(^{14}\text{C}\)] SB-649868

<table>
<thead>
<tr>
<th>[M+H](^+) (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
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<td>803.2423</td>
<td><img src="image" alt="Proposed Structure" /></td>
<td><img src="image" alt="Fragment Ions" /></td>
</tr>
</tbody>
</table>

Empirical formula of [M+H]\(^+\): C\(_{35}\)H\(_{42}\)FN\(_6\)O\(_{10}\)S\(_2\)

\[\text{Error: 1.00 ppm}\]

| m/z 785: [M+H]\(^+\) - H\(_2\)O |
| m/z 767: [M+H]\(^+\) - 2H\(_2\)O |
| m/z 710: |
| m/z 656: |
| m/z 512: |
| m/z 510: |
| m/z 480: |
| m/z 319: |
| m/z 151: |
The reaction proceeds via nucleophilic addition. The lone pair of electrons on the free thiol of glutathione attacks the δ+ carbon atom of the epoxide, causing the electrons in the carbon-oxygen bond to move to form a new bond with any protons available, forming an OH group on the adjacent carbon. Since nucleophilic attack by the sulphur lone pair can occur on either of the carbon atoms in the epoxide, two positional isomers are possible for the hydrated glutathione.

It is likely that both isomers are present in the analysis described here and co-elute to form the single peak shown at the retention time of 3.9 minutes. In future work, it would be prudent to redevelop the chromatographic method to try to resolve these two positional isomers. Since these two isomers are extremely similar in terms of structure, it is likely that this could be difficult to achieve. A mechanism describing the formation of the hydrated glutathione is shown in Figure 4.16.

![Mechanism for the formation of a hydrated glutathione conjugate of SB-649868](image-url)

**Figure 4.16: Mechanism for the formation of a hydrated glutathione conjugate of SB-649868**
4.4 Detecting covalently bound protein-adducts of SB-649868 using in vitro systems

The formation of covalently bound adducts of SB-649868 with proteins was investigated by incubation of CYP3A4 Supersomes™ and human liver microsomes with SB-649868 as described in Experimental Section 2.2. As with the data generated on acetaminophen in Chapter 3, the quantitative covalent binding data in Tables 4.1 and 4.2 indicate the likely formation of covalent adducts of SB-649868 to proteins within the incubation mixture. In addition, the metabolism and glutathione trapping data generated in Sections 4.2 and 4.3 predict that the most likely reactive species generated is the formation of an epoxide species, which can be further metabolised to dihydrodiol metabolites (as shown in Table 4.3) or can be trapped by glutathione to form a hydrated glutathione conjugate (as shown in Table 4.4). The trapping of the epoxide with a soft nucleophile such as glutathione suggests that the most likely amino acid target on proteins would be cysteine residues, which have a free thiol group, although binding to lysine residues, which have a free lone pair of electrons present on the free amine, could also be a possibility. Based on the relative nucleophilicity of atoms, where R₂S is a more reactive nucleophile than R₂N, epoxides are more likely to react with cysteine residues than lysines. This could be investigated further using trapping experiment with glutathione and a surrogate amine trapping agent such as semicarbazide (369).

For simplicity, the primary focus of investigation for these additional experiments involved using the simpler CYP3A4 Supersomes™ incubations, however data were also generated using the human liver microsomes incubations. Whilst incubations were conducted for 1, 2 and 4 hours and the proteins retained for further investigation, the primary focus was for the 4 hour incubations as the extent of binding was shown to be highest in these incubations, therefore the likelihood of identifying and characterising protein adducts should be greatest in these incubations. Due to the rapid turnover observed in these in vitro incubations (as shown in Figure 4.10), only the no drug incubation was used as a control, since metabolites were detected in the 0 hour incubation, and also could not be definitively excluded from the no cofactor incubation.

Based on the experimental data from Chapter 3, the LC/MS analysis of the intact protein to monitor for any shift in molecular mass due to adduction by SB-649868 was not attempted due to the nature of the cytochrome P450 proteins and the difficulty of solubilising these membrane-bound proteins without the aid of detergents. As alluded to Chapter 3, the effectiveness of these experiments may be improved by the use of mass spectrometric
compatible surfactants; however this was not attempted during the course of this investigation and would therefore be an area of future study.

4.4.1 Detection of modified peptides by LC/MS

The experimental procedures describing incubation, protease digestion and LC/MS analysis are described in Experimental Sections 2.2, 2.5 and 2.15, respectively. As discussed in Section 3.1.3.1, trypsin was selected as the protease because the percentage protein coverage observed for trypsin was superior to the percentage protein coverage observed for Lys-C and Asp-N.

After incubation of SB-649868 with CYP3A4 Supersomes™ and human liver microsomes, each incubation mixture was centrifuged to separate the protein content from the rest of the mixture. This protein pellet was retained and washed three times with phosphate buffer to ensure the complete removal of any un-bound drug-related material before resolubilisation in 0.1% (w/v) RapiGest SF in 50 mM ammonium bicarbonate and digestion overnight at 37°C with trypsin. Sample incubations prepared in the absence of drug were prepared and analysed to eliminate false positives during data processing.

As described in Chapter 3 and Experimental Sections 2.5 and 2.15, initial analyses were conducted using in-solution digestion with analysis by LC/MS using a chromatographic method on a Waters Acquity UPLC system coupled to a Waters QTof Premier mass spectrometer, with an MS² method devised for the identification of the digest peptides analysed. LC/MS data generated on the tryptic digestion of the 0, 1, 2 and 4 hour incubations, with the no drug incubation used to provide a comparison, were processed and compared using BioPharmaLynx.

The BioPharmaLynx method used the protein sequences of human cytochrome P450 3A4 (195), NADPH cytochrome P450 reductase (298) and cytochrome b5 (299) and trypsin as the digest reagent with 2 missed cleavages allowed. The mass tolerance was 50 ppm for the MS match and 100 ppm for the MS² fragment ion match. For incubations with [14C] SB-649868, the addition of [14C] SB-649868 to cysteine and lysine residues (+495.2 Da), and the loss of water from this addition (+477.1) were included as variable modifications. For incubations using non-labelled SB-649868, these values were + 493.2 Da and +475.1 Da respectively. The inclusion of the loss of water from the addition of SB-649868 to cysteine residues as a modification was based on the glutathione trapping experiment where water was lost very readily from the hydrated glutathione conjugate in the source, resulting in the dehydrated [M+H]⁺-H₂O ion.
being more abundant in the spectra than the [M+H]+ ion itself. As with acetaminophen, carbamidomethylation on cysteine residues (+57 Da) was included as a modification due to the alkylation of tryptic peptides using iodoacetamide during the sample preparation process. All modifications were set as variable modifications, with no maximum given, i.e. all cysteines were allowed to be either modified or unmodified, with more than one modification allowable for each peptide. In addition to the automatic data interrogation by BioPharmalynx, manual data interrogation was carried out. This involved looking for postulated modified peptides by extracting relevant ion chromatograms and comparing against the equivalent chromatograms for the control sample. The ions of interest were based on in silico digestion of cytochrome P450 CYP3A4 using BioLynx™ (Waters Ltd.) and assuming modification of the cysteines in the protein sequence. The ions evaluated are shown in Experimental Section 2.29, Tables 2.8 and 2.9.

Initial attempts to identify SB-649868-modified peptides in an in-solution tryptic digest of the CYP3A4 Supersome™ incubation were unsuccessful, with no modified peptides identified. As discussed in Chapter 3, this is most likely due to the poor sensitivity of the QTof Premier MS5 method employed. As shown in Table 4.1, the quantitative covalent binding data suggest that the degree of protein modification was around 2%, indicating that the abundance of modified peptides compared to unmodified is very small. In addition, the highly reactive nature of the postulated epoxide intermediate may lead to modifications on multiple cysteine residues, thereby making detection of individual modifications more difficult.

Several of the methods described in Chapter 3 to improve the detection of covalent adducts have been applied for SB-649868 and are discussed further here.

4.4.2 In-gel digestion, LC/MS analysis and analysis by MALDI MS

The rationale for the separation of a protein mixture using SDS-PAGE followed by in-gel digestion of the protein bands of interest is discussed in Chapter 3 Section 3.5.3. The major advantage of this kind of experiment is that it can increase the dynamic range of the peptide MS experiment, making the detection of lower abundant peptides easier as the MS analysis of each protein band will be significantly less complex than analysis of the whole sample.

The experimental procedures describing the incubation, SDS-PAGE and in-gel digestions are described in Experimental Sections 2.2, 2.16 and 2.17. The coomassie-blue stained gel prior to in-gel digestion is shown in Figure 4.17.
Lanes E-H of the gel shown in Figure 4.17 show the separation of the protein components in the CYP3A4 Supersome™ incubation, primarily NADPH cytochrome P450 reductase (Mw 76690 Da, accession number P16435 (298)), cytochrome P450 3A4 (Mw 57343 Da, accession number P08684 (195)), and cytochrome b₅ (Mw 15330 Da, accession number P00167 (299)). All three proteins appear in the correct order and with a reasonably accurate molecular mass compared to the molecular weight marker protein used (shown in the outer two lanes). Lanes A-D represent the separation of the protein components for the human liver microsome incubations with SB-649868 and show a far more complex profile of proteins than observed for the CYP3A4 Supersome™ samples, as expected, particularly in the region between 40 and 220 KDa. Due to the complexity of the human liver microsome SDS-PAGE gel, the focus of further investigation was on the simpler CYP3A4 Supersome™ incubations (lanes E-H). All three gel bands from each lane were excised and subjected to trypsin digestion as described in Experimental Section 2.17. Two different analytical methods were used to examine the protein digest generated by in-gel digestion of the bands of interest. LC/MS analysis was conducted using the same method as was used for analysis of the in-solution digests described in Section 4.4.1 (see Experimental Section 2.15 for the analytical details). In addition, MALDI MS analysis was conducted using a Bruker ultraFleXtreme III (Bruker Daltonik GmBH, Bremen, Germany) (see Experimental Section 2.28 for the analytical details).
Figure 4.17: Coomasie-blue stained SDS-PAGE gel for the protein pellets from the human liver microsome and CYP3A4 incubations prior to in-gel digestion. A Novex® sharp pre-stained molecular weight marker was used for molecular weight comparison on each of the outer gel lanes. Lanes A-D represent the human liver microsome incubations with SB-649868 at 0, 1, 2 and 4 hour time points, respectively. Lanes E-H represent the CYP3A4 Supersome™ incubations with SB-649868 at 0, 1, 2 and 4 hour time points, respectively. The control incubations (no drug and no cofactor) were analysed using an additional SDS-PAGE gel.

The data from the LC/MS analysis of the digested SDS-PAGE were processed using the BioPharmaLynx method described in Section 3.2, as well as manually by extracting ion chromatograms based on the in silico digestion of the three proteins of interest, incorporating the mass shifts expected for the addition of SB-649868 to cysteine residues. The UltraFleXtreme is a MALDI ToF/ToF mass spectrometer (370), with the advantage that it allows the high mass resolution separation of both precursor and fragmentation product ions. The use of MALDI ionisation rather than electrospray ionisation also has its advantages for protein and peptide analysis. MALDI ionisation is generally less susceptible to ion suppression caused by interfering compounds present in the sample (371) and has been found to favour the ionisation of peptides containing basic and aromatic amino acids, whereas electrospray...
ionisation favours peptides containing aliphatic and hydroxy amino acids (372), therefore MALDI has been shown to identify peptides that were not detected in electrospray LC/MS analyses. MALDI ionisation also tends to produce singly-charged ions rather than the multiply-charged ions seem in electrospray analyses, which can simplify data processing.

The data from the MALDI MS analysis was processed manually by direct comparison of the ions observed in the 1, 2 and 4 hour incubations with the ions detected in the no compound control sample. Any ions detected in the 1, 2 and 4 hour samples which were not present in the control sample were compared to the ions expected from an *in silico* digestion of the three proteins of interest, with and without modification by SB-649868, and were also subjected to CID fragmentation.

Unfortunately, no modified peptides were detected in either the LC/MS or MALDI MS analyses. As postulated in Chapter 3, the failure to identify any adducted peptides in either of these analyses suggests that the sensitivity of the MS methods used are not sufficient to identify the low abundant peptide adducts which should, based on the quantitative covalent binding data, be present in the sample.

### 4.4.3 Radiodetection of adducted peptides

Incubations were performed with $[^{14}\text{C}]$-labelled SB-649868 in an attempt to generate a radiochromatogram for the LC separation of a tryptic digest of the proteins from the *in vitro* CYP3A4 Supersome™ incubation. As with the acetaminophen incubations described in Chapter 3, while fraction collection and off-line radiodetection with TopCount™ was attempted for these samples, it was not sufficiently sensitive to generate a useful radiochromatogram, therefore, radiodetection using accelerator mass spectrometry was used.

Once again, due to the high cost and labour intensive nature of the sample preparation process for AMS, only one sample, a tryptic digest of the 4 hour incubation of $[^{14}\text{C}]$ SB-649868 with CYP3A4 Supersomes™, was selected for AMS analysis. The sample was prepared using the protocols described in Experimental Section 2.19 by AMS experts (Adrian Pereira and Claire MacIntyre for sample preparation, and Steven Corless for sample analysis.). For this analysis, a total of 96 fractions (collected at 25 second intervals between 5 and 45 minutes of the LC analysis) were analysed by AMS. The reconstructed radiochromatogram is shown in Figure 4.18.
The presence of numerous peaks in the reconstructed AMS radiochromatogram appears to reinforce the hypothesis that the highly reactive epoxide intermediate binds to several residues on the proteins contained in the sample. When compared to Figure 4.12, the representative radiochromatogram for the metabolites observed after a 4 hour incubation of $[^{14}\text{C}]$ SB-649868 with CYP3A4 Supersomes™, analysed using the same LC/MS method as for the AMS radiochromatogram, the peaks observed in the AMS radiochromatogram do not appear to correspond to any of the major metabolite peaks. This is as expected, since the pellet was extensively washed with phosphate buffer, prior to digestion, to remove any unbound drug-related material.

Based on the retention time data from the AMS radiochromatogram, the LC/MS data previously generated in Sections 4.4.1 and 4.4.2 were re-interrogated manually at the retention times corresponding to the radio peaks, and compared with the no drug control; however, no modified peptides were detected.

The radiochromatogram for the $[^{14}\text{C}]$ SB-649868 tryptic digest is in contrast to that generated for acetaminophen (Chapter 3, Figure 3.26) which shows one peak, indicating modification of...
the CYP2E1 at only one position. The presence of numerous peaks in the radiochromatogram for SB-649868 is concerning in terms of the sensitivity of the MS analyses currently available; the drug appears to have modified the proteins at numerous positions; therefore, the abundance of each individual modified peptide will be much lower, than if modification had occurred in only one position. These numerous modifications could also be an indication of several mechanisms of modification occurring, although the glutathione trapping data does not support this.

The presence of numerous modifications of the proteins in the sample does potentially pose a problem in terms of their detection; however, analysis of the constituent amino acids should circumvent this issue. Assuming that modification generally occurs on cysteine residues and, in each case, by the same mechanism, i.e. a similar mechanism to that proposed in Figure 4.16, complete digestion of the proteins at the amino acid level would mean that all of the modified cysteines should co-elute, thereby negating the decrease in sensitivity seen in the peptide analysis due to multiple modifications. The protein pellets from the incubations with human liver microsomes and CYP3A4 Supersomes™ were therefore digested using pronase and analysis of the resultant amino acids conducted. In addition, as described in Chapter 3, derivatisation of the amino acids with 6-aminoquinolyl N-hydroxysuccinimidyl carbamate was used to increase the hydrophobicity of the amino acids, improve the chromatographic retention, and increase the MS sensitivity compared. The characteristic loss of m/z 171 as a result of CID fragmentation of each derivatised amino acid (See Figure 4.20) was utilised to develop more a sensitive precursor ion scanning MS method.

4.4.4 Digestion with pronase to amino acids and LC/MS analysis

CYP3A4 Supersome™ and human liver microsome incubations with SB-649868 were prepared using the method described in Experimental Section 2.2. These were digested to their constituent amino acids with pronase and derivatised with 6-aminoquinolyl N-hydroxysuccinimidyl carbamate using the methods described in Experimental Section 2.20 and 2.21, respectively, prior to analysis by LC/MS method as described in Experimental Section 2.22 and Chapter 3.5.5, monitoring the precursor ions of the common fragment ion m/z 171. LC/MS analysis was conducted on a triple quadrupole API Sciex 5500 instrument, which was capable of performing tandem MS scanning experiments such as precursor ion scanning and multiple reaction monitoring. This was coupled to a Waters Acquity UPLC system, using the LC method utilised in Chapter 3. An amino acid standard mix was purchased from Waters Ltd. and derivatised using the method described in Experimental Section 2.21, to test the system.
suitability before each run of samples. A representative extracted ion chromatogram showing the chromatographic separation of this standard is shown in Chapter 3, Figure 3.30.

Figure 4.19: LC/MS analysis of pronase digestion of protein pellets retained following incubation of SB-649868 with CYP3A4 Supersomes™, showing the extracted ion chromatograms for ion m/z 785, the hydrated cysteine conjugate, from the precursor ion scanning MS data for (A) the no drug control, and (B) the 4 hour incubation.
Figure 4.20: Loss of the 6-aminoquinolyl N-hydroxysuccinimidy carbamate fragment from a derivatised hydrated cysteine conjugate of SB-649868

Extracted ion chromatograms for the SB-649868 hydrated cysteine conjugate (m/z 785, isolation window 1 Th) for the 4 hour CYP3A4 sample incubation and the no drug control are shown in Figure 4.19, with the postulated structure of the modified cysteine shown in Figure 4.20. The data show the presence of a signal peak at 8.2 minutes in the sample which is not present in the no drug control corresponding to the expected m/z of a hydrated derivatised cysteine. This suggests that modification of proteins occurs only on cysteine residues and supports the hypothesis that the modification proceeds via a reactive epoxide. Unfortunately, this peak could not be verified by full scan MS analysis due to the relatively inferior sensitivity of a full mass range scanning method compared to targeted precursor ion scanning, once again reinforcing the hypothesis that the key to the successful detection of covalent adducts is the sensitivity of the method used. The lack of a method for the chemical formation of an epoxide of SB-649868 with which to produce a reference standard, as utilised in Chapter 3 for acetaminophen, also makes the authentication of the peak as a genuine modification of a cysteine residue difficult, however, since the peak does not appear in the no drug control, despite other endogenous peaks being present in both samples, there is no reason to suspect that the peak is an artefact of the analysis.

4.4.5 Digestion to peptides and LC/MS analysis using MRM

The successful application of highly sensitive tandem MS methods to the identification of covalently modified peptides was demonstrated in Chapter 3 with the identification of an acetaminophen-modified T56 peptide of CYP2E1. For SB-649868, a precursor ion scanning method was successfully used to identify SB-649868-modified cysteine residues in the amino
acid analysis described in Section 4.4.4., therefore MRM methods were developed based on the in silico digestion of cytochrome P450 CYP3A4, with trypsin, with two missed cleavage sites allowed, and taking into account the mass change associated with carboxymethylation (from the reduction and alkylation with iodoacetamide applied to peptides prior to trypsin digestion). This in silico digestion was performed using BioLynx™ (Waters Ltd.) and was based on the sequence described in the Uniprot database, as described previously. Based on the amino acid data generated in Section 4.4.4, all cysteine containing peptides were selected and the mass modified by the addition of SB-649868 (+493.1 Da, to represent addition of the peptide across the epoxide), and the addition of SB-649868 minus the facile loss of water (as observed in the glutathione trapping spectra, +475.1 Da), to predict the resultant m/z value of the potentially modified peptides. The +2, +3, +4 and +5 charge states of each peptide were considered up to m/z 2000. As described in Chapter 3, since it was impossible to predict the largest fragment ion expected for each peptide in order to optimise each individual MRM experiment, the $y_1$ ion was selected. Since trypsin was used as the protease of digestion, the $y_1$ ion used was either m/z 175 for arginine terminal peptides, or m/z 147 for lysine terminal peptides, with the exception of the terminal peptide. The MRM transitions were divided into batches of approximately 15 experiments per analysis.

CYP3A4 Supersome™ and human liver microsome incubations with SB-649868 in the presence of a NADPH cofactor were prepared as detailed in Experimental Section 2.2. The protein pellet from these incubations were digested with trypsin, as described in Experimental Section 2.14, and then analysed using an LC/MS system comprising an Acquity UPLC and API Sciex 5500 mass spectrometer, and using the chromatographic and MRM MS methods described in Experimental Section 2.29. Controls containing no SB-649868 were also analysed for comparative purposes.

The analysis of digested proteins from the CYP3A4 Supersome™ incubations using MRM experiments produced a minor peak for the modification of one peptide of cytochrome P450 3A4, as shown in Figure 4.21. The peak at 32.9 minutes corresponds to the addition of 493 Da to the doubly charged T9 peptide (EC*YSVFTRN). No additional modified peptides were observed in these analyses, or in the analyses of human liver microsome incubations. Again, the low intensity of this peak supports the lack of data using less sensitive full scan LC/MS analyses on the QTof Premier.
Figure 4.21: Extracted MS data showing the MRM transition m/z 806→175 from the LC/MS analysis of the pronase digestion of the protein pellet following incubation of CYP3A4 Supersomes™ where (A) shows the no drug control, and, (B) the 4 hour incubation with SB-649868

Whilst this peak was detected in the 4 hour CYP3A4 Supersome™ incubation sample and is absent from the no drug control, it was not detected by full scan LC/MS therefore it was not
possible to provide any other structural data to support its assignment. Furthermore, the retention time of the peak (approximately 33 minutes) does not correspond to any of the larger peaks observed in the AMS radiochromatogram shown in Figure 4.18. This calls into question the reliability of the assignment and suggests that this peak may not be a drug modified peptide at all. This uncertainty highlights the importance of having additional data to support the assignment of modified peptides. For example, in the assignment of an acetaminophen-modified peptide of CYP2E1, described in Section 3.5.1.3 of Chapter 3, the assignment was supported by comparison of the retention time of the peak with the peak detected in the AMS radiochromatogram, and also by the use of a chemically generated modified peptide standard. Unfortunately, it was not possible to produce a chemically generated epoxide standard of SB-649868 so this comparison has not been possible for this work.

Whilst the added sensitivity of tandem MS methods for the detection of modified peptides is certainly useful for detecting modifications which would be missed using full scan experiments on the QTof Premier instrument, the lack of supporting structural information generated certainly can call into question any subsequent peptide assignments. The use of the more sensitive Waters Synapt G2S instrument described in Chapter 3 was therefore explored.

4.4.6 Digestion to peptides and LC/MS analysis using MS6 on the Synapt G2S

An LC/MS method using an Acquity UPLC coupled to a Waters Synapt G2S was devised incorporating low and high collision energy full scan MS6 functions, the details of which are recorded in Experimental Section 2.26. The experimental procedure for the incubation of CYP3A4 Supersomes™ with SB-649868, and the subsequent digestion of proteins with trypsin, is detailed in Experimental Section 2.2 and 2.14, respectively. 0, 1, 2 and 4 hour incubations were conducted, together with no drug and no cofactor controls for comparison. Data were processed using BioPharmaLynx using the protein sequences of human cytochrome P450 3A4 (195), NADPH cytochrome P450 reductase (298) and cytochrome b5 (299), and trypsin as the digest reagent with 2 missed cleavages allowed. The mass tolerance was 5 ppm for the MS match and 10 ppm for the MS6 fragment ion match, with leucine enkephalin (m/z 556.2771) used for lock mass correction. Carbamidomethylation on cysteine residues (+57 Da), SB-649868 (+493.15 Da), and SB-649868 with the in-source loss of water (+ 475.14 Da), to cysteine residues were selected as variable modifications, with no maximum number of modifications given. In addition, a manual comparison of the sample data, and the no drug control data was carried out.
As seen in Chapter 3, the protein coverage was in excess of 80% for each of the proteins analysed, with an example protein coverage map shown in Figure 4.22. Peptides identified in both the no drug control and the sample (in this case the 4 hour incubation of SB-649868 with CYP3A4 Supersomes™) are shown in green, in blue where they are present in the no drug control only, and in orange if present in the sample only.

<table>
<thead>
<tr>
<th>CYP3A4</th>
<th>Control Coverage (%)</th>
<th>Combined Coverage (%)</th>
<th>Analyte Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>85.0</td>
<td>89.7</td>
<td>84.2</td>
</tr>
<tr>
<td></td>
<td>Control Unique Coverage (%)</td>
<td>Common Coverage (%)</td>
<td>Analyte Unique Coverage (%)</td>
</tr>
<tr>
<td>1:10 to 50</td>
<td>NIPKSFTRL AQRFGPVFTL YVGSQRMVVM HGYKAVKEAL LDYKDEFSGR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:50 to 160</td>
<td>GOLPAFHAR DPGIIPNGNP TWKDRRSSL TLRNYGMGK QGNERSIQRE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:150 to 200</td>
<td>AMFLLEALRK TQGPPDFTF LIGCAPCVNI ADILFRKHFD YNDEKLKLML</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:200 to 250</td>
<td>YLFHNLHLLI STPSLQYNN FFSFLHYLPQ SHRVYIKNVQ EYKVYSEVY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:250 to 300</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1:300 to 350</td>
<td>BTTSSTLFLY GLIIKMKYDF EEEKLHEBID RVVSFRIPQA JKPRQEMPYM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:350 to 400</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1:400 to 450</td>
<td>QEFEDPEKFK PEFLNENEGK FYKSDYKFKE STGKRPVCAGE GLARDELFL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:450 to 493</td>
<td>LCAILOHFNL KPLVDPKDID LSPHIGGC FFPRYKLCVI PRG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.22: BioPharmaLynx output showing the percentage protein coverage for CYP3A4 following LC/MS analysis on the Synapt G2S instrument of the 4 hour incubation of SB-649868 with CYP3A4 Supersomes™

As shown in Figure 4.22, the protein is very well covered by the analysis, with the exception of the first 51 amino acid residues. Only one position of tryptic cleavage (the arginine residue at amino acid position 24) exists in this region. Proteolytic cleavage at this residue should result in two large peptides (24 and 27 amino acids long) which may suffer from reduced MS sensitivity due to that ionisation occurring on a large number of sites on the peptide, resulting in the signal being distributed over several charge states, therefore, it is not surprising that this region is not covered by the LC/MS analysis. Since the 1-51 amino acid region does not contain any cysteine residues, the fact that peptides from this region were not observed should not affect the identification of modified peptides.

Several SB-649868-modified peptides were identified by BioPharmaLynx, however each of these modified peptides were also detected in the no drug control sample and were therefore considered to be false positives. No genuine SB-649868-modified peptides were detected. This
is disappointing given the improved sensitivity of the Synapt G2S instrument. However, considering that the quantitative levels of binding are low (approximately 2% modification of total protein by SB-649868, from Table 4.1) and the number of modified peptide peaks observed in the trypsin digest sample by AMS (Figure 4.18), it is not completely surprising. As discussed, in Chapter 3, efforts to concentrate the sample could include the use of biotinylation of SB-649868 and affinity chromatography.

4.5. Conclusions and future work

The identification of a SB-649868-modified cysteine moiety in the amino acid analysis presents the first confirmation of how the postulated reactive species binds to proteins during in vitro incubation of SB-649868 with metabolising enzymes. The presence of an ion in the precursor ion scanning experiment (Section 4.4.4) at m/z 785 corresponds to the formation of a hydrated cysteine conjugated, which has been derivatised with 6-aminoquinolyl N-hydroxysuccinimidy carbamate. This confirms the hypothesis suggested by the glutathione trapping data that binding to proteins occurs on cysteine residues, with a mechanism consistent with the nucleophilic addition of the free thiol across the epoxide, to form a hydrated cysteine.

Whilst a modified amino acid was observed, detection of modified peptides after in vitro incubation and trypsin digestion was not successful, even with the most sensitive instrument available within GSK, the Waters Synapt G2S. The quantitative levels of binding for SB-649868 were similar to the level observed for acetaminophen (both approximately 2% protein modified), however, the AMS chromatogram generated for SB-649868 revealed that covalent modification had occurred on several peptides (possibly on several proteins), in contrast to acetaminophen, where only one peak was observed. In terms of sensitivity, this represented a significant decrease in abundance of each individual modified peptide. The data in this chapter again highlight the limitations of current instrumentation in identifying the covalent adducts of reactive metabolites to protein targets and the difficulties in identifying and characterising any modifications observed. This was made more difficult for SB-649868, having no chemical means of promoting the formation of a reactive epoxide, which could then be used to produce reference standards for glutathione trapping, amino acid and peptide LC/MS experiments.

Whilst a small peak corresponding to the potential modification of the T9 peptide of CYP3A4 was identified during the tandem MS MRM analyses, the retention time of the peak does not correspond to any of the major radio peaks in the AMS chromatogram. This, coupled with the lack of a reference modified peptide, or the sensitivity to conduct confirmatory full scan LC/MS experiments casts doubt on the reliability of the assignment.
Attempts within GSK to form the benzofuran epoxide of SB-649868 using electrochemical oxidation (see Chapter 5) were unsuccessful, with the drug mostly unchanged with increasing voltage, (unpublished work, Mike Nash, GSK). One possibility for the chemical formation of epoxides could be the use of the Fenton’s reagent (373), which is a combination of hydrogen peroxide with an iron (II) catalyst. The reaction is described in the equation: 

\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{OH}^{-} + \text{OH}^{\cdot} + \text{Fe}^{3+} \]  

where hydrogen peroxide oxidises iron (II), resulting in the formation of a hydroxy radical which can go on to act as an oxidising agent. Fenton’s reagent has been used successfully by Panzella et al. (374) to produce epoxides of retinoic acid, and by Brunmark et al. (375) for the formation of epoxides of benzoquinones. Zbaida and Kariv (376) reported the use of Fenton’s reagent to simulate the hepatic metabolism of stilbene, an acyclic olefin and successfully isolated and characterised epoxide products. The exploration of the use of Fenton’s reagent as a means to produce the reactive epoxide of SB-649868, or other drugs of interest, and how these chemical epoxides can be used to mimic binding of enzymatically formed epoxides would be very interesting as a focus of future study.

The importance of maximising sensitivity and the application of non-enzymatically produced reactive species as a means to investigate covalent binding to proteins is further explored in the next chapter.
Electrochemical oxidation of amodiaquine and detection of covalent protein-adducts using LC/MS

Amodiaquine (trade names Camoquin or Flavoquine) is a 4-aminoquinoline, used for the treatment and prevention of malaria, which was withdrawn from general prophylactic use because of incidences of agranulocytosis and hepatotoxicity (377, 378) and is now only recommended for the treatment of chloroquine-resistant malaria infections. The exact mechanism for this toxicity is not completely understood although many hypotheses have been put forward in the literature. While direct dose dependent toxicity (379, 380) may be a factor, it has been suggested that oxidative activation of amodiaquine to a reactive metabolite, with subsequent covalent binding to large macromolecules could initiate an immune response, which would be consistent with the high incidence of anti-amodiaquine antibodies in patients suffering from adverse reactions to amodiaquine (381-384).

Upon oral dosing of amodiaquine to humans, the drug is rapidly absorbed and extensively metabolised with very little parent drug detected in the plasma (385). White et al. (386) have shown in in vivo pharmacokinetic studies that extensive first-pass biotransformation of amodiaquine is the primary route of elimination in humans, with the liver being the major site of this metabolism. The major circulating and urinary metabolite is N-desethylamodiaquine (387) as shown in Figure 5.1; minor metabolites include the 2-hydroxy-desethylamodiaquine and N-bisdesethylamodiaquine. While the formation of desethylamodiaquine is rapid, Winstanley et al. (385) have shown that its elimination is slow, with a half-life in excess of 100 hours. Since both amodiaquine and its desethyl metabolite have been shown to have antimalarial activity, and as amodiaquine itself is rapidly cleared, it can almost be considered a pro-drug.
Figure 5.1: The major metabolites of amodiaquine in human urine and plasma (387)

Numerous *in vitro* and *in vivo* studies have shown that amodiaquine is highly susceptible to oxidation. Maggs *et al.* (384) demonstrated the spontaneous auto-oxidation of amodiaquine in air at physiological pH and also in the presence of horseradish peroxidases, as well as oxidation due to incubation with human liver microsomes in the presence of NADPH. The major oxidative product was demonstrated to be the potential reactive metabolite amodiaquine quinone imine (AQMI) (Figure 5.1). The authors were able to show the irreversible binding of a ¹⁴C-labelled analogue to bovine and human serum albumin, suggesting the liability of AQMI to covalently bind to protein. Tingle *et al.* (388) demonstrated a similar oxidation of amodiaquine to AQMI and the subsequent covalent binding to protein in *in vitro* incubations with stimulated polymorphonuclear leucocytes (PMN). This is particularly significant as drug hypersensitivity reactions such as agranulocytosis have been attributed to the formation of reactive metabolites by PMN. They have also shown the formation of glutathione adducts of amodiaquine from endogenous glutathione, with a corresponding depletion of cellular levels of glutathione indicating that the oxidation of amodiaquine is taking place intra-cellularly. This is significant as it may offer an explanation for the direct toxicity which is associated with the administration of amodiaquine to humans.

Studies *in vitro* with recombinant human cytochrome P450 isoforms have identified CYP2C8 as the major cytochrome P450 enzyme in the liver responsible for the metabolism of amodiaquine to its major metabolite N-desethylamodiaquine (389). However, for all the evidence for the formation of amodiaquine covalent adducts with proteins, the detection of
such adducts remains problematic. The isolation of a single protein from microsomal incubations or in vivo samples requires complex chromatographic separation methods (390), while the extent of modification of a protein by a small molecule drug is very low. Therefore, an alternative approach using electrochemistry to produce oxidative reactive metabolites has been devised (135, 136). The use of electrochemistry to mimic cytochrome P450 mediated oxidation reactions is already in widespread use within academia and the pharmaceutical industry to give an insight into potential Phase I drug metabolism reactions (391-394). Phase II reactions, including the conjugation of potentially reactive metabolites to glutathione, have also been simulated (395, 396). The advantage to simulating Phase I and II metabolism using electrochemistry is that it gives the analyst the opportunity to produce sufficient quantities of the desired metabolite to obtain definitive spectroscopic data for its structure, i.e. by using LC/MS and NMR, and therefore to formulate a possible mechanism for the metabolite’s formation. A disadvantage is the assumption that the metabolites formed using the electrochemical process are the same metabolites observed using in vitro enzymology methods, or that the mechanism for their formation is similar, which may not always be the case.

This chapter describes the use of electrochemistry, on-line with LC/MS, to produce metabolites of amodiaquine and an assessment of their reactivity by the demonstration of binding to glutathione and large molecular weight (MW) proteins such as bovine serum albumin (BSA) and CYP2C8. While Lohmann and Karst (136) have previously demonstrated and characterised the binding of electrochemically oxidised amodiaquine to β-lactoglobulin A (approx. 15 KDa) in their 2008 paper, binding to large MW proteins such as BSA or to metabolising enzymes, has not previously been fully characterised. BSA was selected as a model protein as its molecular mass of approximately 67 KDa closely resembles the average molecular mass of the liver metabolising enzymes such as the cytochrome P450s. Due to numerous disulfide bridges, BSA has only one free cysteine residue, and therefore only one anticipated binding site. CYP2C8 was selected as a tool protein because it is the major cytochrome P450 enzyme in the liver responsible for the metabolism of amodiaquine, however there is evidence that the formation of reactive metabolites of amodiaquine may occur via a number of mechanisms including autooxidation, peroxidises and hydroperoxidases (384).

5.1 Generation of potential metabolites by electrochemical oxidation

The generation of electrochemical oxidation products of amodiaquine using the Coulochem III electrochemical cell, and the MS experiments to generate data on these products, are
described in Experimental Section 2.30. Briefly, a solution containing 0.1 mM concentration of amodiaquine in 80:20 (v/v) 20 mM aqueous ammonium acetate in water: acetonitrile was pumped through the ESA Coulochem III electrochemical cell and the potential on the cell increased stepwise (50 mV steps) from 0 to 700 mV. The effluent from the cell was infused directly into the electrospray source of a Thermo Scientific LTQ Orbitrap. MS and MS/MS data were generated on the electrochemical products of amodiaquine formed due to increase in potential.

The spectra generated at 0 mV (i.e. with no voltage applied to the electrochemical cell) are shown in Figure 5.2. The MS spectrum (A) shows m/z 356.1512 as the base peak, with the exact mass in good agreement (a difference of 3.4 ppm) with the expected mass of the amodiaquine (derived from its empirical formula shown in Figure 5.3). The empirical formula of amodiaquine contains a Cl atom, and the $^{37}$Cl isotope peak can be clearly seen at m/z 358. The proposed fragments of amodiaquine based on the product ion spectra shown in (B) and (C) are summarised in Table 5.1.
Figure 5.2: (A) Mass spectrum generated by direct infusion of the effluent from the electrochemical cell (set at 0 mV) into the LTQ Orbitrap, (B) product ion spectrum of amodiaquine (m/z 356), and (C) product ion spectrum of m/z 283, a fragment ion of m/z 356
At 700 mV, the amodiaquine was almost completely oxidised to amodiaquine quinone imine (AQQI) (as shown in Figure 5.3), a potentially reactive metabolite previously observed by Maggs et al. (384)

![Diagram of amodiaquine to AQQI conversion](image)

Figure 5.3: Amodiaquine quinone imine (AQQI) – the major oxidative product at 700 mV in the electrochemical cell

The structure of AQQI was confirmed via direct infusion of the effluent from the electrochemical cell into the electrospray source of the MS, with the MS data obtained shown in Figure 5.4. The exact mass of the primary product at 700 mV (ion m/z 354.1363) was in good agreement (an error of 1.3 ppm) with the expected mass of the quinone imine, derived from its empirical formula as shown in Figure 5.3. When compared to the product ion spectra for unchanged amodiaquine (shown in Figure 5.2) the product ion spectra shown in (B) and (C) show a decrease in the m/z of fragment ions m/z 283 and m/z 255 to m/z 281 and m/z 253, respectively, a loss of 2 Th in both cases. This is indicative of the electrochemical product formed at 700 mV having two hydrogen atoms less on the nitrogen and oxygen substituted ring than unchanged amodiaquine, consistent with the proposed structure of AQQI. The proposed fragments of AQQI based on the product ion spectra shown in (B) and (C) are summarised in Table 5.1.
amodiaquine 700mV_090824144736 #53  RT: 0.33  AV: 1  NL: 4.03E8
F: FTMS + p ESI Full ms [60.00-1000.00]

(A)

354.1363
C_{20}H_{21}O_{13}Cl = 354.1368
-1.3414 ppm

320.1758
C_{20}H_{21}O_{12} = 320.1757
0.0371 ppm

358.1480

(B)

281.0
283.1
253.1
275.1
290.9
306.2
325.1
354.1
365.3

m/z
Figure 5.4: (A) Full scan mass spectrum generated with the electrochemical cell set at 700 mV, (B) product ion spectrum of m/z 354, the amodiaquine quinone imine, and (C) product ion spectrum of m/z 253, a fragment ion of m/z 354
Table 5.1: Summary of MS data for amodiaquine and AQQI

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<thead>
<tr>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td></td>
<td></td>
<td>m/z 248: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m/z 255: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m/z 238: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m/z 218: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td>354.1363</td>
<td><img src="image" alt="Amodiaquine quinone imine (AQQI)" /></td>
<td>m/z 283: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m/z 253: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m/z 281: <img src="image" alt="Fragment" /></td>
</tr>
</tbody>
</table>

The metabolite appeared to be stable in the MS source, with no reduction back to the parent molecule. This is in contrast to the chemically synthesised quinone imine of acetaminophen NAPQI, discussed in Chapter 3.4, which appeared to convert back to acetaminophen in an aqueous environment. Lohmann et al. (136) reported that the potential required for amodiaquine to undergo electrochemical oxidation was lower than that required for acetaminophen and clozapine, therefore the relatively low potential required to generate the quinone imine product of amodiaquine may suggest that amodiaquine is particularly labile to this oxidation and could be indicative of a potential route of metabolism in vivo.
The potential on the cell was increased to try to generate more oxidation products. The potential was optimised by step-wise (50 mV) increments to 1250 mV, to yield numerous oxidation products. The mass spectrum is shown in Figure 5.5.

**Figure 5.5:** Full scan mass spectrum from the direct infusion of the effluent of electrochemical cell (set at 1250 mV) into the MS source, showing the electrochemical oxidation products of amodiaquine

A summary of the MS and MS/MS fragmentation data (as described in Experimental Section 2.30) on the electrochemical products formed at 1250 mV is shown in Table 5.2 and Figure 5.6, with the spectra detailed in Appendix 4, A4.1 – A4.8.
Table 5.2: Summary of MS data for the electrochemical oxidation products of amodiaquine generated at 1250 mV

<table>
<thead>
<tr>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
</table>
| 297.0424         | ![Proposed Structure](image1) | m/z 269: [M+H]$^+$ - CO   
  m/z 251: m/z 269 - H$_2$O  
  m/z 241: m/z 269 - CO  
  m/z 240: ![Diagnostic Fragment](image2)  
  m/z 206: ![Diagnostic Fragment](image3)  
  m/z 234: ![Diagnostic Fragment](image4) |
| 298.0740         | ![Proposed Structure](image5) | m/z 281: [M+H]$^+$ - NH$_3$  
  m/z 280: [M+H]$^+$ - H$_2$O  
  m/z 270: [M+H]$^+$ - CO  
  m/z 269: ![Diagnostic Fragment](image6)  
  m/z 255: ![Diagnostic Fragment](image7)  
  m/z 253: ![Diagnostic Fragment](image8) |
<table>
<thead>
<tr>
<th>[M+H]$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(m/z)</td>
</tr>
<tr>
<td>299.0580</td>
</tr>
</tbody>
</table>

**Proposed Structure**

![Proposed Structure](image)

**Diagnostic Fragment Ions (m/z)**

- m/z 297:
  ![Fragment Ions](image)
- m/z 271: m/z 299 – CO
- m/z 269:
  ![Fragment Ions](image)
- m/z 251: m/z 269 – H$_2$O
- m/z 253: m/z 271 – H$_2$O
- m/z 243: m/z 271 – CO
- m/z 241: m/z 299 – CO
- m/z 240:
  ![Fragment Ions](image)
- m/z 214:
  ![Fragment Ions](image)
- m/z 206:
  ![Fragment Ions](image)
- m/z 205: m/z 241 – HCl

| 312.0534   |

**Proposed Structure**

![Proposed Structure](image)

**Diagnostic Fragment Ions (m/z)**

- m/z 294: [M+H]$^+$ – H$_2$O
- m/z 284: [M+H]$^+$ – CO
- m/z 267: m/z 284 – NH$_3$
- m/z 256: m/z 284 – CO
- m/z 239: m/z 256 – NH$_3$
- m/z 220: m/z 248 – CO
- m/z 248:
  ![Fragment Ions](image)
<table>
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<tr>
<th>[M+H]$^+$ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
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<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 297: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 270: m/z 296 - HCl m/z 269: <img src="image" alt="Fragment" /></td>
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<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 296: m/z 324 - CO m/z 283: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td>326.1054</td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 298: [M+H]$^+$ - CO m/z 253: <img src="image" alt="Fragment" /></td>
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<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 283: <img src="image" alt="Fragment" /> m/z 218: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 281: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td>340.0844</td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 235: m/z 271 - HCl m/z 228: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 271: m/z 299 - CO m/z 299: <img src="image" alt="Fragment" /></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 243: m/z 271 - CO m/z 253: m/z 271 - H2O m/z 252: m/z 271 - NH3</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Proposed Structure" /></td>
<td>m/z 272: m/z 271 - H2O m/z 271: m/z 299 - CO</td>
</tr>
</tbody>
</table>
AQQI is still detected in the spectrum at m/z 354.1362, however this is oxidised further at the higher potential (m/z 368.1156), as shown in Figure 5.6. The major oxidation product (m/z 340.0844) appears to be an oxidised form of the N-desethamodiaquine quinone imine (NDAQI). NDAQI was also detected at m/z 326.1054, together with the N-bisdesamodiaquine quinone imine (NBDAQI) (m/z 298.0740, exact mass data shown in Appendix 6) and its oxidised form at m/z 312.0534. These assignments are consistent with the fragmentation data described in Table 5.2. Electrochemical dealkylation at heteroatoms has also been noted for other compounds, most notably N-deethylation of lidocaine (397). The formation of both NDAQI and NBDAQI and their further oxidised products are significant as they are structurally similar to the major metabolites of amodiaquine detected in both human plasma and urine (36-39), re-enforcing the idea that the products of the electrochemical process have some correlation to the products seen via metabolic processes in vivo.
Figure 5.6: Electrochemical oxidation products of amodiaquine at 1250 mV. All structures are supported by the data shown in Table 5.2
5.2 Generation of glutathione adducts by electrochemical oxidation and MS analysis

The potential on the working electrode of the electrochemical cell was initially set to 1000 mV in order to try and trap the AQQI product formed at this potential with glutathione, thus demonstrating the reactive nature of the quinone imine and its potential to bind to protein, and mimicking protection mechanisms in the liver (as described in Experimental Section 2.31). Briefly, a solution containing 0.5 mM amodiaquine and 0.1 mM glutathione in 80:20 (v/v) 20 mM ammonium acetate: acetonitrile was passed through the electrochemical cell, with the potential set to 1000 mV. The effluent from the cell was infused directly into the electrospray source of the MS. The experiment was then repeated at the higher electrochemical potential of 1250 mV to try and trap some of the additional quinone imine species observed in Section 5.1 (Figure 5.5). A summary of these data are shown in Table 5.3 with the spectra detailed in Appendix 4, A4.9 – A4.12. These structures are supported with exact mass data and fragmentation data on the electrochemical products generated.

The mass spectrum shown in Figure 5.7 (A) was generated at 1000 mV and shows an exact mass ion at m/z 661.2201, an addition of 307.0833 Da to the [M+H]^+ of AQQI. This correlates well with the empirical formula C_{30}H_{38}O_{7}N_{6}ClS (with an acceptable error of 0.7033 ppm). The addition of 307.0833 Da is (within experimental error) indicative of the addition of C_{10}H_{17}N_{3}O_{6}S (MW 307.0838) to the AQQI molecule indicating a glutathione group attached, most likely, to a carbon-carbon double bond.
amodiaquine 1000mV + GSH 090824144736 #62-81  RT: 0.50-0.65  AV: 20  NL: 3.40E7
F: FTMS + p ESI Full ms [105.00-1000.00]

661.2201
C_{30}H_{38}O_{7} N_{6} Cl S = 661.2206
-0.7033 ppm

(1)

amo + gsh msms 661_120714143023 #28-39  RT: 0.26-0.35  AV: 7  NL: 1.11E6
F: FTMS + c ESI Full ms2 661.20@cid30.00 [180.00-1000.00]

588.1

(B)
Figure 5.7: (A) Mass spectrum of the product formed by the electrochemical oxidation (1000 mV) of amodiaquine in the presence of glutathione, (B) product ion spectrum for ion m/z 661, and (C) product ion spectrum for m/z 588, a fragment ion of m/z 661

The product ion spectra (shown in Figure 5.7 (B) and (C) and summarised in Table 5.3) are consistent with the addition of a glutathione moiety to the parent AQQI molecule, with characteristic losses of the glycine moiety (fragment ion observed at m/z 588), and glutamic acid (fragment ion at m/z 459) components of glutathione, and a fragment indicating the addition of a sulphur atom to the amodiaquine fragment ion of m/z 283 (fragment ion at m/z 315).
Table 5.3: Summary of MS data generated on the glutathione adduct of AQQI

<table>
<thead>
<tr>
<th>[M+H]+ (m/z)</th>
<th>Proposed Structure</th>
<th>Diagnostic Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>661.2201</td>
<td><img src="Image" alt="Proposed structure" /></td>
<td><img src="Image" alt="m/z 588:" /> <img src="Image" alt="m/z 459:" /></td>
</tr>
<tr>
<td></td>
<td><img src="Image" alt="Empirical Formula: C30H38ClN6O7S+" /></td>
<td><img src="Image" alt="m/z 532:" /> <img src="Image" alt="m/z 315:" /></td>
</tr>
</tbody>
</table>

While the data are consistent with the postulated glutathione adduct structure shown in Table 5.3, the product ion fragmentation data does not provide any definitive information on the positioning of the glutathione moiety on the amodiaquine molecule. However, based on the structure of the reactive AQQI molecule, the higher electronegativity value of oxygen rather than nitrogen means that the likely site of attack of a soft nucleophile such as glutathione is adjacent to the carbonyl.

Since the MS data was consistent with glutathione conjugation of the AQQI electrochemical product (shown in Figure 5.7), 5 mL of the effluent from the cell was collected into a vial and submitted for preparative LC purification with subsequent 1H NMR analysis. Preparative HPLC, and 1H NMR analysis was conducted on a standard of amodiaquine (Figure 5.8) and an isolated fraction of the postulated glutathione adduct to definitively identify the structure of the glutathione conjugate (Figure 5.9) using the method described in Experiment Section 2.32. Preparative LC of the glutathione adduct was performed by Rita Tailor and the NMR data was acquired by Steven Thomas, both from GlaxoSmithKline, Ware.

The addition of the glutathione moiety to the benzene ring at the position adjacent to the oxygen atom is shown by the absence of proton h from the spectrum (proton h is observed at approximately 7.2 ppm in the 1H NMR spectrum of the authentic standard of amodiaquine. The remaining protons of amodiaquine are largely unchanged, with the exception of protons f and g. The signal for proton g appears as a doublet in the 1H NMR spectrum for the authentic
standard due to proton g coupling with proton h. Since this coupling is absent when glutathione is adducted to amodiaquine, proton g then appears as a singlet. The addition of the S-CH₂-R moiety glutathione has a large shielding effect to protons para to this substitution i.e. proton f. Therefore, proton f moves approximately 0.2 ppm from 7.29 ppm to 7.08 ppm, which is consistent with NMR substitution tables such as those found in Pretsch, Clerc, Seibl and Simon (270). The effect is less pronounced for protons meta to the substitution i.e. proton g. Proton g exhibits a small upfield shift from approximately 7.35 ppm to 7.32 ppm.

The presence of the glutathione moiety is confirmed by the characteristic signals for the cysteine –a and –b protons (at approximately 4.40 ppm for the Cys-a signals, and 3.15 ppm and 3.28 ppm for the Cys-b protons, respectively), the glutamic acid –a, -b and –c protons (at approximately 3.60 ppm for the partially obscured Glu-a signals , and 2.40 ppm for the Glu-c signals, respectively) and the signals for the glycine moiety at approximately 3.58 ppm. The signals for the Glu-b protons are obscured underneath the large background protons signals for acetonitrile at approximately 1.97 ppm. In addition, the signals observed for both the Cys-a protons and the i protons of amodiaquine have been reduced due to their proximity to the water signals at 4.22 ppm, which were reduced by a pre-saturation pulse on acquisition.
Figure 5.8: $^1$H NMR data on a standard of amodiaquine (NMR data generated by Steven Thomas, GSK Ware)
Figure 5.9: $^1$H NMR data on the glutathione conjugate of AQQI (NMR data generated by Steven Thomas, GSK Ware)
A proposed mechanism for the addition of glutathione to a quinone imine group is given in Figure 5.10.

**Figure 5.10: Proposed mechanism for the addition of glutathione to AQQI**

This is a similar mechanism to that observed for acetaminophen toxicity on overdose (see Chapter 3), with both proceeding via the reaction of the sulphur ion of glutathione attacking quinone imine moieties. Glutathione itself is a soft nucleophile and as such will spontaneously react with soft electrophiles in a non-enzymatic environment, as observed in this case.
The data generated at the higher potential of 1250 mV shows the formation of several additional glutathione conjugates, each corresponding to GSH conjugates of the oxidative products shown in Figure 5.6. The mass spectrum is shown in Figure 5.11. For display purposes, the ions are displayed to 1 decimal place. However, exact mass data was generated and is summarised in Table 5.4, with individual mass spectra shown in Appendix 6.

Figure 5.11: Mass spectrum of the products formed by the electrochemical oxidation (1250 mV) of amodiaquine in the presence of glutathione

Several glutathione conjugates were observed in the sample; however a large proportion of glutathione (m/z 308) and several electrochemical products of amodiaquine remain unreacted, despite amodiaquine being in large excess of the (5-fold) in solution passing through the electrochemical cell. This implies that the non-enzymatic conjugation of GSH in solution is not very efficient during the time-scale of the passage of the solution through the electrochemical cell. This could be further investigated in future experiments by decreasing the flow rate from the 50 µL/min used for these experiments to see if the proportion of unreacted glutathione increases. However, GSH adducts for the N-desethylamodiaquine quinone imine (m/z 326, GSH conjugate at m/z 633), and numerous other oxidative product were observed. Product ion spectra for the major glutathione conjugates are shown in Appendix 6 and the data
summarised in Table 5.4. Exact mass data supported the proposed structures with an error of less than 5 ppm. Sites of glutathione conjugation have been assumed based on a similar mechanism of formation as proposed for AQQI in Figure 5.10. The peaks noted at m/z 681.1, 703.1 and 725.1 did not have an isotope pattern indicative of the presence of a chlorine atom and so were likely not related to amodiaquine, although it is possible that electrochemical dechlorination (398) could be taking place.
Table 5.4  Summary of MS data on products formed by the electrochemical oxidation (1250 mV) of amodiaquine in the presence of glutathione

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<td>m/z 447:</td>
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<td>m/z 331:</td>
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<td>[M+H]^+ \  (m/z)</td>
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m/z 588: ![Fragment](image)
m/z 459: ![Fragment](image)
m/z 570: m/z 588 – H₂O
m/z 504: ![Fragment](image)
m/z 444: ![Fragment](image)
m/z 441: m/z 459 – H₂O
m/z 315: ![Fragment](image)
5.3 **Electrochemical synthesis of drug-protein adducts and intact protein analysis**

A solution containing 0.5 mM concentration of amodiaquine in 80:20 (v/v) 20 mM aqueous ammonium acetate in water: acetonitrile was pumped through the electrochemical cell, with the potential set to 800 mV. 1 mL of effluent from the electrochemical cell was collected in to a vial containing 1 mL of a 0.1 mM solution of bovine serum albumin in 8M urea (so that the drug related material was in 5-fold molar excess) and incubated at 37°C in a heated water bath for 30 minutes, as described in Experimental Section 2.33. Unreacted BSA was also incubated at 37°C for 30 minutes as a control. Mass spectra were generated for the intact protein unreacted BSA and the BSA derivative of the electrochemically generated AQQI product after LC/MS analysis (as described in Experimental Section 2.34). The unprocessed data are shown in Figure 5.12, with the Max Ent 1 processed data in Figure 5.13 (Max Ent 1 processing parameters are described in Experimental Section 2.34).
Figure 5.12: Intact protein mass spectra from the LC/MS analysis of (A) unreacted BSA, and (B) BSA reacted with electrochemically generated AQQI (800 mV) at 37 °C for 30 minutes.
Figure 5.13: Deconvoluted mass spectra from the LC/MS analysis for (A) control unreacted BSA and (B) the BSA-AQQI adduct formed after incubation of electrochemically generated AQQI with BSA at 37 °C for 30 minutes
DTHKSE IAHRFKDLGE EHFKGLVLIA FSQYLQOCPF  DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL
HTLFGDELCK VASLRYEYMDA MADCDEKQEP ERNECEFLSHK DDSLDPKLLK PDPNLTLCDEF
KADKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQEQ CCAEDKGAQL LPKRIKMRE KVLAASQR
LRQSIIQFGLERALKAWSVA RLINQKFPKAE FVEVTAVLTD LTKVHEECCH GDLLEDADDR ADLAKICQDNL
QDTISSKLKE CDDKPPLLEKS HADAEVEKDA IPENLPLATA DFAEDKDVCK NYQEAKDAFL GSFLYEYSRR
IVRYTRKVPQ VSTPTLVEVS RSLGKVGRTE CTKPESERMP CTEDYLSLIL NRCVLHHEKT PVSEKVTCC
TESLNRPC FSALTPDETY VPKAFDEKLF TFHADICLTP DTEKIQKKTQ ALVELLHKP KATEEQLKTV
MENFVAFVDK CCADDEKAC FAVEGPKLTV STQTLA

Figure 5.14: Amino acid sequence of BSA, shown with disulfide bridges between cysteine residues (399, 400)

The amino acid sequence of BSA is shown in Figure 5.14. The molecular mass of BSA is quoted in the literature as 66430.3 Da (401). From the deconvoluted spectrum in Figure 5.13, the experimental MW observed 66436 ± 6 Da is in good agreement with the theoretical MW and is within an error of 0.01%, the average expected error for the determination of intact protein mass using ESI-MS (402, 403). The deconvoluted spectra for native BSA and the BSA-AQQI derivative (Figure 5.13) show an increase in the mass of the protein of 350 Da after incubation with AQQI. The mass of amodiaquine quinone imine is 353 Da, thus suggesting the addition of one molecule of drug-related material to the protein structure. The disulfide bridges within the structure of BSA mean that only one cysteine residue is free to bind to drug-related material (399), hence we would expect to see the addition of only one amodiaquine related moiety.

While the change in mass of BSA was 350 Da rather than 353 Da, the error in the original mass calculation was approximately ± 6 Da, therefore this error is reasonable and is within the error limits observed within the experiment. This inaccuracy is most likely due to a combination of errors within the experiment e.g. errors in instrument calibration, in the deconvolution algorithm and subsequent calculation of the mass of the protein. The accuracy of the mass measurement could be improved by the use of a reference standard such as a [Glu1]-Fibrinopeptide B (GluFib) as an external calibrant to correct any minor shifts in mass. GluFib (Mw 1570.60 Da) is a suitable external calibrant with its use well established and documented in the literature (404). Calibration of the ToF based on the MS/MS fragment ions of GluFib would calibrate the MS over the mass range most for the charge-state distribution of intact BSA. Both the untreated BSA and BSA incubated with electrochemically generated AQQI samples were
digested with trypsin to generate further structural information to confirm both the position of binding, and the structure of the bound species, as described in Experimental Section 2.33.

5.4 Trypsin digestion of the electrochemically synthesised drug-protein adduct and LC/MS analysis

Control BSA and BSA incubated with AQQI were digested with trypsin (Experimental Section 2.33), and analysed by LC/MS² to identify the tryptic peptide to which AQQI was bound (Experimental Section 2.35). The data were processed using BioPharmaLynx (Waters). The protein sequence used for BSA was accessed from the UniProt website (400) with trypsin as the digest reagent and 2 missed cleavages allowed. Carbamidomethyl (+57 Da) added as an expected modification (from the alkylation of the protein with iodoacetamide prior to digestion with trypsin) and amodiaquine (+353.1 Da) added as a custom modification. The mass tolerance was 50 ppm for the MS match and 100 ppm for the MS² fragment ion match. These relatively wide variables were selected to do an initial assessment to identify potential candidates of peptides modified with amodiaquine, with the intention that any peptides identified would be subjected to more vigorous investigation such as MS/MS. BioPharmaLynx highlighted several differences between the control and analyte samples. As shown in Figure 5.15, BioPharmaLynx identified two triply charged peaks which correlated to the addition of 353 Da to the T5 peptide in the analyte sample, shown by ions of m/z 930.1 (T5 is the tryptic peptide which, in computer simulated digests (ProteinLynx, Waters, Manchester, UK), is expected to contain the free cysteine residues).
Figure 5.15: Screen-shot of BioPharmaLynx processing results indicating potential modified peptides of T5

Mass chromatograms were extracted from the data for ion m/z 930.1, corresponding to the mass expected for the triply charged T5 peptide modified by the peaks identified by BioPharmaLynx and compared with the equivalent mass chromatogram for the control sample (Figure 5.16)
Figure 5.16: Extracted ion chromatograms for (A) m/z 930.1 (0.1 Th window) for the trypsin digest of the incubation of electrochemically generated AQQI and BSA, and (B) m/z 930.1 (0.1 Th window) for trypsin digest of the unreacted BSA protein control

The peak at retention time 27.2 minutes was immediately discounted as it appeared in both the control and AQQI-modified samples, and so was a false positive in the BioPharmaLynx assessment. The remaining peak at retention time 27.0 minutes was unique to the AQQI-incubated sample and was considered for further evaluation. Extracted ion chromatograms corresponding to the m/z of the T5 peptide plus the mass of AQQI (MW 353 Da) for the [M+2H]^{2+} and [M+4H]^{4+} ions were generated from the mass spectra and compared to the corresponding mass chromatograms for the control BSA digest. No additional unique peaks were detected.

Mass spectra showing the [M+3H]^{3+} ion clusters of T5 (m/z 812.4) at retention time 26.7 minutes and T5 plus AQQI (m/z 930.1) at retention time 27.0 minutes are presented in Figure 5.17.
Figure 5.17: Mass spectra for (A) the unmodified T5 peptide ([M+3H]^{3+}) in the trypsin digest of the unreacted BSA control, and (B) the AQQI modified T5 peptide ([M+3H]^{3+}) in the trypsin digest of the incubation of electrochemically generated AQQI and BSA.

As shown in Figure 5.17 (A), the component yielding the [M+3H]^{3+} ion m/z 812.4 co-elutes with that yielding a doubly-charged peptide ion at m/z 811.4 in the control BSA digest sample.
There was no trace of unmodified T5 peptide in the AQQI modified digest sample, suggesting that all the protein in the sample has been modified by AQQI. The experiment was conducted using a large excess (5-fold) of AQQI compared to BSA. Since BSA has only one free cysteine, it was expected that all of the BSA would react with an excess of AQQI, leaving no unreacted BSA remaining in the samples. This was observed which was in contrast to the experiment with glutathione (Section 5.2), where a proportion of glutathione remained unreacted despite being in solution with a 5-fold molar excess of the electrochemically produced reactive species, implying that the non-enzymatic conjugation of glutathione was inefficient. One reason for this difference may be the comparative time-scales for the reactions. In the glutathione experiment, a solution containing both glutathione and amodiaquine is passed through the electrochemical cell, directly into the MS source. In the experiment with BSA, reacted amodiaquine (AQQI) was collected into a vessel containing BSA and allowed to react at 37°C in a heated water bath for 30 minutes. This may indicate that the reaction of AQQI and a free cysteine moiety such as found in glutathione and BSA occurs relatively slowly and the prolonged incubation time therefore may have allowed for the complete reaction of the BSA protein. This could be investigated further by monitoring the depletion of glutathione from a reaction with AQQI in a heated water bath, over a time-course.

Both the modified and unmodified peptides had a retention time of approximately 27 minutes on the chromatographic system, indicating that the addition of the amodiaquine moiety to the peptide did not significantly alter its retention properties on the C18 column.

The amino acid sequence of the T5 peptide is as follows (K)GLVLIAFSQYLQCPFDEHVK(L). Based on the MS and NMR data generated on the glutathione adduct of AQQI, it was anticipated that the AQQI moiety would bind to the peptide via the cysteine residue. Product ion spectra were generated to confirm that AQQI binds to the cysteine residue (Figure 5.18) and to generate some structural information. Ions m/z 812.4 and 930.1 were selected as precursor ions in the control BSA sample and in the AQQI modified BSA digest, respectively, using a low mass resolution value of 15 arbitrary units in the instrument tuning parameters (on the Waters QTof Premier, this equates to an approximate mass ion selection window of 0.2 Th), which was insufficient to isolate the m/z 812.4 ion of interest from the co-eluting ion observed at m/z 812.4 (which is part of the doubly charged cluster for ion at m/z 810.9) in the control BSA digest. It is likely that some of the unassigned peaks in the product ion spectrum for the control BSA sample are derived from this co-eluting species. These data are shown in Figure 5.18.
Fragmentation of the modified peptide proves that the modification has taken place on the cysteine residue. For the modified BSA digest sample, the y ion series identified in the product ion spectrum remains unmodified up to the y7 position, indicating that the modification must occur on or after the 8th residue from the C-terminus of the peptide. The mass of the y8 ion has increased by 280 Da, indicating that modification has occurred on the cysteine residue which is 9 positions from the C-terminus of the peptide. The same is true for the y9, y10 and y11 ions, as labelled in the spectrum.

The mass increase of 280 Da corresponds to the mass of an amodiaquine molecule minus the diethylamine moiety, suggesting that the amodiaquine molecule has also been fragmented during the product ion fragmentation experiment. The fragmentation of amodiaquine is not surprising as product ion fragmentation data generated on a standard of amodiaquine shows the loss of the diethylamine as the primary fragmentation pathway (see Appendix 6 for spectrum), suggesting that the C-N bond is particularly labile. Despite not seeing an intact amodiaquine moiety on the peptide due to fragmentation, the evidence from the product ion spectrum, coupled with the mass changes observed at both protein and peptide levels indicate the existence of an amodiaquine modified peptide in the digest sample, which is not present in the unmodified BSA sample.

The structure of the unmodified peptide and the proposed structure of the modified peptide are given in Figure 5.19. The structure of the modified peptide is based on structural information generated on the GSH adduct generated by the electrochemical method (see Section 5.2), and subsequently analysed by a combination of MS and 1H NMR and makes the assumption that the AQQI reactive species binds to the free cysteine of the T9 peptide by a similar mechanism. This seems to be a reasonable assumption to make based on the weight of data available. The NMR can be considered unequivocal as it is based on the comparison of the glutathione adduct data with an authentic standard of unmodified amodiaquine. In addition, the mass spectra on the glutathione adduct of amodiaquine all support the structure proposed by the NMR analysis. It is therefore unlikely, from a chemical reactivity point of view, that conjugation of the AQQI moiety with a cysteine on the BSA protein would occur via a different mechanism, to the conjugation with the cysteine portion of glutathione. However this cannot be proved explicitly using the data detailed in this report.

To definitively identify the adducted peptide structure work, it should be possible to isolate 10-20 μg of adducted peptide using preparative chromatography for 1H NMR analysis, however the time and resources required to do this were outside the scope of this project.
GLVLIAFSQYLQQCPFDEHKV

(A)

b2, L, Y, b3, and b4

y2, y3, y4, y7, y8, y10, y11, y12, Z10

m/z 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400

% 0 100
Figure 5.18: Product ion spectra for (A) the unmodified T9 peptide (m/z 812) in the trypsin digest of unreacted BSA control and, (B) AQQI-modified T9 peptide (m/z 930) in the trypsin digest of the incubation of electrochemically generated AQQI and BSA
Figure 5.19: Structure of the amodiaquine modified and unmodified T9 tryptic peptide of BSA
5.5 Trypsin digestion of the CYP2C8 Supersomes™ after incubation with electrochemically generated AQQI and LC/MS Analysis

An LC/MS method using an Acquity UPLC coupled to a Waters Synapt G2S was devised incorporating low and high collision energy full scan MS\(^5\) functions, the details of which are recorded in Experimental Section 2.36. The LC/MS was switched from the Waters QToF Premier MS instrument due to superior sensitivity of the Waters Synapt G2S instrument as discussed in Chapter 3. CYP2C8 supersomes (100 pmol) were incubated with electrochemically generated AQQI (50-fold excess), digested with trypsin and analysed by LC/MS, primarily see if the electrochemically-produced AQQI would bind to a metabolizing enzyme such as CYP2C8, and secondly to identify the modified peptide of CYP2C8 to which AQQI was bound (as described in Experimental Section 2.36). In contrast to the series of experiments with BSA described above, analysis of the intact protein of CYP2C8 was not attempted, based on the experiences described in Chapters 3 of this thesis (280). The presence or absence of modification of the CYP2C8 protein was based solely on comparison of tryptically-digested peptides from incubation with electrochemically generated AQQI compared to a control sample of tryptically-digested unreacted CYP2C8.

The data were processed using BioPharmaLynx, using the protein sequence of CYP2C8 as accessed from the UniProt website (405) and trypsin as the digest reagent, with 2 missed cleavages allowed and leucine enkephalin (m/z 556.2771) was used for lock mass correction. The mass tolerance was 5 ppm for the MS match and 10 ppm for the MS\(^6\) fragment ion match due to the improved mass resolution and accuracy of the instrument, with carboxyamidomethylation on cysteine residues (+57 Da, added due to alkylation of cysteine with iodoacetamide which was part of the trypsin digestion protocol described in Experimental Section 2.36) and amodiaquine (+353.13) as a variable custom modification, with no maximum number of modifications given. In addition, a manual comparison of the sample data, with the no drug and no cofactor control data was carried out.

BioPharmaLynx identified two triply charged potential AQQI-modified peptides at m/z 695.98; both possible modifications of the T61 peptide of CYP2C8 (see Figure 5.20 for BioPharmaLynx screen-shot) with retention times of 14.6 and 17.3 minutes, as shown in Figure 5.21. The doubly-charged unmodified T61 peptide (m/z 895.48) was observed at retention time 30.7 minutes, as shown in Figure 5.22. From an initial assessment of the data and comparison with the data observed for amodiaquine-modified BSA, the modified peptides identified by BioPharmaLynx were likely to be false positives. Ions corresponding to the addition of amodiaquine incorporating the $^{37}$Cl isotope were not present on manually interrogation of the
data. A significant shift in retention time was observed for the candidate modified peptides compared to the unmodified T61, suggesting a significant difference in polarity between the modified and unmodified peptides and in direct contradiction to the lack of change in polarity observed for the modified peptide of BSA compared to its unmodified counterpart. In addition, the potential modified and unmodified peptides observed have different charge-states (triply-charged for the modified T61 and double charged for the unmodified), again in contradiction to the information generated using BSA as a tool protein for modification with amodiaquine.
Figure 5.20: Screen shot of BioPharmaLynx processing results indicating two potential modified peptides of T61
Figure 5.21: Mass chromatogram of the postulated modified T61 peptides ([M+H]^{3+} m/z 695.98, 0.01 Th window) in the CYP2C8 Supersome™ incubation with electrochemically generated AQQI

Figure 5.22: Mass chromatogram of the unmodified T61 peptide ([M+H]^{2+} m/z 895.48, 0.01 Th window) in the CYP2C8 Supersome™ incubation with electrochemically generated AQQI
These initial observations were confirmed by generating product ion spectra on both the m/z 895.48 and the m/z 695.98 ions.

Figure 5.23: Product ion spectrum of the unmodified T61 peptide ([M+H]\(^+\) m/z 895.48, at retention time 14.6 minutes) in the CYP2C8 Supersome™ incubation with electrochemically generated AQQI

The product ion spectrum for the unmodified T61 peptide (Figure 5.23) correlates well with the expected product ion spectrum for a peptide of composition GIVSLPPSYQICFIPV. Fragment ions representing b\(_{14}\), y\(_{11}\) and internal fragments modified with carbamidomethylation of the cysteine residue are observed as expected (due to the alkylation of free cysteines using iodoacacetamide, prior to digestion), together with smaller fragments such as the prominent y\(_2\) and b\(_2\) ions.

The product ion spectrum for the two postulated modified T61 peptides are shown in Figure 5.24 and Figure 5.25. As shown in the spectra, the product ion spectra for these peptides bear little resemblance to the spectra observed for the unmodified T61 peptide, particularly with the absence of the y\(_2\) and b\(_2\) ions which would be expected to be present, if the spectra are related to the T61 peptide structure. The product ion spectrum observed is not sufficient to de novo sequence the peptide to determine its structure; however the presence of m/z 175 in both spectra may suggest they are both arginine terminal tryptic peptides.
Figure 5.24: Product ion spectrum of the postulated modified T61 peptide peak ([M+H]$^{3+}$ m/z 695.98 at retention time 14.6 minutes) in the CYP2C8 Supersome™ incubation with electrochemically generated AQQI.

Figure 5.25: Product ion spectrum of the postulated modified T61 peptide ([M+H]$^{3+}$ m/z 695.98, retention time 17.3 minutes) in the CYP2C8 Supersome™ incubation with electrochemically generated AQQI.
It is likely therefore, that the two modified peptides identified by BioPharmaLynx are false positives. As discussed previously, peak assignment in BioPharmaLynx is primarily based on the exact mass values of peaks observed in the deconvoluted spectra. While MS\textsuperscript{E} fragmentation data is taken into account for the larger peptides to confirm the peptide assignment, covalently-modified peptides tend to be of low abundance which means any MS\textsuperscript{E} fragmentation data generated is either partial and therefore not definitive, or not distinguishable over other background fragment ions. This is exacerbated by the lack of chromatographic resolution between peptides in a complex digestion mixture. Finally, BioPharmaLynx is only able to search and assign known protein sequences that have been entered into the search parameters. This differs from other processing software such as Mascot™ or SeQuest™ which can search and assign unknown proteins based on internet protein databases. Due to the nature of Supersomes™ and the fact that they also contain cDNA-expressed human P450 reductase and human cytochrome b\textsubscript{5}, and are manufactured using over-expressed baculovirus infected insect cells, it is feasible that the two peptides identified as modified T61 could be peptides belonging to other unassigned proteins within the sample.

### 5.6 Conclusions and future work

The lack of modified peptides in the incubation with CYP2C8 Supersomes™ is disappointing. The reaction of electrochemical AQQI to BSA in solution would suggest that reaction with other proteins might be expected. One reason for this disparity may be the relative difference in hydrophobicity of water soluble proteins such as BSA, and hydrophobic membrane proteins such as CYP2C8, making it easier for the polar AQQI moiety to interact with BSA in solution. Brandon et al. (254) reported that one of the disadvantages of using UGT Supersomes™ to investigate metabolism in vitro is that the active site is shielded by a hydrophobic barrier, thereby affecting the extent of glucuronidation observed, however it must be acknowledged that this observation was related to the enzyme in terms of its metabolising capability via the active site rather than access of substrates to the protein itself.

However, the data shown in Chapter 3 using chemically generated NAPQI (generated via an oxidation reaction of acetaminophen with silver oxide) covalently bound to CYP2E1, shows that it should be possible to react an isolated CYP Supersome™ with a chemically reactive species, in the absence of metabolising co-factors such as NADPH. A feature of the cytochrome P450 super-family of enzymes is that they are all similar in size, amino acid composition and tertiary structure (406) but differ in the size of the active site (221) as defined by their crystal
structure, CYP2E1 having one of the smallest active sites of the cytochrome P450s at 190 Å and CYP2C8 significantly larger at 1536 Å. In the absence of metabolism, it is difficult to rationalise how this would have an effect. One experiment which could be conducted to investigate the difference observed would be to incubate CYP2C8 with chemically produced NAPQI, and CYP2E1 with electrochemically produced AQQI, to see if covalent peptide adducts could be formed in either case. This would give an indication of how amenable both proteins are to non-enzymatic reactions with chemically-reactive species in solution.

Despite the lack of success in binding electrochemically-produced AQQI to CYP2C8, the work in this chapter demonstrates the potential to use electrochemistry to simulate the formation of reactive species, usually formed via metabolism, to investigate how they might reactive with proteins. Two recent papers by Melles et al. (407) and Jahn et al. (408) have successfully used the techniques described to investigate the electrochemical oxidation of aniline, and para-phenylenediamine, respectively, to reactive species, and the subsequent binding of these reactive moieties to proteins such as human serum albumin and β-lactoglobulin, so there is increasing evidence to support the application of this technique. As discussed in the previous chapters, one of the major technical challenges of investigating how reactive metabolites covalently bind to proteins is that the abundance of these adducted proteins is relatively very low, making detection, even with the most sensitive MS instruments, problematic. One of the advantages of this methodology is that very large quantities of stable reactive species can be produced and incubated with GSH or tool proteins, making detection by MS and even NMR possible. This technique could also potentially be applied to produce adducted peptide standards which could either provide an HPLC retention time marker for additional in vitro or in vivo experiments, or could be used to develop more sensitive MS experiments, such as multiple reaction monitoring, to maximise instrument sensitivity, thereby making detection of covalent adducts easier.

An important area of future work would be to apply the methods described in this chapter to other compounds with different structural moieties, which are linked to reactive metabolism. Kalgutkar (77) describes a comprehensive list of structural groups in his review of 2005. For example, troglitazone is a thiazolidinedione containing drug which is known to cause heptotoxicity for which the mechanism is currently unknown. Toxicity could either be mediated via metabolism of the chromane ring to a quinonemethide or oxidative ring-opening of the thiazolidinone. Tahara et al. (409) were able to demonstrate the electrochemical formation of a reactive quinone methide which could potentially be used for further investigational studies with target proteins. Aniline groups such as procainamide can be
activated using electrochemistry to a nitroso metabolite which can be trapped by GSH and is capable of binding to protein \textit{in vivo} (410). This information could be applied to one of its structural analogues such as dapsone. While a few groups have already been successful in using electrochemistry to investigate how reactive species bind to proteins, as described above, the missing piece of the puzzle seems to be how to prove that these mechanisms are relevant to what happens \textit{in vitro} and ultimately \textit{in vivo}.

In terms of future work on amodiaquine, based on the observation by Tingle \textit{et al.} (388) that amodiaquine is oxidised to AQQI \textit{in vitro} incubations with stimulated polymorphonuclear neutrophils (PMN), which then subsequently binds to HSA acting as a target protein, it would be interesting to investigate the binding of AQQI to neutrophil proteins themselves. Isolated neutrophils could be incubated with electrochemically-generated AQQI to induce binding, with separation of the proteins using 1- or 2-D gel electrophoresis, as discussed in Chapters 3 and 4, in-gel digestion and LC/MS to identify any adducted peptides present.

The use of electrochemistry in this way is not appropriate for all compounds of interest. The degree of conversion to reactive species varies significantly from compound to compound, with some compounds unable to undergo electrochemical oxidation at all (393, 411-413). For example, dehydrogenation to form quinones, quinone imines and quinone di-imines have been well documented (135), aromatic and aliphatic hydroxylation is rarely observed (393), with dimerisation being favoured. In addition, the electrochemical products generated for some compounds may have no physiological relevance or may be produced in far greater abundance in the electrochemical cell than would ever be seen in humans or preclinical species. The methodology must therefore be applied with care, and with knowledge of the metabolism and reactive metabolite risk factors associated with a compound to avoid making false assumptions on the reactivity.
6 Conclusions

As discussed in the Introduction, the formation of covalent adducts between reactive metabolites and proteins have, for several drugs, been linked to unpredictable toxicity in humans and animal species. At the start of this project, direct detection of the covalent protein adducts was very rare with only a few references in the literature, with researchers mainly relying on circumstantial evidence from trapping experiments and metabolite identification to postulate the mechanism of binding. It was hoped that by directly identifying reactive species while bound to protein targets, further insight into the binding mechanisms could be gleamed and this information could be used by medicinal chemists to design out these reactive moieties in future drug molecules. Information about the protein targets of these reactive species would also be useful in understanding the physiological mechanisms of toxicity observed, which could in turn be used to better protect patients by earlier termination of drug candidates in development.

The decision to investigate in vitro methods of generating reactive species to bind to simple protein systems was taken to maximise the chances of seeing drug modification, with a hope that the methods developed during this project could then be applied to more complex in vivo experiments. Using the tool substrate acetaminophen described in Chapter 3, this was largely successful with novel covalent adduct of the T56 peptide of CYP2E1 identified for the first time and was partially successful for covalent adducts SB-649868 (Chapter 4) and amodiaquine (Chapter 5). Throughout this thesis, the focus has been on the different analytical methods that can be applied to the detection of these covalent adducts and their relative merits from a sensitivity and specificity perspective.

The failure to detect modification of the cytochrome P450 protein CYP2E1 using LC/MS of the intact protein was very disappointing, as this was anticipated to be one of the simplest methods of demonstrating drug binding to protein (as shown by the BSA-amodiaquine data in Chapter 5). The most likely cause of the failure of this experiment was due to the presence of detergents causing MS ion suppression when the native sample was analysed, and an inability to resolubilise the proteins once these detergents were removed. Future work to overcome these issues could focus on the use of mass spectrometry compatible surfactants such as (1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) (282) and n-dodecyl-β-D-maltoside (DDM) (283).

One of the over-riding findings of this project has been the sensitivity required to reliably detect these low level protein adducts. Due to the nature of metabolism and the binding of
reactive metabolites, even when large concentrations of parent drug are used in experiments, the degree of covalent binding is dependent on the percentage metabolic conversion of a drug to a reactive metabolite and the propensity of this reactive metabolite to bind to proteins, rather than be converted into other metabolites via further oxidation or conjugation. For example, some downstream metabolites of the reactive epoxide metabolites, such as the dihydrodiol, were still noted in the glutathione trapping experiments with SB-649868, suggesting that not all of the reactive epoxide bound to the proteins on formation. An interesting piece of future work could be conducted to examine the reactivity of species such as epoxides and investigate the percentage binding to protein versus the percentage undergoing further metabolism and how this changes depending on variables such as drug structure, drug concentration and protein concentration. If some reactive metabolites are more reactive than others, this may have value in explaining why some drugs which have the potential to form reactive metabolites exhibit lower than expected levels of protein binding.

In terms of sensitivity, drug-protein adducts have been identified in this project only where highly sensitive and selective methods and instrumentation have been used. The application of tandem MS/MS methods such as multiple reaction monitoring and precursor ion scanning have been used to successfully identify modified amino acids and peptides, however their use requires a careful application of the information gathered from metabolism identification and reactive metabolite trapping experiments. The disadvantage of using these types of targeted methods is that they do not provide any fragmentation information which could be used to confirm structural assignments. The methods are based on assumptions around the mass and amino acid target of the modification, so there is a danger of missing modifications which occur either via unexpected mechanisms or to unexpected amino acid residues and as such, they tend to confirm what is already expected. The lack of material available for more data-rich full scan mass spectrometric methods has been circumvented in part in this work, by the chemical or electrochemical generation of reactive species which can be used to create more abundant reference standards. The use of chemically-generated NAPQI in Chapter 3 to bind to CYP2E1 Supersomes™ to create a more abundant acetaminophen-modified peptide adduct for retention time comparison and CID fragmentation, is a good example of how this may be used for future drug substrates. The lack of a chemical method for the formation of a reactive epoxide of SB-649868 means that the modified peptide observed in CYP3A4 Supersomes™ could not be confirmed. The recent introduction of the Synapt G2S instrument, which potentially offers sensitivity similar to the tandem MS/MS experiment, while operating in full scan MS mode so that structural information such as exact mass is retained, is a step forward
in offering the solution to this. The option of employing ion mobility to simplify and refine MS data generated on the Synapt G2S may also be of future use in identifying low level modified ions. Due to time-constraints, this wasn’t investigated fully during this project and so investigation into the use of ion mobility for the detection of covalent protein-adducts would constitute an important part of any future work.

As discussed in Chapters 3 and 4, further purification and hence simplification of the complex digestion samples could be achieved by using affinity chromatography. Tagging of the drug substance with, for example, biotin could provide a method to retain any drug-related material, including drug-modified peptides or amino acids, while removing non-drug related components from the samples. This would have the advantage of dramatically reducing the complexity of the background and a potentially significant improvement to the signal to noise ratio of any drug-bound components. Several injections of the same sample could be retained on the column to concentrate the sample before elution, thereby increasing the amount of drug-modified peptide available for analysis. Some important considerations would have to be taken into account however, during method development, most importantly, that the presence of the biotin tag does not significantly alter either the types of metabolic routes seen, or the extent of metabolism observed. Since cytochrome P450 metabolism is driven by structural recognition of the substrate by the enzyme, this is an important consideration, however, as the cytochrome P450s enzymes tend to metabolise a wide range of substrates of various size and composition, it is unlikely that the presence of a biotin tag would obstruct interaction with these metabolising enzymes completely, although the specificity for a particular enzyme may change. This is likely to be heavily influenced by the positioning of the biotin tag on the drug structure. The use of affinity chromatography offers an exciting possibility for the enrichment of covalently bound species within complex samples and would make a good focus for future work in this area.

Accelerator mass spectrometry has been used very successfully in this project to understand the extent of modification of proteins in terms of the number of modified peptides present. This has aided the explanation as to why protein adducts were identified in some cases and not in others, despite the percentage modification being similar. For example, for both acetaminophen and SB-649868, the P450 enzyme was modified by approximately 2% in both cases, however, for acetaminophen, only one peak was detected in the AMS radiochromatogram for [14C] SB-649868. This suggests that the 2% modification is split over several peptides, making the detection of each individual modification more problematic. The very low limit of detection of accelerator mass spectrometry makes it the perfect technique for
the detection of low level drug-modified peptides and can be a useful tool for understanding the stoichiometry of binding, even where individual adducts are not detected, however, the requirement for [14C] labelled drug material, the scarcity of instruments in both academia and industry and the high cost and technical difficulty of sample preparation mean it may not be suitable for widespread use.

If the sensitivity of protein adduct detection can be improved by some of the methods described here, the natural next step in investigating covalent binding of reactive metabolites to proteins would be the development of methods for the quantitative assessment of covalent binding without the use of a [14C] radiolabel. Within GSK, the high cost of synthesis means that [14C] labelled drug material is generally only prepared once the drug has undergone initial toxicity testing in animals and there is a commitment to dosing to humans. Generally, this is much later in the drug development process and radiolabelled material for definitive ADME investigations is only synthesised after proof-of-concept studies in patients, often forming part of the Phase II clinical programme. At this point a significant amount of resource, both in terms of money and scientist effort, has been spent on developing the drug candidate. New data generated to characterise covalent binding mechanisms would come far too late to change the structure of the drug itself to potentially eliminate the dangerous structural moieties and can only aid to terminate its development. Whilst this is hugely important in terms of patient safety, it can be detrimental to the company itself, with the time, effort and money used to develop that drug effectively wasted. These kinds of investigations would be better placed earlier in the drug development process, where either the drug can be terminated before patients have been exposed to potentially reactive moieties and before a huge amount of resource has been expended on it, or changes to the structure, while retaining pharmacological activity, can be implemented in back-up drug development programs.

Several methods may lend themselves to being used to quantify the extent of covalent binding without the use of [14C] radiolabelled drug. iTRAQ (414-417) quantification techniques involve the use of isotopically-labelled tags which covalently bind to free amines in lysine residues and on the N-terminus of peptides. Fragmentation of these labelled peptides result in characteristic reporter ions which appear in the low mass region of the product ion spectrum. Incubation samples could be labelled with one isotopic label, while the no drug control could be labelled with another and the two samples mixed for co-analysis. Fragmentation of the modified and unmodified peptide of interest would then reveal different reporter ions, the integrals of which could be compared to give an assessment of the relative extent of binding to the protein. The advantage of this kind of experiment is that it requires no previous knowledge
of the modified peptide in order for the method to be used. The disadvantage is that only a relative assessment of modification can be achieved.

Label-free methods such as the use of AQUA peptides (418, 419) or specially designed concatenated proteins (QConCat) (329, 420-422) could also be applied. AQUA peptides are synthetic stable labelled peptides which can be spiked into a tryptic digest sample at known concentrations; however, each individual modified peptide of interest would require the synthesis of its own stable-labelled AQUA peptide analogue. These could then be spiked into the sample at known concentrations and the peptide peak for the modified peptide and the stable-labelled analogue integrated. Since the ionisation efficiency and retention characteristics of the stable-labelled peptide should be identical, the amount of the modified peptide observed can be directly correlated to the amount of stable-labelled peptide present. The advantage of this method is that it is relatively simple; however, any sample losses during sample digestion would not need to be taken into account and each modified peptide would require the synthesis of an individual AQUA peptide. Several suppliers of customised AQUA peptides are currently available including Sigma-Aldrich and Thermo Fisher. The QConCat method involves the design and synthesis of a stable-labelled small protein which incorporates the peptide of interest, based on an earlier assessment of the modified peptides in the sample. The QConCat protein can be spiked into the sample at a known concentration and is designed to yield the peptide of interest after trypsin digestion of the sample as a whole. By spiking into the sample prior to digestion, any losses occurring in the sample as a result of the digestion process should affect the QConCat protein to the same extent as the protein of interest, thereby not affecting the quantification. Stable-labelled peptides should have exactly the same retention characteristics and MS ionisation efficiency as their non-labelled counterparts, therefore integration of these spiked stable-labelled peptides can be directly correlated to the non-labelled peptides of interest. Whilst absolute quantification of the levels of covalent binding could be achieved by this method, it does require previous knowledge of the peptides of interest and assumes that all of the modified peptides will be visible by LC/MS analysis.

In summary, the data contained in this thesis demonstrate the application of sensitive analytical techniques for the identification and characterisation of adducts formed via the covalent binding of reactive metabolites to proteins, with the major success being the first reported identification of an acetaminophen- adduct of the CYP2E1 Supersome™ enzyme after in vitro incubation. A combination of analytical techniques have been utilised, most notably sensitive MS and MS/MS methods, using the Synapt G2S and Sciex 5500 triple quadrupole mass spectrometers, respectively. Assignment of any covalent adducts identified have been
reinforced by the use of chemical and electrochemical methods to produce large amounts of reactive metabolites which have then been used to bind to proteins, and produce appropriate reference standards. The use of adduct standards of high concentration has significant future utility if additional chemical or electrochemical methods can be developed for the synthesis of other reactive moieties.

As discussed previously, the difficulties encountered for the identification of drug protein-adducts using even the most sensitive mass spectrometric techniques currently available suggest the possibility that a major factor in characterising these adducts may be the absolute amount of adduct present in the sample, rather than the techniques used for identification. Therefore the primary focus of the initial future experiments conducted would be an exploration of methods to increase the amount of drug-protein adduct available. *In vitro* experiments with SB-649868 described in this thesis failed to identify any peptide adducts with CYP3A4 Supersomes™, most likely due to the low abundance of the likely several adducted peptides. However, liver tissue from an *in vivo* study where rats are dosed with [14C] SB-649868 could potentially generate several milligrams of adducted protein. A larger scale preparative method using a method such as preparative scale SDS-PAGE (423) could be used to isolate protein bands of interest, with subsequent in-gel digestion and analysis using LC/MS as described in this thesis. The combination of a more abundant sample of adducted protein with the sensitive mass spectrometric methods devised in this project could provide the breakthrough necessary to finally identify SB-649868 adducted peptides, and could aid the application of these methods for other compounds in drug development.

Ultimately, it is hoped that the methods described in this thesis can be applied to aid the understanding of the mechanisms of toxicity observed where covalent binding of drugs to proteins is suspected, helping to eliminate these drugs early in the drug development process and ultimately improve the safety of future drugs for patients.
7 References


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